Chapter 1
General Introduction
Neuronal synaptic transmission

The brain is undoubtedly the most complexly build organ of the human body with estimates of cell numbers close to 86 billion neurons⁰ and even more non-neuronal glial cells. A complex interplay between neurons in different areas of the brain is widely believed to be at the basis of thoughts, moods and behaviors. Fast transmission of signals between neurons is achieved via specialized organelles interfacing axon terminals and dendrites of neurons, referred to as synaptic transmission. The estimated number (>10¹⁵) of these synapses is staggering and is believed to underlie the immense computational power of the human brain. Synapses have long been subject of study; being first recognized by Cajal in his early anatomical observations of 1888, and referred to these as dendritic spines². Since then, numerous studies have been carried out to understand the mechanisms of synaptic transmission and it is now well accepted that modulation of the efficacy of synaptic transmission is crucial to learning and memory at the cellular, system and behavioral levels.

`Decades of neuroscience research have revealed various fundamental mechanisms of synaptic neurotransmission. Generally speaking, fast synaptic transmission in the brain can be inhibitory or excitatory, i.e., leading to inhibiting or facilitating postsynaptic neuronal responses. In line with this, synapses are generally classified as excitatory or inhibitory, based on the principle neurotransmitter involved. Glutamate for excitatory and γ-aminobutyric acid (GABA) for inhibitory synapses, mediated by glutamate receptors and GABAₐ receptors, respectively. Apart from these fast synaptic responses, metabotropic receptors present on neurons are responsible for slower and long-term changes mediated via G-protein-coupled signaling.

Importantly, synapses respond to changes in neuronal activity and are able to adapt the strength of signal transmission in an activity-dependent manner, a process commonly referred to as synaptic plasticity. Several mechanisms, either on the presynaptic³,⁴ or on the postsynaptic side⁵,⁶ regulate synaptic strength operating at short (millisecond to minutes, short term plasticity), and long (minutes to days and even years, long term plasticity) time scales. This modulation of synaptic strength affects the activity that is propagated in neuronal circuits and changes the functional properties of the neuronal network. These synaptic plasticity processes, thereby can act at short- and long-term memory and other higher cognitive processes. Additionally, abnormalities in synapse function, often caused by genetic defects and commonly referred to as synaptopathies, have been implicated in a large number of
neuropsychiatric disorders. The translation of synaptic dysfunction to brain disorders continues to be an area of active research in the field of neurobiology.

**Structure of the synapse**

Excitatory glutamatergic synapses are the most commonly studied because of their large abundance in the brain and their relative ease of biochemical purification\textsuperscript{7-9}. They comprise of the presynaptic element, which makes contact on the dendritic spine on dendrites of postsynaptic cell. The membranes of the pre- and postsynaptic cell are separated by the synaptic cleft. The pre- and postsynaptic compartments are held together tightly by many trans-synaptic adhesion molecules\textsuperscript{10}.

**Presynaptic architecture**

The presynaptic element is a specialized structure dedicated to exocytosis. It comprises a large number of synaptic vesicles of which a relatively small number is docked at the active zone. Synaptic vesicles are initially synthesized in the cell body and transported in the axons along microtubule tracks by kinesin motor proteins to reach their target presynaptic element\textsuperscript{11}. Neurotransmitters are then loaded into these vesicles by the action of transmitter pumps at the vesicle membrane\textsuperscript{12}. The synaptic vesicles release their neurotransmitter content after fusion with the plasma membrane. This process is triggered by Ca\textsuperscript{2+} entry into the presynapse via Ca\textsuperscript{2+} channels\textsuperscript{15} opening at depolarization of the presynaptic membrane. Vesicle fusion and release of transmitters at the active zone is mediated by a complex interplay of proteins associated on the synaptic vesicle, the active zone membrane, as well as the cytosol, as summarized in Fig. 1 (refs\textsuperscript{13-15}). Following fusion, vesicles are retrieved either via clathrin-mediated endocytosis\textsuperscript{16-18} or activity-dependent bulk endocytosis\textsuperscript{19-21}. Several proteomics studies have aimed to elucidate the composition of the different presynaptic machineries, including the active zone\textsuperscript{22-24}, synaptic vesicle\textsuperscript{25} or set out to quantify the entire presynaptic proteome and its constituents\textsuperscript{26}. 
Figure 1. Architecture of the excitatory synapse. Schematic of the molecular architecture of the excitatory synapse showing both pre- and postsynaptic elements. The coordinated protein-protein interactions within the synapse regulate complex synaptic biology as exemplified by the various steps of the synaptic vesicle cycle on the presynaptic side, involving the release of neurotransmitters in the synaptic cleft. Neurotransmitters bind to postsynaptic receptors anchored in the postsynaptic membrane by a plethora of PSD proteins. PSD proteins are involved in signal transduction processes and regulation of receptor density. (Reprinted from Chua et al, 2010 with rights and permissions.

Postsynaptic architecture

The presynaptic released neurotransmitters bind to neurotransmitter receptors on the postsynaptic membrane. The postsynaptic element forms a distinct protrusion on the neuronal dendrite, the spine, comprising of the spine neck, endocytic zone and an electron-dense structure in alignment with the presynaptic zone, called the postsynaptic density (PSD) (Fig. 2) This dominant electron-dense structure is characteristic of excitatory glutamatergic synapses, whereas inhibitory synapses lack this prominent thickening.

The dendritic spine head is a micro compartment (0.5 to 2 µm) separated from the rest of the dendrite by the spine neck which limits the diffusion of molecules and organelles depending on activity-dependent changes in the spine neck architecture. Dendritic spines undergo activity-dependent structural changes. These changes are correlated with the induction of long term potentiation (LTP), or long term depression (LTD), inducing increase or shrinkage of the dendritic spine head, respectively. Consistent with these findings, many synaptopathies, such as established for autism spectrum disorders, schizophrenia and fragile X mental retardation are associated with alteration in spine architecture.

In addition, membrane bound organelles, such as, smooth endoplasmic reticulum (SER) polyribosomes, mitochondria and clathrin-mediated endosomal compartments are found in or at the base of the dendritic spine. The SER functions in regulating protein and lipid availability at the spine heads, and polyribosomes have been observed to move towards dendritic spine heads in response to neuronal activity serving local protein synthesis.

Finally, the PSD consists of an elaborate scaffold of proteins, which serves to organize neurotransmitter receptors, allows alignment with the presynapse, and hosts many signal transduction proteins that link synaptic transmission to downstream signaling cascades via membrane-associated protein kinases, phosphatases and G-protein coupled receptors.
Composition of the PSD

Due to its abundance in the brain and suitability for biochemical enrichment, the PSD has been subject of intense biochemical analyses\textsuperscript{41–46}. In particular, the advent of mass spectrometry, yielded excellent coverage of proteins of the PSD, and a large set of approximately 1500 proteins has been observed in PSD preparations\textsuperscript{47,48}. These proteins functionally include, e.g., synaptic membrane receptors, kinases and phosphatases, scaffolding proteins, cytoskeletal elements, GTPases, cell adhesion molecules\textsuperscript{39,49}. Of all protein constituents of the postsynaptic density, quantitative estimates revealed that this structure is largely dominated by CaMKII (an estimated 5600 copies), followed by the scaffolding DLG family member proteins (estimated 400 copies), followed by SynGAP (approximately 360 copies)\textsuperscript{43,50}. Although the function of these high levels of enzymes, such as CamKII and SynGAP, in the PSD is unknown, one might speculate that apart from their roles as kinase and RasGAP, respectively, they have a structural role in the PSD.

The PSD contains large amounts of protein interaction organizing scaffold proteins, such as Dlg4 (Also known as PSD-95), Gkap1 (GKAP/SAPAP), Shank (Shank1-3 and Homer (Homer1-3) proteins, each of these represented by multiple isoforms and showing brain region and neuronal subtype-specific expression patterns\textsuperscript{43,50}. One of the most commonly found protein domains in members of the PSD family is the PDZ domain; a 90 amino acid module, which is present in multiple copies in many PDZ domain containing proteins and allows these to interact with a multitude of proteins. For instance, PDZ binding domains of Dlg4 are responsible for tethering N-methyl-D-aspartate receptor (also known as the NMDA receptor or NMDAR) within the postsynapse\textsuperscript{51}. Furthermore, PSD95 also interacts with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (also known as AMPA receptor, AMPAR) interacting protein Cacng2 (also known as Tarp γ2 or Stargazin) and Shisa6, thereby playing a role in the stabilization of AMPA receptors in the synapse\textsuperscript{52–54}. Thus, Dlg4 has a direct role in regulating the availability of AMPARs and NMDARs at the postsynaptic density and thereby directly affects plasticity. PSD scaffolding proteins also bind cell adhesion molecules, which determine the specificity of axo-dendritic interactions and drive synapse formation, maturation and maintenance. Typical examples are the interactions of the presynaptic (Nrxe1-3) Neurexins and postsynaptic (Nlgn1-3) Neuroligins, which bind presynaptic CASK scaffolds and postsynaptic Dlg4\textsuperscript{55}. The PSD-localized Dlg gene family members are similarly involved in the localization of other synaptic adhesion molecules, such as SALMs (synaptic adhesion-like molecules) and LRRTMs (leucine-
rich repeat transmembrane neuronal proteins). The ionotropic glutamate receptors, AMPAR and NMDA receptors are present in tenths of copies, and in particular AMPARs are highly dynamically regulated. Whereas, the AMPA receptors, show a large extent of dynamic regulation, and move in and out of synapses, NMDA receptors are kept in the PSD and are far less mobile than AMPA receptors.

Even after a few decades of biochemistry, the number of true PSD residents is still unknown. Partly this is due to the limitations of biochemical purification adequately distinguishing true PSD-enriched proteins from contaminants. A proteomics strategy based on high enrichment of the PSD components and identification using high-resolution mass spectrometry has the potential to shed light on yet undiscovered PSD proteins. This is a focus of chapter 2. Despite advances in characterizing PSD proteomes, the function of many of these proteins in the PSD is unclear.

**Glutamate receptors in synaptic transmission**

Glutamate receptors in the mammalian brain are central to neurotransmission. These receptors can be further grouped into metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs) based on their mechanism of action. Whereas iGluRs, such as the AMPARs and NMDARs, are ion channels that activate upon agonist binding, leading to the rapid influx of Na+ and Ca2+ ions and to rapid membrane depolarization of (in most cases) the postsynaptic membrane, mGluRs act more slowly by modulating second messenger signaling pathways. Undoubtedly, understanding the regulation of mGluRs and iGluRs by studying the composition of their interacting partners and their functions serves to better understand the properties and functions of excitatory transmission mediated by glutamate in the brain. Therefore, mGluR5, a typical member of the mGluR family, as well as the AMPARs and their interacting proteins are the focus of my thesis studies (chapters 3 and 4, respectively).

**Metabotropic Glutamate receptors**

mGluRs are members of the G-protein coupled receptor (GPCR) superfamily, which are activated by glutamate and signal intracellularly via interactions with G-proteins. Genes encoding eight types of mGluR have been identified, which fall into 3 groups (Table 1).

All mGluRs, which are known to be present as dimers, share a large extracellular venus flytrap domain, which contains the binding site for glutamate (Fig. 2) and plays an important role in receptor dimerization and binding to cations (Mg2+ and Ca2+).
Ca$^{2+}$) to potentiate the receptor$^{60,61}$. The cysteine-rich domains are responsible to transfer the conformational changes induced in the venus fly trap domain to the transmembrane domains. The transmembrane domains consist of a hepta-helical architecture. Furthermore, the intracellular C-terminus is important for modulating G-protein coupling. Furthermore, it can be alternatively spliced, and as such is used to modulate protein-protein interactions.

Group-1 mGluRs have been subject of intense study because of their potential involvement in neuropsychiatric disorders, such as autism, fragile X mental retardation and schizophrenia$^{63–65}$. The expression pattern of mGluR1 and mGluR5 has been shown to be either unique or in overlap$^{66}$. At the subcellular level, these GPCRs are known to localize to the postsynaptic element, specifically in a perisynaptic zone close to the PSD$^{67}$. mGluR1 and -5 act by coupling to Gq/G11 and both activate phospholipase C (PLC), which results in the formation of inositol 1, 4,5-triphosphate (IP3) and subsequent protein kinase C (PKC) activation. However, apart from this signal transduction route, the group 1 mGluRs have been shown to act via activation of MAPK/ERK and MTOR/p70 S6 kinase pathways$^{62}$, thereby affecting postsynaptic plasticity mechanisms. Interestingly, the mechanisms of activation of mGluR-dependent LTD in hippocampus and cerebellum differ$^{68}$, however, the downstream effect of the two leads to the endocytosis of AMPA receptors. The mechanism of mGluR-activated LTD in cerebellum granule cells is induced by Ca2+-dependent activation of PKC, leading to the AMPAR GluA2 subunit phosphorylation at Ser880, and resulting in a reduced affinity for GRIP, which leads to AMPA receptor endocytosis. On the other hand, mGluR-dependent LTD in CA1 synapses of the hippocampus is induced by the activation of striatal-enriched tyrosine phosphatase (STEP), which shows increased synaptic translation after mGluR activation$^{69}$. This in turn induces AMPAR dephosphorylation$^{69}$ and endocytosis. More specifically, mGluR5-dependent LTD in hippocampal CA1 synapses$^{70}$ is thought to act through activation of MMP tumor necrosis factor-α converting enzyme (TACE) and the subsequent ectodomain cleavage of the neuronal pentraxin receptor (NPR). Cleaved NPR clusters AMPARs through extracellular interactions and stimulates their endocytosis$^{70}$, leading to AMPAR accumulation in endosomes. Importantly, mGluR1/5 activation also triggers the translation of several neuronal proteins via the activation of the translation of mRNA granules in spines; a process that is inhibited by the fragile X mental retardation protein (FMRP). Loss of function of FMRP, as in the case of fragile x syndrome (FXS), leads to loss of translational repression of mRNAs, thereby causing a hyper-activated protein...
translation machinery, which is believed to be a causal factor of altered spine morphology, one of the hallmarks of FXS. Thus, pharmacological inactivation, alteration of expression or even genetic deletion of mGluR5 holds promise for the treatment of FXS\textsuperscript{71}. Under the glutamatergic hypothesis of schizophrenia and the underlying NMDAR hypofunction, mGluR5 positive allosteric modulators hold promise for the amelioration of symptoms of schizophrenia\textsuperscript{65}.

Thus, the understanding of regulation and expression of group-1 mGluRs is of utmost importance. Several studies have addressed the interaction proteins of mGluR1/5 and their functions as summarized in Table \textsuperscript{2}\textsuperscript{72–76}. A full understanding of stably interacting mGluR partners using a knockout-controlled strategy has the potential to elucidate potential mechanisms of mGluR1/5 regulation in the brain.

Table 1. Classification of metabotropic receptors. mGluRs fall into 3 groups, namely Group 1–3. The receptor name as well as the gene name is indicated.

<table>
<thead>
<tr>
<th>Group</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Receptor</td>
<td>mGluR1</td>
<td>mGluR5</td>
<td>mGluR2</td>
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<tr>
<td>Gene</td>
<td>GRM1</td>
<td>GRM5</td>
<td>GRM2</td>
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Table 2. Summary of the well-characterized group-1 metabotropic glutamate receptor interacting proteins and some of their studied functions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
<th>Detection Method</th>
<th>Function</th>
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| Aβ + PrPC       | Ji Won Um et al., 2013     | HEK cell screen  | 1. mGluR5 links Aβ + PrPC to calcium channels and protein translation via Fyn kinase activation.  
2. Alzheimer’s mouse model memory deficits and synapse loss are rescued by mGluR5 antagonist. |
| NHERF-2         | Paquet M et al., 2006      | mGluR1 and mGluR5 C-terminal domain screen | NHERF-2 prolongs mGluR5 mediated calcium mobilization.                                                                                   |
| Tamalin/GRASP   | Kitano J et al., 2002      | Yeast two hybrid screen | Modulates surface expression and localization of mGluR1/5.                                                                               |
| Grid2 and PKCy  | Kato et al., 2012          | Proteomic approach | Interact with mGluR1 and alter surface expression, subcellular localization and prolongs time course of mGluR1 in parallel fiber Purkinje cell synapses. |
| Homer1          | Kammermeier et al., 2013   | Heterologous expression | Localization and coupling to IP3 signaling.                                                                                              |
Ionotrophic glutamate receptors

Ionotrophic glutamate receptors (iGluRs) are transmembrane proteins composed of four large subunits that form a central ion pore. iGluRs are subdivided into 4 types based on their sequence similarity, namely, AMPA receptors, NMDA receptors, Kainate receptors and delta receptors. Table 3 summarizes the subunit composition of each of these receptor subtypes. The subunit architecture of iGluRs shares many common features, such a large amino-terminal domain (ATD), a ligand binding domain (LBD), a transmembrane domain (TMD) and a C-terminal domain (Fig. 3a). The resolved atomic structure of iGluRs shows an internal two-fold radial symmetry (Fig. 3b) and shows that glutamate receptors assemble as dimers of dimers. AMPA-type and Kainate receptor subunits can form both homo and heteromers, whereas delta receptors form homomeric receptors. Functional NMDA receptors are assembled as tetramers of two GluN1, with either GluN2 or GluN3 subunits. Whereas, AMPAR and Kainate receptors are activated by only glutamate, NMDA receptors require both glutamate and glycine.

In this thesis, I examined the role of proteins that regulate AMPA receptors and their associated proteins in more detail.

Figure 3. Subunit architecture of ionotropic glutamate receptors. a) Cartoon depicting the domains of a typical iGluR comprising of the extracellular N-terminal ATD and LBD, followed by 4 transmembrane units M1-M4 and a C-terminal domain. b) Subunit interface between ATD and the LBD as viewed from the top reveals a 2-fold axis of symmetry whereas the TMD has a fourfold axis of symmetry. (Adapted from Traynelis et al, 2010 with rights and permissions).
Table 3. Summary of ionotropic glutamate receptor subtypes, their amino acid sequence lengths, and gene expression pattern in the brain. iGluRs show brain region specific expression patterns, namely the Gria4 subunit of the AMPAR is highly enriched in the cerebellum. Grin1 is widely expressed throughout the brain, whereas Grin2a is hippocampus specific and Grin2c is cerebellum specific. Grid1 is expressed all through out, while Grid2 mainly in the cerebellum. Source; Allen brain atlas probes shown in white.
AMPARs: subunit structure

AMPARs are responsible for glutamate-induced depolarization of neurons. They are the most abundant and ubiquitously expressed iGluRs in the brain. Their name was derived from the fact they are activated by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA). AMPARs are primarily localized on the postsynaptic dendritic spine membrane and the cell body of neurons and open their channel upon binding of glutamate to the ligand-binding domain (LBD). Depending on the subunit composition, AMPARs are permeable to Na\(^+\) and/or Ca\(^{2+}\) ions. Opening of AMPARs leads to an increase in postsynaptic membrane potential and potentially to action potential propagation in the postsynaptic neuron. The crystal structure of the AMPA receptors revealed that these are tetrameric assemblies, comprising of highly homologous GluA1-A4 subunits. Both the extracellular domain of AMPARs (ATD) and the LBD are responsible for receptor tetramerization. The transmembrane domain (TMD) of each subunit comprises 4 hydrophobic α-helical segments (M1-M4), one of which forms a reentrant loop and the pore of the receptor (M2). The M2 reentrant loop is also the site for Q/R RNA editing in the GluA2 subunit, which affects calcium permeability of the AMPARs. Almost all hippocampal AMPA receptors contain GluA2 and are thus calcium impermeable. The cytoplasmic C-terminal domain (CTD) of the AMPAR is the site of extensive posttranslational modifications and binding of the AMPAR to its C-terminal interacting proteins. The CTD of AMPARs has been implicated in anchoring to postsynaptic proteins. PDZ domain containing scaffolding proteins interact with the AMPAR via proteins, such as GRIP1 and PICK1 and thereby regulate synaptic AMPA receptor pools. Furthermore, PSD95 directly binds with the C-terminus of the AMPAR interacting proteins TARP γ2 and Shisa6, which affects AMPA receptor lateral mobility in synapses. Thus, the study of proteins that regulate AMPA receptor localization, from cell soma biosynthesis to the localization in the PSD, is of prime importance in understanding AMPA receptor physiology and function.
Figure 4. Post-translational modifications on the AMPA receptor. The amino acid sequence of the extreme C-terminal tail of GluA1–4 is shown with phosphorylation sites by PKA, PKC, CamKII, src kinases and Jun kinase on serines and threonine as they regulate AMPAR insertion in the plasma membrane\(^{94}\), synaptic plasticity\(^{95}\) and alter channel function\(^{96,97}\). Palmitoylation regulates AMPAR membrane insertion rate\(^{98}\) and affects PKC phosphorylation\(^{99}\). (Adapted from Traynelis et al 2010\(^{59}\) with rights and permissions).

The complex life of the AMPA receptor

Like all protein receptors in neurons, the AMPA receptors undergo a long journey before they reach their final destination, for instance at the PSD (Fig. 5). One of the first steps in the life of the AMPAR is the biosynthesis of subunits and their assembly as dimers in the endoplasmic reticulum, after which they form tetrameric AMPARs\(^{100}\). The precise mechanisms of endoplasmic reticulum synthesis and subsequent trafficking to the Golgi apparatus are not completely understood, however, a few AMPAR interacting proteins such as Cornichon 2 (Cnih2), Transmembrane AMPA receptor interacting proteins (TARP) family proteins and Cpt1c are known to be involved in this process\(^{101–103}\).

Next, AMPARs are either directly inserted in the plasma membrane by vesicle fusion or they are loaded onto trafficking vesicles and move via kinesin motor proteins along microtubule tracks to reach distal dendrites by binding to the AMPAR interacting protein Grip1\(^{104}\). It has been shown that kinesin-1 not only regulates the number of AMPARs but also the type of receptor\(^{105}\). Exocytosis mechanisms of the AMPARs, needed for insertion at the plasma membrane, were shown to depend on their interaction with NSF in a SNARE-dependent manner\(^{106,107}\). Of interest is that the sites of AMPAR exocytosis have been observed in the extrasynaptic membrane, and that this pool of extrasynaptic receptors shows exchange with receptors at synaptic sites by lateral diffusion in the plasma membrane\(^{108}\). AMPA receptors can get trapped at
synaptic locations, thereby affecting the total number of AMPARs at synapses. The diffusion of AMPARs is regulated by a number of factors, including membrane lipid composition, intracellular scaffolding proteins of the PSD and the extracellular matrix. Each of these mechanisms dictates AMPAR availability at synapses and hence affects short- and long-term synaptic potentiation. Conversely, endocytosis of the AMPA receptors underlies long term depression and predominantly takes place in somatodendritic compartments, as well in an endocytic zone close to the PSD, in a clathrin-dependent manner. Endocytosed AMPARs can either undergo reinsertion in the plasma membrane or are targeted for degradation. The precise mechanisms of proteins mediating degradation of AMPARs are unclear. Proteins, such as UBE3A and Nedd4, have been shown to play a role in AMPAR degradation in an Arc-dependent manner.

Such a complex regulation of the AMPAR life cycle is achieved by precise control dependent on subunit composition, post-translational modification and on a large set of AMPAR interacting proteins. Understanding regulatory mechanisms has led in many studies to identification and characterization of each of these proteins, also referred to as AMPAR

**AMPAR interacting proteins**

The observation that native AMPARs in the brain exhibit different channel properties from those expressed in heterologous systems, led to the idea that AMPARs in the brain are associated with other proteins that potentially alter these properties. Moreover, fitting the notion of the complex life cycle the AMPARs, a multitude of regulatory proteins are predicted to guide localization. With the advent of yeast two hybrid screening methods and interaction proteomics tools, a large set of AMPA receptor interacting proteins has been identified. A model describing the AMPA receptor protein complexes into inner and outer core proteins, based on affinity to the AMPAR, exists. A defined set of criteria to classify associated proteins as ‘auxiliary proteins’ of the AMPA receptor have emerged. The auxiliary subunit should: 1) be a non-pore forming subunit, 2) have a direct and stable interaction, 3) be able to modulate channel properties and/or trafficking in heterologous systems, and 4) have a function in vivo. Although several proteins have been classified as AMPAR interaction partners, only a few, namely proteins of the TARP family (TARP γ2, 3, 4, 7, and 8), the Cnih2/3, the Shisa 6 and 9/CKAMP44 proteins fulfill all criteria of an auxiliary subunit.
Figure 5. The complex life of the AMPA receptor. The AMPA receptor undergoes biosynthesis in the cell body and after passing quality control in the ER and Golgi, is trafficked into the dendrites along microtubule tracks. Insertion in the plasma membrane occurs extra-synaptically or peri-synaptically from where AMPARs can laterally diffuse to the PSD and anchor there. Furthermore, endocytosis/exocytosis as well as local synthesis in the spines maintain the available pool of AMPARs for synaptic transmission. (Adapted from Shepherd and Huganir, 2007 with rights and permissions)
TARPs (Transmembrane AMPA receptor regulator proteins, Gene name Cacng)

The discovery of TARP $\gamma_2$ as the first stably associated protein with the AMPA receptor was made in ‘Stargazer’ mice, which were shown to lack functional AMPA receptors in the cerebellum, thereby giving them the typical ataxia phenotype\textsuperscript{118}. TARP$\gamma_2$ was shown to bind to PSD95 via its C-terminal domain and alter the AMPAR lateral diffusion in the plasma membrane\textsuperscript{53}. Also, TARP $\gamma_2$ slows AMPAR deactivation and desensitization\textsuperscript{119}, and it affects AMPAR trafficking to the plasma membrane by regulating AMPAR maturation and ER exit\textsuperscript{120,121}. TARP $\gamma_2$ is phosphorylated by CamKII, triggered by NMDAR activation, leading to diffusion trapping and enhancement of synaptic AMPAR levels\textsuperscript{122,123}. Other members of the TARP family, TARP $\gamma_3$, 4, 7, and 8 also slow AMPAR deactivation and desensitization rates and alter AMPAR recovery from desensitization\textsuperscript{124}.

Although TARP $\gamma_2$ is the predominant TARP family member expressed in the cerebellum, TARP $\gamma_8$ is widely associated with AMPARs in the hippocampus\textsuperscript{101}. Whereas TARP $\gamma_2$ is highly localized within the PSD, TARP $\gamma_8$ is distributed over synaptic and extrasynaptic sites\textsuperscript{125} and similar to TARP $\gamma_2$, contains a PDZ-binding domain which is important for synaptic trapping of AMPA receptors\textsuperscript{126}, as well as promoting surface expression of AMPARs in a glycosylation dependent manner\textsuperscript{127}. TARP interactions with the AMPAR are resistant to strong detergent treatment and therefore TARP proteins have been proposed to be the part of inner core of the AMPAR complexes\textsuperscript{115}.

Cornichon family of proteins

The cornichon family (Cnih2 and -3) of AMPAR interactors were identified by performing immunoprecipitations of native AMPARs in the brain\textsuperscript{128}. Cnih2 has been shown to facilitate surface expression of AMPARs and to slow AMPAR deactivation and desensitization\textsuperscript{129,130}. In addition, Cnih2 knockdown induces a selective loss of GluA1 containing AMPA receptors as well as affects ER exit of AMPARs\textsuperscript{131}. Furthermore, it has been shown that Cnih2-containing AMPARs dictate slow decay not only in heterologous systems, but at least also in hilar mossy cells of the hippocampus\textsuperscript{132}. Together with TARPs, Cnih2 has also been proposed to be part of the inner core complex of the AMPA receptor and like TARPs, Cornichons also show brain region specific expression patterns\textsuperscript{116,133}.
The Shisa family of proteins

The first member of the Shisa family of proteins discovered interacting with the AMPA receptor was Shisa9/ CKAMP44, which was also identified in a proteomics screen of AMPAR interacting proteins\textsuperscript{134}. Shisa9 expression is highly expressed in the dentate gyrus granule cell layer and has been shown to interact stably with the AMPA receptor. Furthermore, Shisa9 has been shown to slow deactivation, to accelerate desensitization and to prolong recovery from desensitization both in heterologous cells and neurons\textsuperscript{134}. The C-terminal PDZ binding site of Shisa9 also binds to PSD95 and disruption of PDZ binding domain of Shisa9 has effects on deactivation and recovery from desensitization\textsuperscript{135}. Shisa6, another member of the Shisa family of proteins also directly interacts with the AMPAR, is enriched at the PSD, prolongs decay time of AMPARs and affects the lateral diffusion of AMPAR by trapping AMPAR receptors at synaptic sites\textsuperscript{136}. Compared with Shisa9, Shisa6 shows distinct properties, namely it slows desensitization and has no effect on recovery from desensitization.

Receptor complexes are frequently built of combinations of proteins

Until recently, studies on AMPAR auxiliary subunits were focused on ‘one to one’ characterization of their functions with the AMPA receptor, treating Cnih2 and TARPs as if they were to form completely distinct protein complexes\textsuperscript{128}. However, recent evidence suggests that TARP \(\gamma\)8 and Cnih2 can belong to the same complex of the AMPAR in a noncompetitive manner\textsuperscript{129,131}. Additionally, Shisa9 and TARP \(\gamma\)8 have also been shown to be part of one AMPAR complex, however have opposing effects on short term synaptic plasticity in hippocampal neurons\textsuperscript{137}. Thus, it appears that TARP \(\gamma\)8 plays a central role in regulating AMPAR function in hippocampus which opens up possibilities of many more of such permutations and combinations of AMPAR interacting proteins. Leaving the question, which of these are truly made \textit{in vivo}.

The ‘other’ proteins

A number of interacting proteins of the AMPAR have been identified\textsuperscript{115,133}, many of which might not meet the criteria of auxiliary subunits, but do have specific roles to play in the AMPA receptor life cycle. Only a few of these proteins have been characterized in terms of their role in AMPA receptor function, namely GSG1\textsuperscript{138,139}, Cpt1c\textsuperscript{103,140}, Porcupine\textsuperscript{141} and an overview of the interactors is given in Table 4. However, a large set of these proteins remains uncharacterized. Their identification is the first step towards functional analysis.
Considering the fact that several of these proteins could potentially occur in various combinations, classical approaches of interaction proteomics might not be sufficient.

Table 4. Functions of AMPAR interacting proteins identified in interaction proteomics studies with respect to AMPA receptor.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
<th>Function with respect to AMPAR</th>
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| 1 TARP γ2 | Cheng et al., 2001; Tomita et al., 2005; Kessels et al., 2009 | 1. Alters lateral diffusion via binding to PSD95.  
2. Regulates Surface expression and ER exit.  
| 2 TARP γ8 | Rouach et al., 2005; Sumioka et al., 2011; Zheng et al., 2005. | 1. Synaptic targeting via PSD95 binding.  
2. Promotes surface expression.  
| 3 Cnih2 | Schwenk et al., 2009; Herring et al., 2013; Boudkkazi et al., 2014 | 1. Facilitates surface expression and ER exit.  
2. Slows deactivation and desensitization. |
| 4 Shisa6 | Klaassen et al., 2016 | 1. Alters lateral diffusion of by binding PSD95.  
2. Prolongs deactivation and desensitization. No effect on recovery from desensitization. |
| 5 Shisa9 | von Engelhardt et al., 2010; Karataeva et al., 2014 | 1. Binds PSD95 and alters deactivation and desensitization.  
2. Reduces recovery from desensitization. |
| 6 Gsg1l | Shanks et al., 2012; McGee et al., 2015 | 1. Prolongs decay and recovery from desensitization of PSD enriched AMPA receptors.  
2. Reduces the single-channel conductance and calcium permeability of recombinant AMPARs while increasing polyamine-dependent rectification. |
| 7 Cpt1c | Gratacos-Batlle et al., 2015; Fado et al., 2015 | Modulates AMPAR surface levels and transmission in the ER. |
| 8 Porcn | Erlenhardt et al., 2016 | Regulates AMPAR and TARP γ8 levels in the ER. |
Proteomic methods to molecularly dissect the AMPAR interactome

A classical approach to identify the AMPAR protein interactome has been to perform pull down or immunoprecipitation (IP) experiments using multiple antibodies directed against different subunits of the AMPA receptor\textsuperscript{115}. In a seminal study, Schwenk and co-workers\textsuperscript{115} performed a knockout-controlled IP experiment from whole brain lysates extracted using different detergents to identify 34 high confidence proteins that stably associate with the AMPA receptor. This study was extended to identify and quantify brain region specific AMPAR differences in the interactome\textsuperscript{116,133}. Based on this data, they proposed a model of the AMPAR interactome based on the affinity of AMPAR interacting proteins, which poses that AMPAR interacting proteins are divided into inner core and outer core proteins. The model does not take into account the possibility that different AMPAR complexes might be present in very different stoichiometries based on other characteristics than affinity, such as regional expression, posttranslational modifications, etc. Another limitation of the model is that it assumes that the interacting proteins of the AMPAR are always organized in a similar fashion throughout the neuron; a view that is hard to conceive given the specific modalities of AMPAR function in the neuron. A novel model would ultimately use data of the existence of AMPAR subcomplexes based on biochemical data. Thus, this called for development of novel multi-dimensional proteomics strategies to discern protein subcomplexes. Obviously, this would assist in identifying the roles of these proteins and their modulatory effects on AMPAR function given the nature of their specific combinations.
Aim and scope of the thesis

The main aim of the thesis was to analyze and identify glutamate receptor complexes from the brain using different, novel, proteomics strategies. This study has the intention to lay the foundation for understanding the complex regulation of glutamate receptors, which manifests in many aspects of central nervous system functioning in health and disease.

First in Chapter 2, I aimed to create a comprehensive quantitative map of different synaptic subfractions isolated from cortex and hippocampus, to aid the interpretation of the subcellular localization of proteins and protein complexes. Using label free proteomics, I demonstrated that many neuronal proteins are enriched in specific biochemical fractions. This allowed identification of novel postsynaptic density proteins, which were further validated using super-resolution microscopy. This data set might be a resource to the synaptic research community.

In Chapter 3, I explored the interactomes of type-1 metabotropic receptors using an interaction proteomics strategy. A knockout control strategy was used to identify true interacting proteins of mGluR5 and to identify and verify mGluR1 as a binding partner of mGluR5 both in cortex and hippocampus.

In Chapter 4, I extensively analyzed AMPAR interacting protein complexes in the hippocampus. High-density reverse IP experiments were used to provide an overview of AMPA receptor subcomplexes including new insights into constituents of distinct AMPAR complexes. In addition, we developed novel IPBN-MS (Immunoprecipitation combined with Blue native page and Mass spectrometry) as a strategy to separate protein subcomplexes based on their molecular weights, a universally applicable method to discern protein subcomplexes from native tissue. Proteins from a number of subcomplexes of the AMPAR were analyzed using super-resolution microscopy, showing that these distribute over different subcellular compartments. This study forms a framework for interaction proteomics strategies in the future and may guide studies into AMPAR regulation by auxiliary proteins in complexes of different protein composition.

In Chapter 5, I focus on a previously uncharacterized secreted AMPAR interacting protein, Noelin1, to assess its role in AMPA receptor function. Using a variety of tools, such as interaction proteomics, surface plasmon resonance and heterologous expression I characterized the direct interaction between Noelin1 and AMPA receptors. To functionally characterize the interaction, electrophysiology, super-
resolution microscopy and single particle tracking analysis were performed. In particular, we determined the effect of Noelin1 on AMPA receptor lateral diffusion.

Finally, in Chapter 6, I summarize the results of our interaction proteomics strategies to characterize glutamate receptor complexes and I discuss the implications, challenges and the future of these.
Chapter 2

Hidden proteome architecture of the synapse revealed by correlation profiling of proteins in biochemical sub-fractions of the mouse brain

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Abstract

Synaptic proteins are main organizers of synaptic function. Here we performed a quantitative proteomics analysis of five different biochemical fractions generated from synaptic preparations isolated from mouse hippocampus, cortex and cerebellum. Using mass spectrometry, approximately 2000 proteins were identified and quantified in each fraction. Subsequent correlation profiling analysis confirmed the co-occurrence of several well-known pre- and postsynaptic proteins. Various AMPA receptor interactors were found differentially enriched in synapse sub-fractions suggestive of the existence of distinct AMPA receptor subcomplexes. In addition, a correlation profiling approach suggested that two proteins, Adgra1 and Plekha5, are corelated with canonical PSD constituents. Using super-resolution microscopy their localization in the PSD of hippocampal primary neurons was confirmed.
Introduction

Neuronal synapses are functional units for fast signal transmission, and integration. In addition, activity-dependent plasticity processes take place, which are at the basis of neuronal cellular and circuitry adaptation, such as those becoming apparent in learning and memory\textsuperscript{46,142}. Understanding synaptic transmission and plasticity requires insight in the proteins that sub serve these processes. To comprehend the molecular diversity in various synapse subdomains and organelles\textsuperscript{47,48}, to identify multi-protein assemblies\textsuperscript{116,143}, and to capture adaptations that occur during plasticity\textsuperscript{142}, mass spectrometry-based proteomics has been frequently applied. In an ideal experiment a morphologically defined synaptic domain, e.g. an endosome or post-synaptic membrane, would be biochemically isolated from selected brain tissue and would yield a specific set of proteomics data of the domain’s protein constituents. A well-known caveat of this is that, whereas the biochemical fractionation enriches proteins of the targeted subcellular compartment, the distinct biophysical properties of proteins combined with the specific isolation procedure can also harness proteins from different subcellular structures. In particular, the choice of detergents and sequential centrifugation steps in biochemical extraction procedures determine the outcome of such an experiment. Here we reasoned that proteins, being part of complexes and sharing physical properties or subcellular environments, are likely co-isolated. Multidimensional separation followed by correlation profiling potentially yields increased power to detect co-isolated proteins that are part of the same biophysical environment, e.g. subcellular structure, or take part in the same complex.

To test this idea and to reveal the biochemically defined proteome architecture by correlation profiling in synaptic preparations of the mouse brain, we performed a comprehensive quantitative proteomics analysis on five sequentially isolated synapse sub-fractions. Unlike previous studies\textsuperscript{45,48}, we isolated these fractions from three distinct brain regions; namely the hippocampus, the cortex and the cerebellum. These brain regions are functionally different, have divergent protein expression profiles and demonstrate protein expression dependent divergence in biochemical pathways\textsuperscript{133,144}. Indeed, correlation profile analysis of proteins across the synapse sub-fractions showed the expected co-enrichment of canonical pre- and postsynaptic proteins. We then used correlation profile analysis to derive proteins that co-isolated with these. The analyses suggested the presence of distinct receptor subcomplexes, and indicated novel synaptic proteins, two of which the postsynaptic localization was confirmed by super-resolution microscopy on primary hippocampal neurons.
The comprehensive dataset of a total of 4000 proteins identified from synaptic sub-fractions together provides a novel resource to interrogate proteins in hippocampus, cortex, and cerebellum with correlated biochemical extraction properties.
Results

First we generated three biological replicates of five distinct biochemically isolated fractions from the hippocampus; namely 1) hippocampal microsome, 2) P2, 3) synaptosome, 4) synaptic membrane and 5) postsynaptic density as shown in Supplementary Fig. S1. The proteomics analyses of these five biochemical fractions were performed using label free quantification. We first assessed the biological variation in the three replicates of the biochemical fractions (Fig. 1). Three replicates (Batch1, 2, 3) of synaptic membrane (SYM) fractions yield an $R^2$ of ~0.9 (Fig. 1a), which is representative for all synapse sub-fractions (Supplementary Fig. S2). Overall, the mean co-variation (CV) of each of the synaptic fractions is 0.3 (Fig. 1b); this variation is typical for data-dependent analysis, as reported previously for other biological systems.145

In this study 4162 proteins were identified in total. The dynamic range of intensities of the 2000 proteins identified in each fraction spans at least 4 orders of magnitude (Fig. 1a). To investigate the quantitative distribution of each protein across the synapse sub-fractions, we used the rowsum normalization approach, i.e., for each protein, its summed MS1 intensities across all fractions were taken as 100% and the individual fractions were calculated as percentage thereof. As a measure of abundance of each protein, iBAQ protein values were calculated as an in-silico approximation of absolute protein abundances.146 For each protein, we divided its intensity (the sum of all assigned peptide MS1 peak intensities) by the number of theoretically observable peptides. The complete list of proteins with iBAQ values and subfraction distribution are shown in supplementary Table S1.

To ascertain good fractionation of our synaptic preparations, we looked at canonical pre- and postsynaptic proteins in our hippocampal dataset.15,27 The presynaptic compartment contains protein machinery that drives and regulates release of synaptic neurotransmitter vesicles, and the retrieval and reloading of these. Several proteins were highly abundant with iBAQ values >10000 (Fig. 3a), including Syn1, Snap25, Vamp2, Syp, Stxbp1, Stx1b, Rab3a, Syt1 and Atp6vod1. Proteins reported to be present in subsets of synaptic vesicles, or involved in alternate releasing pathways, as Vamp4, Snap29, Vti1, as well as exocytosis regulators Stxbp5 and Stxbp5l were present at >100 fold lower amount than these canonical presynaptic proteins (Fig. 2a). The distribution patterns of the pre-synaptic proteins are in agreement with their known subcellular localization. Compared to the P2 fraction, these proteins showed increased enrichment in synaptosome to synaptic membrane, and were decreased in the PSD.
fraction to the level of being hardly detectable. Of these, Syn1 and Rab3a were the exceptions. Syn1 has been reported in the PSD preparation, which is attributed to its non-specific binding to PSD\textsuperscript{44}. Furthermore, nearly all presynaptic proteins were also enriched in the microsome fraction as compared to the P2. Thus, the presynaptic proteins show biphasic enrichments in microsome and synaptosome (Fig. 2a).

The PSD is the main location site of ionotrophic glutamate receptors, which are partially held in place by various postsynaptic scaffold proteins. Due to its compact structure with tight protein-protein interactions the PSD is not soluble in regular non-ionic detergent, such as Triton-X 100. Therefore, the Triton X-100 insoluble fraction of the synaptosome or synaptic membrane input is generally referred to as PSD proteins. When compared to the P2 fraction, the classical PSD proteins were indeed highly enriched in the PSD fraction, with very low level in synaptosome or synaptic membrane and almost non-detectable in the microsome fraction (Fig. 2b). For example, the canonical PSD residing NMDA receptor is represented by subunits Grin2a and Grin2b, 85% and 78%, respectively. Similarly, the scaffold proteins were highly enriched. For example, Dlg2, -3, and -4 were present at 73%, 67% and 66%, respectively, in the

Figure 1. Reproducibility of the measurement. a) Correlation plot of 3 biological replicates (batch1_HC_SYM, batch2_HC_SYM, batch3_HC_SYM) of synaptic membrane fractions from hippocampus. X- and Y-axis indicate log10 protein iBAQ abundances. Diagonally opposite boxes indicate the regression value for each pairwise comparison. b) Box-whisker plots denoting coefficient of variation in three biological replicates of different fractions of mouse hippocampus. H: homogenate, M: microsomes, P2: pellet 2, SYN: synaptosome, SYM: synaptic membrane, PSD: postsynaptic density; n: number of quantified proteins.
PSD. Dlg1/Sap97 is a major exception with only 12% present in PSD; it was found enriched in the synaptosome, synaptic membrane and microsome fractions (23%, 22%, 26%, respectively; Fig. 2b). The PSD scaffolding proteins Dlg2, -3, -4 were the most abundant proteins, present with several fold higher amount than the NMDA receptor subunits, which in turn were about tenfold more abundant than kainate receptor subunits. The non-PSD-enriched Dlg member Dlg1 was more than tenfold less abundant than the other Dlg members. The guanine nucleotide exchange factor lqsec1 has recently been reported to have a PSD localization. Whereas lqsec1 is indeed a PSD protein, being 43% present in PSD (Fig. 2b), it also has a wider distribution than the typical PSD proteins across all fractions.

To illustrate the power of correlation profiling, we used the top 30 most correlated proteins with the well-established synaptic vesicle protein Sv2b. The top most abundant proteins in this list are Syt1, Atp6v0d1 and Dnaj5. Syt1 is regulated by direct interaction with Sv2b, Atp7v0d1 is a constituent of the synaptic vesicle proton pump, while Dnajc5 is a key protein involved in the synaptic function mediating exocytosis and regeneration of synaptic proteins. Lower down the list, proteins such as Rab3d, Sept, Rab14 and Rab1a represent sets of GTPase proteins involved in exocytosis of vesicles. Thus, the top most profile correlated proteins with Sv2b represent proteins that are functionally and subcellularly related to it.

Correlation profiling using Dlg4 yields a top-30 group that contains many of the classical PSD proteins, such as Syngap1, Grin2b, Grin1, Homer1, Dlgap2 (Fig. 3b). These proteins are observed at very low level in synaptosome and synaptic membrane fractions and are almost not detectable in the microsome fraction (Fig. 3b). For example, the typical PSD-localized NMDA receptor subunits Grin2b and Grin1 were present in the PSD fraction with a correlation equal to 1, but are present in the synaptosome and synaptic membrane fraction as well, with 81% and 77% in the PSD fraction, respectively. Similarly, the scaffolding proteins Dlg2, -3 and -4 were present in PSD at 79%, 77% and 75%, respectively.
Figure 2. Representation of canonical synaptic proteins in hippocampus: Rowsum normalized heatmap profile for known presynaptic proteins from Chua et al (2010)\textsuperscript{27}, Sudhof (2012)\textsuperscript{15}, Morciano et al (2009)\textsuperscript{147} (a) and postsynaptic proteins (b) arranged in decreasing order of their IBAQ abundance. Numbers in boxes indicate fraction of total protein abundance in the preparation. Numbers beside protein names indicate IBAQ abundance. Scale bar: rowsum normalized protein intensity
Figure 3. Correlation profiling using presynaptic Sv2b and postsynaptic Dlg4 as bait. Rowsum normalized heatmap profile for the top 30 most correlated proteins with a) Sv2b and b) Dlg4. Numbers in boxes indicate fraction of total protein abundance in the preparation. Numbers beside protein names indicate summed IBAQ abundance in all fractions for a protein. Scale bar: rowsum normalized protein intensity.
Figure 4. Enrichment of AMPA receptor interacting proteins\textsuperscript{115,116,133} in synaptic subfractions. Correlation profile of rowsum normalized data for AMPA receptor interacting proteins in the hippocampus (a), cortex (b) and cerebellum (c). Numbers in boxes indicate fraction of total protein abundance in the preparation. Numbers next to protein names indicate IBAQ abundance. Scale bar: rowsum normalized protein intensity.
Chapter 2: Proteome architecture of the synapse

Brain region specific differences across biochemical fractions

Whereas basic machineries for synaptic neurotransmission generally are thought to be conserved, brain region specific alteration of synaptic protein abundancy may underlie, at least in part, its functional specification. To assess brain region-specific synapse sub-fraction proteomes, we analyzed, besides hippocampus, samples from cortex and cerebellum. The canonical presynaptic proteins in cortex and cerebellum also show enrichment in the synaptosome and depletion in the PSD fraction, while the canonical post-synaptic proteins are highly enriched in the PSD fraction, while being low expressed in other fractions. In particular, when examining IBAQ values, the abundances of the top most abundant proteins is conserved across all three brain regions (Supplementary Fig. S3a-d) likely indicating a conservation of the stoichiometry of proteins of the basic presynaptic machinery across brain regions.

Although the core synaptic machinery remains conserved across the brain regions, we hypothesized that complexes known to have a specialized functions may show brain region specificity, reflecting the distinct functionalities between brain regions. To examine this, we investigated the relative abundance over the different fractions of the AMPA receptor and its auxiliary proteins, which are known to regulate AMPA receptor localization and channel properties, features that underlie neuroplasticity. For this, we selected proteins known to be present in AMPA receptor interactome.

With respect to AMPAR subunits themselves, we observed that Gria1 and Gria2 showed distinct subcellular distribution patterns and exhibited different enrichments in the PSD in the hippocampus versus the cortex and cerebellum (Fig. 4a-c). Whereas Gria1 and Gria2 showed strong enrichment in the PSD fraction in cortex and cerebellum (> 65%) and were highly correlated with Dlg4 (> 0.9), they were less PSD-enriched in the hippocampus. Furthermore, IBAQ values for AMPAR subunits demonstrate brain region specific expression patterns, in which Gria4 abundance is almost equivalent to Gria1 and 2 in the cerebellum, and is much less abundant in hippocampus and cortex.

In the hippocampus, the AMPA receptor interactors exhibited highly variable distribution patterns across subfractions (Fig. 4a). Four remarkable features can be mentioned about the known AMPA receptor interactors. (1) The PSD-enriched group consists of Cacng2 and 3, Lrrtm4, Olfm2, Shisa6 and 7. (2) The microsome-enriched group consists of Abhd6 and 12, Cpt1c, Frrs1I and Sacm1I. Interestingly, the microsome-enriched group of AMPA receptor interacting proteins was highly correlated
with several ER markers, such as Ergic1 and Wfs1. (3) A group that is synaptic membrane-enriched and of which members may also be present in other fractions consisted of Prrt1, -2 and -3, Gsg1l. (4) A set that was slightly enriched in the PSD fraction, but also present in other fractions, was Cnih2 and Olfm1. Together, this suggests that, based on the distribution pattern of AMPA receptor interactors, there may be multiple AMPA receptor complexes located in different neuronal subdomains.

With respect to brain region specific differences in AMPAR interacting proteins, as demonstrated in Chen et al, 2014, we observed specific interacting proteins more abundant in subcellular fractions in different brain regions. For example, Cnih2 is abundantly expressed in the PSD and microsome fraction in hippocampus and cortex, respectively, whereas it is non-detectable in cerebellum. Similarly, Lrrtm4 and Frrs1l showed absence in the PSD fraction in cerebellum, largely reflecting brain region specific differences in expression. Interestingly, Cacng8, a highly abundant AMPAR interactor in the cortex and hippocampus is much more correlated with Dlg4 in the cortex than in the hippocampus, just like is the case for the AMPAR receptor subunits Gria1 and Gria2.
Discovery of proteins new to a subdomain

Given that the PSD is typified by a high correlation of proteins, this infers that correlation profiling could be used to reveal novel PSD proteins. To explore this possibility, we focused on the profile of Grina to reveal PSD-residing proteins. Hundreds of proteins have a very high correlation with the distribution pattern of Grin2a (Fig. 5a), implicating them to be PSD proteins. Table 1 highlights proteins with correlation with Grin2a of >0.99. The dataset showing correlation of all proteins is shown in supplementary Table S2.

Table 1: Proteins having a higher than 0.99 correlation score with respect to Grin2a. The table classifies 223 potential PSD-localized proteins indicated by their gene name, their relative abundance over 5 biochemical fraction, and their corresponding correlation with Grin2a. This potential set of PSD proteins contains the previously non-detected Adgra1 (row 63) and Plekha5 (row 175).

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Figure 5: Discovering novel PSD-enriched proteins using correlative expression profiling of Grin2a. a) Top 500 most co-related proteins with Grin2a in hippocampal fractions. Note that Table 1 shows a portion of these proteins. Scale bar: rowsum normalized protein intensity.
Proteins with the highest correlation with Grin2a are typical scaffold proteins residing in the PSD, such as Shank, Homer1 and Dlg proteins. PSD proteins that regulate synapse morphology ranked also high in the list, including Adgra1 (also known as Gpr123), an adhesion G-protein coupled receptor. Adgra1 is exclusively found in the PSD fraction with an IBAQ value 300 fold lower than Gria1, implicating that its copy number in the PSD fraction is low, and it might have been missed in previous studies because of its low abundance level. Another putative PSD protein is Plekha5 (Fig. 5b). It has been identified from the human PSD, but validation of its PSD localization was not yet reported.

**Validation of novel PSD proteins by super-resolution microscopy**

To confirm that the subcellular localization of Adgra1 and Plekha5 is the PSD, we performed super-resolution structured illumination microscopy (SIM) for these proteins by co-staining with a well-known PSD marker, Homer1. We observed excellent overlap between Adgra1 with Homer1 in primary hippocampal cultures (Fig. 6a, e). Also, line scan analysis on synapses showed nearly perfect co-localization of Homer1 and Adgra1 (Fig. 6b, c). We also quantified the percentage of Adgra1 positive synapses, which revealed a 96.54% of Homer1 positive puncta for Adgra1. Plekha5 also shows excellent co-localization with Homer1, but is present in a fraction of ~50% of synapses.
Figure 6. Super-resolution imaging microscopy validation of novel PSD-enriched proteins, Plekha5 and Adgra1. a,e) Left panel shows SIM imaging of primary cultured hippocampal neurons at DIV19 for Plekha5 and Adgra1 (green) along with Gria2 (red) and Homer1 (blue), the latter as a marker for the PSD. Right panel shows zoom-in of the marked area (scale bar, 1 µm). b,c) Line scan analysis on inset from panels a and e shows co-localization between the three proteins at postsynaptic sites. d) Bar graph showing percentage (mean±sem) of Homer1 puncta (PSD localization) that are positive for Plekha5 (dark green, 48.61 ± 4.92, n = 10), Adgra1 (light green, 96.54 ± 1.02, n = 7) and Gria2 (red, 89.20 ± 2.08, n = 17)
Discussion

The present proteomics analysis revealed the quantities of synaptic proteins in biochemically defined sub-fractions of three brain regions. The samples showed high reproducibility with respect to biological replicates. With respect to canonical proteins of the pre- and postsynapse, we observed high enrichment of presynaptic proteins in the synaptosome and synaptic membrane fractions, whereas PSD proteins were enriched in synaptosome with respect to P2 fraction, and were most enriched in the PSD fraction, which validates the quality of our biochemical preparations and the downstream proteomics pipeline.

We found that proteins known to be part of a functional entity follow a similar distribution pattern over the synapse sub-fractions, e.g., protein machineries for neurotransmitter release are enriched in synaptosome or synaptic membrane and microsome fractions, whereas protein machineries that receive and transduce the transmitter signaling are found mainly in the PSD fraction. With respect to the brain region specific expression of canonical synaptic proteins, we cannot comment on direct comparisons of the protein abundances between brain regions because of the lack of absolute quantification. We did however observe small differences in abundance ratios and expression across brain regions. For example, by arranging the canonical proteins in the order of their IBAQ values across different brain regions, we observed that the order remained largely conserved (Fig. 3a,b, Supplementary Fig. S4). This indicates that ratiometric these proteins appear unchanged compared with each other, and therefore likely the mechanisms of synaptic release they are taking part in have remained well conserved across brain regions.

Correlation profiling suggested the presence and spatial segregation of protein subcomplexes. For example, proteins auxiliary to the AMPAR may exist in different composition in various synapse sub-domains. A strong indicator of this is the segregation of the majority of AMPAR interacting proteins in mainly two domains, the microsome-enriched proteins, such Frrs1l, Sacm1l, Cpt1c and the synaptic-enriched group comprising of Cacngs, Shisas, and Olfms. Given that the synaptic functions of the TARPs (Cacng family) and Shisa family of proteins are becoming increasingly clear, the discovery of the non-synaptic enriched AMPAR complexes allow us to suggest a role for these proteins in endoplasmic reticulum and Golgi apparatus such as that for Cpt1c103.

The IBAQ values provided an indication of the protein relative abundancies in each of the synapse sub-fractions. We demonstrated that the core proteins (Vamp2,
Syn1, Syt1, Stxbp1) in a synapse sub-fraction, such as the synaptosome and synaptic membrane fraction, generally were the most abundant ones. Correlation profiling can be extended to reveal novel synaptic proteins, by virtue of their high correlated expression over fractions with known PSD-localized proteins. Doing so, we found two additional PSD proteins, Adgra1 and Plekha5.

Adgra1 (Gpr123) is present on chromosome 10q26.3 and is a 7-TM domain containing protein belonging to the adhesion family of G protein-coupled receptors (GPCRs). It is predicted to have a PDZ binding domain in the C-terminal of the protein. However, it is the only member of the adhesion GPCRs that lacks a cleavable GPCR auto-proteolysis–inducing (GAIN) domain. Due to its widespread expression in the brain, it is suggested that Gpr123 may have an important role in the regulation of neuronal signal transduction. It has been reported in complex with PSD95, which is in line with its localization within the PSD. Further research on Gpr123 should elucidate the mechanisms in which it is involved.

Plekha5 is a cytosolic protein belonging to the PLEKHA family and is a Pleckstrin Homology (PH) Domain containing protein that is involved in binding with phosphatidylinositol (3, 4, 5)-trisphosphate (Pip3). Previous PSD protein identification studies from human postsynaptic densities have identified Plekha5 to be present in PSD preparations, but its PSD localization using immunohistochemical approaches was not yet reported. Interestingly, Plekha5 belongs to chromosome 12p12, and SNPs associated with this locus are associated with early-onset bipolar disorder. Like other PH domain containing proteins, Plekha5 might get recruited to the plasma membrane upon PIP3 formation in the postsynaptic compartment, however, the exact role that it plays in the PSD remains to be determined.

In conclusion, in this study we provide a valid resource of synapse sub-domain proteomes for neuroscientists. This database can be interrogated in different ways that may help to generate hypothesis regarding novel protein localization related to synapse function.
Materials and Methods

Preparation of synapse subfractions

All animal experiments were performed in accordance with relevant guidelines and regulations of the VU University. The animal ethics committee of the VU University approved the experiments.

Subcellular fractions were prepared from 3-month-old C57BL6 mice as described previously with minor modifications (Supplementary Fig. S1a). In brief, mouse hippocampi, cortex and cerebellum were dissected and stored at -80 °C until used. The brain regions were pottered separately in homogenization buffer (0.32 M Sucrose, 5 mM HEPES pH 7.4, Protease inhibitor cocktail (Roche)) on a dounce homogenizer (potterS; 12 strokes, 900 rpm) and spun at 1000xg for 10 min at 4 °C. Supernatant 1 (S1) was centrifuged at 20,000xg for 20 min to obtain pellet 2 (P2) and supernatant 2 (S2). The S2 fraction was ultracentrifuged at 100,000xg for 2 h; the pellet was recovered as microsomal fraction. S1 was subjected to ultracentrifugation in a 0.85/1.2 M sucrose density gradient at 100,000xg for 2 h. Synaptosomes were recovered at the interface of 0.85/1.2M sucrose. The hypotonic shock of synaptosomes in 5 mM HEPES pH 7.4 with protease inhibitor for 15 min yielded the synaptic membrane fraction, which was subsequently isolated by sucrose gradient ultracentrifugation as stated above at the interface of 0.85/1.2M fraction. To obtain the PSD, the synaptosome fraction was extracted in 1% Triton X-100 for 30 min, layered on top of 1.2/1.5/2M sucrose, centrifuged at 100,000xg for 2 h, and recovered as PSD-I at the interface of 1.5/2M sucrose. PSD-I was subjected to second extraction in 2% Triton X-100 for 30 min, subjected to sucrose gradient ultracentrifugation as stated above, and recovered at the 1.5/2M sucrose interface. The PSD-II fraction was then pelleted in 5 mM HEPES pH 7.4 by centrifuging at 100,000xg for 30 min.

Gel separation and in-gel digestion:

Unless otherwise stated, 2.5 ug of each fraction was dissolved in Laemmlli buffer and boiled at 98°C for 5 min. 5 μl 30% acrylamide was then added and vortexed for 30 min. at room temperature to form a fixed modification of Cys-S-beta-propionamide. Samples were run on a 10% SDS-PAGE gel, which was stopped when the front reached halfway of the gel. The gel was fixed overnight in 40% ethanol / 3% phosphoric acid, and stained briefly for about 30 min with colloidal coomassie blue. Each lane was cut into two slices, chopped into 1 mm by 1 mm pieces followed by a sequential incubation in
50% acetonitrile/ 50 mM NH$_3$HCO$_3$ – 100% acetonitrile – 50 mM NH$_3$ HCO$_3$ - 50% acetonitrile/ 50 mM NH$_3$HCO$_3$ – 100% acetonitrile as described previously$^{133}$. The gel pieces were dried in a speedvac, rehydrated in trypsin solution in 50 mM NH$_3$HCO$_3$ (500 ng per gel slice) at 37 °C overnight, extracted with 200 µL 0.1 M acetic acid, and the supernatant was transferred to an Eppendorf tube and dried in a speedvac. The tryptic peptides were dissolved in 17 µL 0.1 M acetic acid and analyzed by LC-MS/MS.

**MS acquisition and data analysis**

Peptides were analyzed by nano-LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific) coupled to the TripleTOF 5600 mass spectrometer (Sciex). Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 µm i.d., 5 µm particlesize, from Dionex) and fractionated on a 200 mm Alltima C18 column (100 µm i.d., 3 µm particle size). The acetonitrile concentration in the mobile phase was increased from 5 to 30% in 90 min, to 40% in 5 min, and to 90% in another 5 min, at a flow rate of 500 nL/min. The eluted peptides were electro-sprayed into the TripleTOF MS. The nano-spray needle voltage was set to 2500V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (m/z 350–1200, 250 ms) followed by a top 25 MS/MS (85 ms per MS/MS, precursor ion > 90 counts/s, charge state from +2 to +5) with an exclusion time of 16 s once the peptide was fragmented. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 10 eV.

The MS raw data were imported into MaxQuant (version 1.5.2.8)$^{156}$, and searched against the UniProt mouse proteome (SwissProt+Trembl February, 2016 release) with Cys-S-beta-propionamide as the fixed modification and Methionine oxidation and N-terminal acetylation as variable modifications. For both peptide and protein identification a false discovery rate of 0.01 was set, MaxLFQ$^{30}$ normalization was enabled with a LFQ minimal ratio count of 1. The minimal peptide length was set to 6, further MaxQuant settings were left at default. The MaxQuant search results are provided in Supplemental Table S1_IBAQ Abundances.

Proteins identified from the MaxQuant contaminant FASTA database were removed. External contaminants, ribosomal and nuclear proteins were identified and excluded from downstream analysis by matching a case insensitive regular expression to the fasta headers of each protein group: “ig \S+chain\keratin\GN=(try|krt|igk|igg|igkv|ighv|ighg|nduf|hist|rpl|rps|hnrn)".
Next, we collapsed protein groups that shared the same gene name. All (majority) protein accessions in a protein group were matched against the fasta database, their gene names extracted from the fasta headers and the set of unique gene names for each protein group was stored. The protein abundance matrix was built by summation of MaxLFQ normalized protein intensities of protein groups that map to the same unique set of genes. These protein intensities were converted to iBAQ pseudo-absolute abundances using the number of digestible peptides provided by MaxQuant. Finally, we scaled the columns to account for differences in the size of each subcellular fraction (e.g. synaptosomes are a subset of the P2 fraction). The scaling factor is the total protein amount in each sample divided by the mean of all sample totals. This data is provided in Supplemental Table S1_Scaled Abundances.

**Immunostaining of primary neurons**

Primary hippocampal and cerebellar neurons were obtained from E18 rat pups as described previously\textsuperscript{157,158}. Briefly, 18,000 cells were grown in neurobasal medium supplemented with B27 on poly D-Lysine coated coverslips. The cells were used for staining at DIV14-16. The coverslips were fixed with ice-cold methanol for 10 min, followed by three washes in ddH\textsubscript{2}O and PBS. The neurons were then blocked and permeabilised with blocking buffer (5% FCS, 0.1% Triton X-100, and 0.1% Glycine in phosphate buffer saline, pH 7.4) for 1 h. Next, the neurons were incubated with anti-Gpr123 (1 in 250, cat. no. sc-390311, Santacruz) or anti-Plekha5 (1 in 250, cat. no. sc-390311, Santacruz) and anti-Gria2 (1 in 500, cat. no. 182 103, Synaptic systems) and anti-Homer1 (1 in 1,000, cat. no. 160 004, Synaptic systems), diluted in blocking buffer overnight at 4 °C. After three times washing in PBS, the cells were incubated with an Alexa-conjugated secondary antibodies for 1 h at room temperature (anti-mouse Alexa 488 (1 in 1,000), anti-goat Alexa 488 (1 in 1,000), anti-rabbit Alexa 568 (1 in 1,000), anti-Guinea pig Alexa 647 (1 in 1,000) (Molecular Probes) and subsequently washed and fixed on glass slides (Superfrost Plus, Thermo) using Moviol. Images were taken using a LSM Elyra SIM microscope with 63x Oil immersion lens (N.A. 1.4) and analyzed using ImageJ. Line scan analysis was performed as described previously\textsuperscript{157}.

**3D-SIM microscopy**

Imaging was performed using a Zeiss Elyra PS1 system. 3D-SIM data was acquired using a 63x 1.4NA oil objective. Diode lasers (488, 561, 642 100 mW) were used to excite the fluorophores together with, respectively, a BP 495-575 + LP 750, BP 570-650 + LP 75 or LP 655 excitation filter. For 3D-SIM imaging a grating was present in
the light path. The grating was modulated in 5 phases and 5 rotations, and multiple z-slices were recorded with an interval of 110 nm on an Andor iXon DU 885, 1002x1004 EMCCD camera. Raw images were reconstructed using the Zeiss Zen software.

Reconstructed 3D-SIM images were analyzed with imageJ\textsuperscript{159} extended in the FIJI framework\textsuperscript{160}. Analysis was performed on maximum projections of the z-stack. A threshold, determined by the moments algorithm\textsuperscript{161} was applied on the Homer1 signal. Particles larger than 10 pixels were detected and marked as region of interest (ROI) and mean Gria2 and Adgra1 or Plekha5 signals inside the ROIs were measured. A Homer1 particle was counted as positive for Gria2 and/or Adgra1 or Plekha5 if their mean intensity was more than three times above their respective backgrounds. For analysis of cerebellar staining, we used PSD-95 as a PSD marker, instead of Homer1 and analysis was done as stated above.

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Author Contributions

N.J.P. performed the experiments. N.J.P., F.K., A.B.S. and K.W.L. wrote the manuscript; F.K. and N.J.P. performed the data analysis of MS data. J.A.S and N.J.P performed data analysis of imaging data. N.J.P, F.K., K.W.L designed the experiments. A.B.H, A.B.S. and K.W.L. supervised the research. All authors reviewed the manuscript.

Additional Information

Smit holds shares in Alea Biotech BV. The author(s) declare no competing financial interests.
Supplementary figures and legends (Fig. S1-S3)

Supplementary Figure S1. Schematic of the biochemical isolation of subfractions of mouse brain regions. Frozen dissected mouse brain tissue from hippocampus, cortex and cerebellum was homogenized to obtain homogenate, which was spun to obtain pellet 1 (P1) and supernatant 1 (S1). S1 was further centrifuged to obtain P2 and S2, or it underwent sucrose density centrifugation to obtain the synaptosome fraction. S2 was centrifuged at 100,000xg, to obtain the microsome fraction. Synaptosomes were further osmotically shocked and again subjected to sucrose density centrifugation to obtain synaptic membrane fraction or doubly extracted in Triton X-100 to obtain the PSD fraction.
Supplementary Figure S2: Correlation plots of 3 biological replicates of hippocampus (batch1_HC, batch2_HC, batch3_HC) of a) microsome (M), b) pellet 2 (P2), c) synaptosome (SYN), and d) postsynaptic density (PSD) fractions from hippocampus. X- and Y-axis indicate log10 protein iBAQ abundances. Diagonally opposite boxes indicate the regression value for each pairwise comparison.
Supplementary Figure S3: Rowsum normalized heatmap profile for canonical pre- and postsynaptic proteins in cortical (a, b) and cerebellar (c, d) subcellular fractions. Numbers in boxes indicate fraction of total protein abundance in the preparation. Numbers beside the protein names indicate IBAQ abundance. Scale bar: rowsum normalized protein intensity.
Chapter 3

Group 1 metabotropic glutamate receptors 1 and 5 form a protein complex in mouse hippocampus and cortex

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Abstract

The group 1 metabotropic glutamate receptors 1 and 5 have been implicated in mechanisms of synaptic plasticity and may serve as potential therapeutic targets in autism spectrum disorders. The interactome of group 1 mGluRs has remained largely unresolved. Using a knockout-controlled interaction proteomics strategy we examined the mGluR5 protein complex in two brain regions, hippocampus and cortex, and identified mGluR1 as its major interactor in addition to the well-described Homer proteins. We confirmed the presence of mGluR1/5 complex by (1) reverse immunoprecipitation using an mGluR1 antibody to pulldown mGluR5 from hippocampal tissue, (2) co-expression in HEK293 cells followed by co-immunoprecipitation to reveal the direct interaction of mGluR1 and 5, and (3) super-resolution microscopy imaging of hippocampal primary neurons to show co-localization of the mGluR1/5 in the synapse.
Introduction

Group 1 metabotropic glutamate receptors are G-protein coupled receptors comprising the closely related mGluR1 (GRM1) and 5 (GRM5), where mGluR1 exists in two isoforms differing only at the C-termini due to alternative splicing of the gene\(^{162}\). Metabotropic GluR5 is primarily localized at the postsynapse, where it regulates short- and long-term synaptic plasticity, in particular long-term depression\(^{163}\). Activation of mGluR1/5 leads via Gq/G\(_{11}\)-proteins to the initiation of phospholipase-C\(_{\beta}\) thereby eliciting IP3 and diacylglycerol signaling\(^{62}\). In addition, mGluR1/5 activation can lead to the activation of MAPK/ERK and MTOR/p70 S6 kinase, which are involved in synaptic plasticity\(^{62,164}\). Non-synaptic mGluR5 also exists, and was recently shown to activate different signaling systems than synaptic mGluRs\(^{165}\). Negative or positive allosteric modulators of mGluR5 have therapeutic potential for a number of brain disorders including fragile X mental retardation and schizophrenia\(^{166}\). Accordingly, mGluR5 is considered a promising drug target aimed at alleviating various neurological and psychiatrically disorders by pharmacological intervention of the receptor activity\(^{62,65,167}\).

Besides G-proteins, also phosphatases (PP1\(_{\gamma}\))\(^{168}\), kinases (PKC)\(^{169}\), scaffolding proteins (NHERF-2, Tamalin/GRASP)\(^{74,170}\) and ion channels (Grid2)\(^{75}\), have been reported as being part of the group 1 mGluR signaling complexes, and driving diverse cellular processes, such as, subcellular localization and Ca\(^{2+}\) responses presumably in a brain region-specific manner. mGluR5 is abundantly present in the hippocampus and mGluR1 has a higher expression in cerebellum\(^{171}\). As functions of mGluRs critically depend on their interacting proteins, it is important to elucidate the constituents of these protein complexes. Attempts have been made to elucidate the mGluR1/5 interactome, but with variable success due to the use of only an in vitro model and/or ambiguity of some interactors\(^{172,173}\).

In the present study, we used a knockout controlled interaction proteomics analysis to examine the interactome of mGluR5 in hippocampus and cortex. Apart from the known interacting proteins, Homer1-3, two mGluR1 isoforms, mGluR1a and 1b, form a complex with mGluR5. We confirmed the presence of the mGluR5-1a/b complex in hippocampus by reverse immunoprecipitation, revealing the direct interaction of mGluR1/5 by co-immunoprecipitation from HEK293 cells co-expressing both receptors, and demonstrating the co-localization of mGluR1/5 at the postsynapse by structured illumination super-resolution microscopy (SIM) imaging.
Results and Discussion

In the present study, we employed interaction proteomics to reveal the mGluR5 interactome. Generally, antibody-based interaction proteomics experiments are noisy, i.e. they are populated by consistently present background protein contaminants as well as by a large number of proteins that appear sporadically in the individual IPs. Here, we used an mGluR5 knockout mouse as a negative control to remove false positives, which in addition should address the problem of (potential) cross-reactivity of the antibody. We carried out multiple IP replicates to filter out the sporadically occurring proteins. As we focused on stable mGluR complexes, the protein constituents of the complexes should be present in most, if not all, IPs. We took a stringent filter to select interacting proteins, which must be present in ≥ 3 out of 4 IPs from WT samples and have an enrichment factor of ≥10 fold. From the approximately 300 identified proteins in the mGluR5 IPs from extracts of hippocampus and cortex (excluding external contaminants, such as keratins, antibodies, trypsin and bovine proteins), 14 proteins pass this filter (Table 1). The bait protein mGluR5 has the highest intensity, followed by Homer1 and mGluR1. mGluR1 is present at a much lower amount than mGluR5, suggesting that a fraction of mGluR5 protein complex contains mGluR1. Proteins of lower intensity in the IP table mostly represent high abundant proteins in the original extract materials, notably mitochondrial proteins and proteins involved in energy metabolism, both of which are often considered as the major sources of contaminants in typical IP experiments. In case a protein passed the filter in one brain region (for example Rab3gap1 and Atp1a2), but was detected in KO samples from another brain region at a level of those of WT, they may present false positives and therefore should be considered with caution (see also Table 2)

The present, as well as our previous studies, identified 200-300 proteins from a single IP. To reveal true positives, stringent filter(s) were applied, together with the use of negative control(s), such as the appropriate knockout mouse samples or alternatively the antigen peptide blocking approach. Furthermore, reverse IP was used to confirm the interaction. In this context a recent interaction proteomics study highlighted a similar example in which 495 proteins were identified from an IP, which eventually was filtered to 16 interactors. Recently, using the same strategy we examined the interactome of an adhesion molecule, Caspr2 (CNNTP2), and revealed tens of proteins as genuine interactors. Here, we revealed a simple composition of mGluR5 interactome with Homer1, -2 and -3 and mGluR1a/b as main interactors. This interactome is simple in contrast to, for instance, the complex interactome of the
ionotropic AMPA-type glutamate receptors, which is reported to contain up to 30 associated proteins\textsuperscript{115,177,178}. A G-protein coupled receptor, such as mGluR5, may exhibit many transient interactions during its activation as exemplified by the interaction with G-protein subunits. These short-lived interactions of presumably relatively low affinity may not be recovered in IP. This might explain that only a small but stable mGluR5 interactome is revealed under the present experimental conditions.

Homer1, -2 and -3 were previously described as mGluR5 interactors, and indeed were recovered from our IPs at high scores (> $10^8$). Homer1, -2 and -3 the homer family, exhibited a distinct brain region-specific mGluR5 interaction pattern, which reflects closely their differential gene expression patterns in these brain regions. In particular, Homer1 and -2 are abundantly expressed in cortex and hippocampus, whereas Homer3 is higher expressed in hippocampus but low in cortex. It is of notice that differential spatial expression occurs even within a brain region; in hippocampus Homer1 and -2 show major expression in the CA1 region, whereas Homer3 has highest expression in CA3 (see Allen Brain Atlas Mouse Brain ISH for Homer1, -2, -3). Future studies will be needed to resolve these protein complexes in order to reveal their similarity/differences.

Whereas mGluR5 is abundantly present in the post-synapse, it is also found extra-synaptically in endoplasmic reticulum as well as in astrocytes\textsuperscript{179}, and that the activation of intracellular dendritic pool of mGluR5 can mediate Ca$^{2+}$ responses in dendrites and are sufficient for mediating LTD in hippocampal neurons\textsuperscript{180}. To examine if there is difference of mGluR5 protein complexes present in distinct subcellular structures, we performed IP on biochemically fractionated hippocampal sub-fractions, namely the P1 fraction enriched in cell bodies, the synapse-enriched P2 fraction, and the microsome fraction which is enriched for endoplasmic reticulum, Golgi and vesicles\textsuperscript{181}. The mGluR5 complex composition was similar across different fractions (Table 2).

It will be interesting to explore the subcellular localization of mGluR5-Homer complex that might be present outside the postsynapse in order to confirm the biochemical fractionation data. Specific interactions may underline distinct functions, such as receptor trafficking or receptor-regulating roles. The role of Homer1 in stabilization of mGluR5 scaffolds has been well documented, however the functional implications of mGluR1-mGluR5 complexes have not been studied in much detail. Several potential interacting proteins were found (Table 1). Glud1, Atp6ve1, hemoglobin and Scl3a2 were found in empty bead controls in this experiment.
2), and/or not consistently observed in mGluR5 IPs and therefore likely are false positives. In this study, we identified two isoforms of mGluR1 in the mGluR5 IPs from cortex and hippocampus extracts. mGluR1a and 1b are generated by alternative splicing and differ at the C-termini; mGluR1b differs from mGluR1a at residues 887-906 with the substitution of mGluR1a peptide sequence NSNGKSWSVEPGRQAPKG to KKRQPEFSPSSQCPSAHVQL, and the remaining amino acid sequence (residues 907-1199) in mGluR1a is missing. As a consequence, there is only 1 unique (tryptic) peptide RQPEFSPSSQCPSAHVQL (Supplementary Fig. S1) for the mGluR1b isoform, compared to multiple unique peptides for the 1a isoform. Even so, the intensity of the mGluR1b isoform was generally higher than the 1a isoform. This suggests that the mGluR1b form may be the predominant mGluR5 interactor.
Table 1: Intensities of mGluR5 interactors immunoprecipitated from cortex and hippocampus. Maxquant MS1 peak intensities for the proteins quantified for unique peptides obtained from the mGluR5 IPs are indicated. Proteins are at least 10 fold enriched in WT, and observed at least 3 out of 4 times in WT. Gene names labeled in red are present in the empty beads experiment in Table 2, and thus most likely represent false positives. KO: mGluR5 IP from knockout mice, WT: mGluR5 IP from wild type mice.

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>CORTEX</th>
<th>HIPPOCAMPUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KO-1</td>
<td>KO-2</td>
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<tr>
<td>Grm5</td>
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<td>1E+06</td>
</tr>
<tr>
<td>Homer1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Grm1 (isoform b)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homer3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Homer2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Grm1 (isoform a)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Grm3</td>
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<td>Clu</td>
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<td>-</td>
</tr>
<tr>
<td>Rab3gap1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Atp1a2</td>
<td>-</td>
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<td>Slc3a2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu1</td>
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<td>-</td>
</tr>
<tr>
<td>Atp6v1e1</td>
<td>-</td>
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</table>
Table 2: Intensities of mGluR5 interactors immunoprecipitated from hippocampal subfractions. Maxquant MS1 peak intensities for the proteins quantified for unique peptides obtained from the mGluR5 IPs are listed here. Proteins labeled in red are putative mGluR5 interactors shown in Table 1, however, these are present in the empty bead controls and most likely represent false positives. EB: Empty bead control, P1: Pellet 1, P2: Pellet 2.

<table>
<thead>
<tr>
<th>Gene Names</th>
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<th>P2</th>
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<td>IP_2</td>
</tr>
<tr>
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<td>3E+08</td>
<td>-</td>
</tr>
<tr>
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<td>4E+05</td>
<td>-</td>
</tr>
<tr>
<td>Grm1 (Isoform b)</td>
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</tr>
<tr>
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<td>- 7E+06</td>
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<td>-</td>
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<td>Homer3</td>
<td>- 5E+05</td>
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<td>Atp6v1e1</td>
<td>4E+05</td>
<td>7E+05</td>
<td>5E+05</td>
</tr>
<tr>
<td>Glud1</td>
<td>3E+05</td>
<td>3E+05</td>
<td>-</td>
</tr>
<tr>
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<td>2E+06</td>
<td>2E+06</td>
<td>6E+05</td>
</tr>
<tr>
<td>Slc3a2</td>
<td>- 2E+05</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 1. mGluR1 co-immunoprecipitates with mGluR5 in HEK293 cells. Upper panel: anti-mGluR1 immunoblots show immunoreactivities from inputs containing mGluR1, but not from input containing only mGluR5. Immunoprecipitation with anti-mGluR5 antibodies precipitate both mGluR1 and 5 in the HEK293 cells co-expressing mGluR1 and 5, but did not precipitate mGluR in HEK293 cells expressing only mGluR1. Lower panel: anti-mGluR5 immunoblots show immunoreactivity from inputs containing mGluR5 and did not show cross-reactivity with mGluR1. Co-immunoprecipitation with the anti-mGluR1 antibody precipitated both mGluR1 and 5.
Table 3: mGluR1a IP from hippocampus confirms the mGluR1-5 interaction. Maxquant MS1 peak intensities for the proteins quantified for unique peptides obtained from the mGluR1 IPs are listed. BD: IP performed with mGluR1a Antibody from BD Biosciences. Ig: IP performed with non-mGluR1 antibody as negative control.

<table>
<thead>
<tr>
<th>Protein names</th>
<th>Gene names</th>
<th>HIPPOCAMPUS</th>
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<tbody>
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<td>Metabotropic glutamate receptor 1</td>
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<td>Ig-1:29026  Ig-2:52842</td>
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<tr>
<td>Metabotropic glutamate receptor 1</td>
<td>Grm1 (Isoform b)</td>
<td>Ig-1:4388  Ig-2:3859</td>
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<td>Metabotropic glutamate receptor 5</td>
<td>Grm5</td>
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<td>Homer protein homolog 3</td>
<td>Homer3</td>
<td>Ig-1:10924 Ig-2:6549</td>
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</table>

Figure 2. Super-resolution imaging validation of mGluR1-5 co-localization. SIM imaging in cultured hippocampal neurons at DIV19 showing an overview or zoom-ins of dendrites (right) or spines (right, inset). mGluR1 (green), mGluR5 (red) and, Homer1 (blue, synaptic marker) are shown. Merge shows color-overlay images of the three channels. Scale bars are indicated. Merge panel: open arrows indicate non-synaptic overlap between mGluR1 and -5, closed arrows indicate synapses positive for mGluR1 and -5. Note that extra-synaptically a part of the mGluR1 is not co-localized with mGluR5.
To confirm the mGluR1-5 interaction specific protein complex, we used a monoclonal anti-mGluR1a antibody for IPs. Several proteins were present in both mGluR1a IPs with >10-fold enrichment compared to the negative controls (Table 3). In hippocampus, the mGluR1a complex contained mGluR1b, mGluR5 and Homer3. These observations are in general agreement with the results of mGluR5 IPs. Homer1 and -2, that were abundantly present in hippocampal mGluR5 IPs, were not detected in mGluR1a IP in the hippocampus. Together, this indicates that (1) mGluR1-specific protein complex harbors primarily Homer3, and (2) hetero-dimerization of mGluR1a-b and mGluR5 can exist in neurons.

To examine whether mGluR1-5 interact directly, we performed co-IP experiments with expression of mGluR1 and/or -5 in HEK293 cells (Fig. 1). We confirmed the direct interaction of mGluR1/5 in the double expression of mGluR1 and -5, in which the IP of mGluR5 precipitated mGluR1, and the IP of mGluR1 precipitated mGluR5. We further confirmed the antibodies specificity; anti-mGluR1 antibody did not stain mGluR5 and did not precipitate mGluR5, and vice versa, anti-mGluR5 antibody did not stain mGluR1 and did not precipitate mGluR1.

If mGluR1 and -5 form a heteromeric complex, they should co-localize. We performed co-immunostaining of mGluR1 and 5 on hippocampal primary neurons and visualized these proteins with SIM imaging. The mGluR1-5 co-localization was observed in synapses, as indicated by their co-localization with the postsynapse marker protein Homer, and was also detected outside synapses (Fig. 2). There was also mGluR1 that did not co-localize with mGluR5, especially outside synapses, indicating that mGluR1 dimerizes with other (G-protein coupled) receptors. Together, these imaging data are supportive to our interaction proteomics data that mGluR1-5 protein complex exists, and that this complex constitutes a sub-population of synaptic mGluR5 (Table 1).

Whereas we demonstrated for the first time the presence of mGluR1-5 protein complex in the brain, interaction of mGluR1 and -5 has been implicated previously. Chemical LTD induced by selective mGluR5 agonist DHPG was abolished in mGluR5 knockout mice\(^{182}\). However, in hippocampus the complete blockade of chemical LTD required the combination of both mGluR1 and -5 receptor antagonists\(^ {183}\), suggesting hetero-dimerization of the two receptors. Time-resolved fluorescence resonance energy transfer with the cell-surface labeling of tagged rat mGluR subunits expressed in a mammalian cell line revealed that group II and III mGluR subunits can produce intergroup heteromeric receptors that are functional, whereas group I mGluR subunits,
i.e. mGluR1 and -5, can interact but do not associate with groups II and III mGluR subunits\textsuperscript{184}. Recently, mGluR2-4 heterodimers have been found in the striatum at the cortico-striatal synapse, and exhibit a distinct pharmacological profile\textsuperscript{185}. Functional interdependence of mGluR1 homodimers and mGluR5 homodimers has been indicated in sympathetic rat neurons co-expressing both receptors\textsuperscript{186}. This study also provided evidence of two pools of mGluR5 in striatal neurons, one of them probably interacting with mGluR1. The discovery of mGluR heteromerization represents a new avenue for the study of mGluR pharmacology and neurobiology, of which the in vivo consequences remain to be investigated.
Materials and Methods

Sample preparation and immunoprecipitation

Whole hippocampus and cortex were dissected from mouse brains, and stored at -80 °C until used. The brain tissue was homogenized in 1% freshly prepared n-Dodecyl β-D-maltoside containing 25 mM HEPES (pH 7.4) and 150 mM NaCl and a protease inhibitor cocktail (Roche), in a PotterS homogenizer with 12 strokes at 900 rpm and incubated for 1 hr at 4 °C. After centrifugation at 20000 xg for 20 min the supernatant was collected and centrifuged again at 20000 xg for 20 min. The final supernatant was used for immunoprecipitation (IP), with the equivalent of 1 hippocampus and 0.3 cortex per IP experiment. 10 µg antibody was added to each sample, and incubated overnight at 4 °C with rotation. 60 µL Protein A/G plus agarose bead (Santa Cruz) was used per IP to capture the antibody. After washing, the agarose beads containing the protein complex were subjected to filter aided sample preparation (FASP) treatment (as described below). The anti-mGluR5 polyclonal antibody was obtained from Genscript (A01493); the anti-mGluR1a monoclonal antibody was obtained from BD Biosciences (clone G209-488).

FASP in-solution digestion of proteins

FASP was used with small modifications. In short, agarose beads from an IP experiment were gently vortexed in 75 µL 2% SDS, 1 µL 50 mM Tris (2-carboxyethyl)phosphine at 55 °C for 1 h. The reduced cysteines were blocked by incubation with 0.5 µL 200 mM methyl methanethiosulfonate for 15 minutes at RT. The sample was centrifuged at 16,000 xg for 20 min. The supernatant was mixed with 200 µL 8 M Urea in Tris pH 8.8, transferred to Microcon-30 (Millipore, Lot R4NA17256), and centrifuged at 14,000 xg for 12 minutes at RT. The addition of 200 µL 8 M urea to the filter and centrifugation were repeated three times. To remove urea, four serial washes were performed with the addition of 200 µL 50 mM NH₄HCO₃ for each wash followed by centrifugation as stated above. Samples were digested with 0.7 µg Trypsin/Lys-C Mix (mass spectrometry grade from Promega) in 100 µL 50 mM NH₄HCO₃ overnight in a humidified chamber at 37 °C. 100 µL of 0.1% acetic acid was added to the filter, and centrifuged. The tryptic peptides were collected, dried in a speedvac, and stored at -20 °C until LC-MS analysis.
Preparation of P1, P2 and microsome fractions

P1, P2 and Microsome fractions were prepared as in Ref 134. Briefly, Hippocampi were homogenized in ice-cold homogenization buffer (0.32 M Sucrose, 5 mM HEPES (pH 7.4)) containing protease inhibitor (Roche). Pellet 1 (P1) was obtained after spinning at 1,000 xg at 4 °C for 10 minutes. The supernatant was spun at 20,000 xg at 4 °C to obtain Pellet 2 (P2). The supernatant from P2 was further centrifuged at 100,000 xg for 2 h to obtain a microsome fraction.

In gel-separation digestion of proteins

IPs were performed as before on an equal amount (2 mg) of P2, microsome and P1 fractions from hippocampus. SDS-PAGE electrophoresis and in gel digestion were performed as described 174.

LC-MS/MS acquisition and data analysis

Peptides were analyzed by two types of nano-LC MS systems, namely LTQ-Orbitrap Discovery (Thermo Scientific) and TripleTOF 5600+ (Sciex) MS.

For the analysis using TripleTOF 5600+ MS, it was coupled with an Ultimate 3000 LC system (Dionex, Thermo Scientific). Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 μm i.d., 5μm particle size, Dionex) and fractionated on a 200 mm Alltima C18 column (100 μm i.d., 3 μm particle size). Acetonitrile concentration in the mobile phase in 0.1% formic acid was increased from 5 to 18% in 88 min, to 25% at 98 min, 40% at 108 min and to 90% in 2 min, at a flow rate of 400 nL/min. Peptides were electrosprayed into the mass spectrometer using an ion spray voltage of 2.5 kV, curtain gas at 35 p.s.i., nebulizer gas at 15 p.s.i. and an interface heater temperature of 150 °C. The MS survey scan range was m/z 350–1,250 acquired for 250 ms. The top 20 precursor ions were selected for 85 ms per MS/MS acquisition, with a threshold of 90 counts. Dynamic exclusion was 16 s. Rolling CID function was activated, with an energy spread of 15 eV.

For the analysis using the LTQ-Orbitrap MS, peptides were loaded onto a nano-LC Ultra system (Eksigent) with the trapping and separation columns as described for 5600+ MS analysis. Acetonitrile concentration in 0.1% acetic acid was linearly increased from 5 to 40% in 80 min and to 90% in 10 min, and electro-sprayed into the LTQ-Orbitrap MS using an ion spray voltage of 1.4kV and heater temperature of 200 °C. LTQ-Orbitrap was operated in the range of m/z 350-2000 at a FWHM resolution of 30,000 after accumulation to 500,000 in the LTQ with one microscan. The five most
abundant precursor ions were selected for fragmentation by CID with an isolation width of 2 Da.

All raw MS data were analyzed by MaxQuant software (version 1.5.2.8) with search engine Andromeda. The Mouse database used was UniProt_2015-02. The fixed modification was MMTS (for FASP) and propioamide (for in gel digestion samples). Peptides for quantitation were set to ‘Unique’. Match between runs with match time window of 0.7 minutes and alignment time window of 5 minutes were used for all analyses. For other parameters the default settings were used.

**Expression plasmids**

Mouse mGluR1, transcript variant 1 (NM_016976.3), was Gateway-cloned into the pReceiver-Lv186 vector, yielding mGluR1-pReceiver-Lv186 (including a C-terminal 3xHA-tag)(GeneCopoeia; Catalog number EX-Mm02865-Lv186). Mouse mGluR5, transcript variant a (NM_001081414), was cloned into the pcDNA3.1(+) vector, yielding mGluR5-pcDNA3.1 (Genscript).

**HEK293 cell culture and transfection**

HEK293 cells (ATCC) were cultured in DMEM medium (Gibco, Life Technologies), 10% FBS (Invitrogen), and 1% Penicillin-Streptomycin (Gibco, Life Technologies) in 10 cm dishes. Cells were 60-70% confluent at the time of transfection and of passage number 14. Medium was refreshed 2-3 h prior to transfection. HEK293 cells were transfected with plasmid DNA (5 µg) using Polyethylenimine (PEI) (25 kDa linear, Polysciences) and incubated for 48 h after transfection.

**Co-precipitation from HEK293 cells**

All steps were performed at 4 °C, with the exception of elution (room temperature). For protein extraction, HEK293 cells were washed with PBS, resuspended in freshly prepared lysis-buffer (1% DDM, 25 mM HEPES (pH 7.4), 150 mM NaCl, and EDTA-free Complete protease inhibitor (Roche)), and incubated for 1 h with gentle end-over-end mixing. The supernatant was cleared of non-soluble debris by two consecutive centrifugation steps at 20,000 xg for 15 minutes. Anti-mGluR1 antibody (4 ug) or anti-mGluR5 antibody (4 ug) was added to the supernatant, incubated O/N, and immobilized to Protein A/G agarose beads (Santa Cruz). The agarose beads were washed 4 times with wash buffer (0.1% DDM, 25 mM HEPES, and 150 mM NaCl), and the bound proteins were eluted by incubation with 2x Laemmli sample buffer. Input
samples were prepared from the supernatant fraction by addition of Laemmlli sample buffer to a 2x final concentration.

**SDS-PAGE and immunoblot analysis**

Protein samples were heated to 55 °C for 45 minutes prior to loading onto a 4–15% Criterion TGX Stain-Free Precast gel (Bio-Rad), with the input samples representing 3% of the lysate used for immunoprecipitation. The gel-separated proteins were imaged with the Gel-Doc EZ system (Bio-Rad), directly transferred onto Immuno-Blot PVDF membrane (Bio-Rad) and probed O/N at 4 °C with mGluR1 antibody (1:1,000) or mGluR5 antibody (1:1,000). Chemiluminescence scans were acquired with the Odyssey Fc system (Li-Cor), and analyzed using Image Studio Lite 5.2.5 software (Li-Cor).

**Protein molecular weight prediction**

The predicted molecular weight was determined using the Expasy online tool (http://web.expasy.org/compute_pi/). The predicted molecular weight for mGluR1 as expressed by the mGluR1-pReceiver-Lv186 plasmid (including a C-terminal 3xHA-tag) was 137.6 kDa. The predicted weight for mGluR5 was 128.3 kDa.

**Immunostaining of primary neurons**

Primary hippocampal neurons were obtained from E18 rat pups. Briefly, 18,000 cells were grown in neurobasal medium supplemented with B27 on poly D-Lysine coated coverslips. The cells were used for staining at DIV14-16. The coverslips were fixed with ice-cold methanol for 10 min, followed by three washes in ddH2O and PBS. The neurons were then blocked and permeabilized with blocking buffer (5% FCS, 0.1% Triton X-100, and 0.1% Glycine in phosphate buffer saline) for 1 h. Next, the neurons were incubated with anti-mGluR1 (clone G209-488, BD Biosciences) or anti-mGluR5 (cat. no. A01493, Genscript) antibodies, and anti-Homer1 (cat. no. 160 004, Synaptic systems), diluted in blocking buffer overnight at 4 °C. After washing three times in PBS, the cells were incubated with an Alexa-conjugated secondary antibody for 1 h at room temperature (anti-rabbit Alexa 488 (1 in 1,000), anti-mouse Alexa 568 (1 in 1000), anti-Guinea pig Alexa 647 (1 in 1,000) (Molecular Probes) and subsequently washed and fixed on glass slides (Superfrost Plus, Thermo) using Moviol. Images were taken using a Zeiss Elyra PS1 SIM microscope with 63x oil immersion lens (N.A. 1.4) and reconstructed images were analyzed using ImageJ.
Acknowledgements

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Additional Information

Smit holds shares in Alea Biotech BV. The author(s) declare no competing financial interests
Multi-dimensional interaction proteomics delineates distinct hippocampal AMPA receptor subcomplexes

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*These authors contributed equally to the manuscript.
Abstract

The AMPA glutamate receptor (AMPAR) is the major type of synaptic excitatory ionotropic receptor in the brain. Regulation of localization and biophysical properties of the AMPAR underlies synaptic plasticity that is key to learning and memory. Recent interaction proteomics analyses have characterized approximately 30 AMPAR interacting proteins with high confidence, which are suggested to form the inner and outer core of the AMPAR complex. Currently, these studies do not accommodate for observations that AMPAR subtypes might fulfill distinct roles in trafficking, mobility and physiology, which each may require a distinct auxiliary subunit composition. Here, we developed a strategy that combines the specificity of immunoprecipitation and the resolution of blue-native PAGE to distinguish distinct AMPAR subcomplexes. We found that in hippocampus the AMPAR is always associated with at least two other proteins, and receptors without interaction partners likely do not exist. AMPAR complexes comprise subgroups over a range of molecular weights, each with different protein constituents. Selected complexes were validated using super-resolution microscopy and heterologous expression. Revealing the existence of distinct AMPAR complexes with different constituents is key to interpretation of synapse physiology, and in particular, will instruct future experiments aimed at defining channel properties of these specific receptor assemblies.
Chapter 4: AMPA receptor subcomplexes

Introduction

AMPA receptors (AMPARs) are glutamate-gated cationic channels underlying the predominant component of fast excitatory synaptic transmission in the mammalian central nervous system. Functional synaptic AMPARs are thought to localize primarily in nano-domains of the postsynaptic density (PSD), in which they are aligned to the glutamate-release sites of the presynaptic active zone. Numbers and biophysical properties of these synaptic AMPARs are regulated in an activity-dependent manner, which is a major postsynaptic contribution to alteration of synaptic efficacy. The molecular and cellular mechanisms of AMPAR dynamics have been extensively studied and shown to crucially depend on AMPAR auxiliary proteins determining trafficking, membrane mobility, localization at the PSD, and affecting conductance properties of the AMPAR channel. In particular, TARP γ2 (Stargazin) and TARP γ8 are known to alter AMPAR surface expression, to affect AMPAR PSD mobility by the interaction with PSD-95, and to prolong AMPAR deactivation and desensitization.

Apart from TARPs, Cornichons (Cnih2/3) can regulate AMPAR channel properties. The Shisa family of proteins (CKAMP44/Shisa9, Shisa6) have also been implicated in affecting AMPAR membrane mobility and channel conductance properties.

Whereas previous studies focused on single, or at most two, auxiliary proteins, recent interaction proteomics analyses reported approximately 30 high confident AMPAR-associated proteins. The AMPAR protein complex was modeled to contain an inner core of stable interacting proteins and an outer core of variable protein composition and number. The model primarily uses ionic strength of protein interaction and assumes affinities as major underlying determinant of AMPAR-auxiliary protein complex formation. Considering that AMPAR trafficking, mobility and physiology show specific spatio-temporal characteristics, and that the AMPAR interactome constituents are brain-region specific, a likely alternative model is the presence of multiple distinct AMPAR subcomplexes with different or overlapping protein compositions. One important caveat to test this hypothesis is the limitation in existing methodology to separate protein subcomplexes and identify their residing components.

In this study, we first used a high-density reverse IP strategy to identify potential subcomplexes from mouse brain hippocampus and observed a large overlap in protein subcomplexes of the AMPARs. We then devised a multi-dimensional protein separation strategy by combining the specificity of interaction proteomics analysis, the resolution of Blue Native polyacrylamide gel electrophoresis (BN-PAGE) to
separate purified protein subcomplexes, and the sensitivity of mass spectrometry to identify components of AMPAR subcomplexes, together termed IPBN-MS. This integrated multidimensional technology enabled the identification of multiple AMPAR subcomplexes, of which individual protein interactions were validated using heterologous expression and of which localization was determined using super-resolution microscopy. IPBN-MS has the potential to be widely applicable in complimenting conventional interaction proteomics experiments to resolve proteins that are present in multiple complexes.
Results

High-density reverse IP for AMPAR interacting proteins reveals overlapping protein complexes

We first applied a high-density reverse IP-based interaction proteomics analysis to acquire co-occurrence data that might aid in clustering of various AMPAR-associated proteins into separate groups, representative of different subcomplexes present in the hippocampus. We used 11 different antibodies (Supplementary Fig. S1a; see selection criteria for reverse IPs in materials and methods section) directed to the AMPAR and its associated proteins (Fig. 1) and performed pull down experiments along with peptide antibody blocking specificity controls, as described previously, followed by LC-MS/MS analysis. IP-MS for GluA2/3 identified nearly all previously reported high confident AMPAR interactors. Conversely, reverse IP-MS of AMPAR-associated proteins identified all AMPAR subunits (GluA1-4). GluA4 was present as minor component due to lower levels in all IP experiments reflecting its low expression in the hippocampus. Interestingly, IP-MS for Noelin1 reveals only a small list of interacting partners, i.e., TARP \( \gamma \)8, Noelin1, -2 and -3 and Nrn1, as compared to IP-MS data of TARP \( \gamma \)8, -\( \gamma \)2, Cnih2, and Prrt1. This IP-MS data set shows considerable overlap of identified proteins in different IPs, indicative of extensive sharing of similar sets of proteins in different subcomplexes. For instance, it is apparent that TARP \( \gamma \)8 is contained in most hippocampal AMPAR complexes, i.e. it is observed in anti-Cnih2, anti-Frrs1l, anti-Prrt1, anti-Noelin1 IPs. Cpt1c and Sac1 are together and exclusively in a distinct AMPAR complex that lacks the most prominent AMPAR interactors, TARP \( \gamma \)2, -\( \gamma \)8 and Cnih2/3. Rap2b is present at low levels in almost all IP experiments, suggesting background binding or low affinity of this protein for all AMPAR complexes.

Taken together, this high-density IP-MS analysis indicates considerable heterogeneity of AMPAR subcomplexes containing overlapping sets of proteins, however, is insufficient to distinguish individual subcomplexes with shared protein constituents.

Therefore, we developed a new approach, IPBN-MS, which first separates AMPAR subcomplexes prior to MS analysis.
To size-separate intact protein complexes, Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE) in principle has high resolving power. To examine the usefulness of BN-PAGE in distinguishing AMPAR subcomplexes, n-Dodecyl β-D-maltoside (DDM) solubilized protein complexes from mouse hippocampus synapse-enriched fraction (P2 + Microsome) were separated on BN-PAGE, transferred to PVDF membrane, and immuno-stained with antibodies to GluA2/3 or TARP γ2, -γ4, -γ8 (Supplementary Fig. S1b). The TARP γ8 immuno-reactive band migrated at higher apparent molecular weight (MW) than the main AMPAR immuno-reactive band, and fell within the AMPAR-containing protein complex apparent MW range of 720–1000 kDa. Thus, this BN-PAGE indicates the existence of multiple distinct AMPAR subcomplexes.

As a next step, we combined the specificity of IP and the resolving power of BN-PAGE to separate subcomplexes and used LC-MS/MS to identify and quantify constituent proteins (hereafter abbreviated as IPBN-MS) (Fig. 2). AMPAR complexes were immuno-isolated using an anti-GluA2/3 antibody, released from the antibody-proteinA/G bead by incubation with 10x excess of peptide-antigen, size fractionated on BN-PAGE, followed by cutting the gel into slices, in-gel tryptic digestion and LC-MS analyses. As expected, peptides belonging to the same protein co-elute (Fig. 3a, b), as exemplified by the tight co-migration profiles of four distinct GluA1 tryptic peptides over the range of BN slices. The estimated MW sizes of the AMPAR complexes from IPBN-MS, agree with those from immunoblotting analysis of tissue extracts run directly on BN, indicative of the stability of the AMPAR complexes throughout the BN procedure. Only a very limited amount of AMPAR subunits detected are devoid of any associated protein (Fig. 3b).
Figure 1: Overview of interaction proteomics analysis revealing overlap in proteins part of AMPAR complexes. Abundances are presented by mean iBAQ values for all high-confident AMPAR-interacting proteins identified in IPs of the AMPAR and of AMPAR interacting proteins, versus values of their corresponding empty bead or peptide antibody blocking controls. Note: colors represent log10-scaled iBAQ values, with all values above the 0.75 quantile ($<10^5$) capped to maximum (red) to prevent the bait protein(s) from dominating the scaling. Separation of protein subcomplexes using IPBN-MS.
Figure 2: IPBN-MS workflow for dissecting protein subcomplexes: IPBN-MS workflow involving multiple steps. I) Isolation of synaptosome + microsome fraction from brain region/tissue of interest. II) Extraction of isolated synaptosome + microsome fraction using n-Dodecyl β-D-maltoside (DDM) to obtain protein extract. III) Immunoprecipitation of proteins of interest using specific antibodies followed by incubation with agarose beads to obtain immuno-purified target complexes. IV) Incubation of immuno-purified target complex with peptide antigen for elution of purified complexes. V) Separation of purified complexes on Blue-Native PAGE. VI) Cutting the gel into 40 or 70 consecutive slices followed by in-gel digestion obtaining tryptic peptides, which are separated on HPLC followed by LC-MS analysis. VII) Obtaining protein and peptide elution profiles using Skyline and identification of subcomplexes.
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Figure 3: IPBN-MS shows intact elution of high molecular weight AMPAR complexes. 

a) Rowsum normalized elution profiles of four different peptides belonging to the GluA1 subunit derived from the IPBN-MS of GluA2/3 complexes shows high degree of overlap. 

b) Rowsum normalized elution profiles for GluA1–4 subunits from a GluA2/3 IPBN-MS experiment show high degree of overlap demonstrating suitability of IPBN-MS in maintaining AMPAR complex integrity. Note the very limited amount of AMPAR not associated with other proteins (slices 16-19). Scale x axis: Slice number relative to 720 kDa. Y axis: Normalized intensity over all slices.

Examination of AMPAR subcomplexes using IPBN-MS

When performing GluA2/3 IPBN-MS we observed many interaction partners of the AMPA receptor, which elute over a broad molecular weight range (Fig. 4). The IPBN-MS on GluA2/3 corroborates the proteins identified in the high-density reverse IP. Pearson correlation clustering identifies at least three MW groups (A-C) of AMPARs and interactors (Fig. 4). Apart from the lowest MW AMPAR complex (Group A), containing the Cnih2-Frrs1l-AMPAR complex, the higher MW AMPAR subcomplex show substantial overlap in proteins and cannot be discerned into distinct complexes using the AMPAR itself as bait (Groups B,C). Therefore, to further dissect the subcomplex we performed high-density IPBN-MS experiments using the AMPAR interacting proteins as baits.
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Figure 4: AMPAR IPBN-MS reveals three distinct yet partially overlapping core complexes. Pearson correlation clustering of rowsum normalized elution profiles of all proteins identified in GluA2/3 IPBN-MS reveals a low molecular weight Cnih2 complex containing Frrs1l and multiple overlapping peaks for the other AMPAR interactors. Scale x axis: Slice number relative to 720 kDa.

Resolving the low molecular weight group-A Cnih2-Frrs1l containing complex

We first interrogated the potential group A Cnih2-Frrs1l-AMPAR complex (cf. Fig. 4) by reverse IPBN-MS using Cnih2, Frrs1l and GluA2/3 as bait proteins. Both Cnih2 and Frrs1l IPBN-MS reveal a distinct peak of the AMPA receptor at the exact same molecular weight (slice +3 with respect to slice 0 at 720 kDa). This data corresponds to the presence of Cnih2 and Frrs1l in the GluA2/3 IPBN-MS. Because no other known AMPAR interactors elute at this low MW position we conclude that this complex is exclusively composed of GluA-Cnih2-Frrs1l (Fig. 5a-f). Importantly, this complex is the major extractable AMPAR complex detected. At higher MW elution of the AMPAR (>720 kDa), Frrs1l and Cnih2 can also be detected, indicating that Frrs1l and Cnih2 are also present in AMPAR subcomplex with higher protein complexity.

Next, we tested whether Cnih2 and Frrs1l can interact directly with the AMPA receptor by co-expression in HEK293T cells. We found the direct interaction between Cnih2 and GluA2 (Fig. 5g) and the direct interaction of Frrs1l and GluA2 (Fig. 5h). Next, we co-expressed the three proteins in HEK293T cells (Fig. 5i), followed by pull
down using the Frrs1l antibody. Again GluA2 and Cnih2, as well as GluA2 and Frrs1l were found to interact. Thus, Cnih2 and Frrs1l can interact directly with the AMPAR. In addition, we observed a weak interaction between Cnih2 and Frrs1l (Fig. 5i).
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Figure 5: Separation of the Frrs1l-Cnih2-AMPAR complex. a,b) Normalized intensity profiles for GluA1-3, Cnih2 and Frrs1l after GluA2/3 IPBN-MS shows co-elution at the slice (0) corresponding to 720 kDa. c,d) Normalized intensity profile for the same proteins in Cnih2 IPBN-MS and e,f) Frrs1l IPBN-MS. Scale bar: rowsum normalized peak intensity from 0 (0%) to 1 (100%). g) Interaction of Cnih2 and GluA2, and h) Frrs1l and GluA2 in HEK293T cells. i) Triple expression of Cnih2, Frrs1l and GluA2 in HEK293T cells shows interaction for Cnih2 and GluA2, Frrs1l and GluA2 and a weak interaction between Cnih2 and Frrs1l. Scale x axis: Slice number relative to 720 kDa.
Resolving the group-B TARP γ8 containing complexes

We next explored the separation of AMPAR complexes using the IPBN-MS for the well-established AMPA receptor interacting protein TARP γ8, in the GluA2/3 IPBN-MS (cf. Fig. 4). IPBN-MS using an antibody directed against TARP γ8 revealed the co-migration of many AMPAR interacting proteins over a wide MW range and larger than 720 kDa (Fig. 4a). Pearson’s correlation (Fig. 4a) showed that the TARP γ8 containing complexes primarily form two groups; group B1 with a peak at slice –2 and group B2 with a peak at slice –7 (with respect to the 720 kDa marker).

The group B1 complex also contains Cnih2. This is in accordance with the Cnih2 and GluA2/3 IPBN-MS (Fig. 4b, c, d) in which TARP γ8 was also found to have a peak at slice –2. Thus, group B1 appears to comprise primarily the TARP γ8-Cnih2-GluA complex.

Group B2 comprised primarily Noelin1, Shisa6, -9 and Prrt1, -2 spread out over a wider mass range (Fig. 4a). Reverse IPBN-MS were performed on Shisa6 and Prrt1, which revealed the presence of GluA1–3, TARP γ8 and Prrt1 in the Shisa6 IP (Fig. 4e), and GluA1–3, Shisa6, -9, and Prrt2 and in the Prrt1 IP, all co-eluting at slice –7 (Fig. 4f). Furthermore, Noelin1 IP data revealed the presence of only Noelin1, TARP γ8 and GluA1–4 (Fig. 1), which was confirmed in TARP γ8 and GluA2/3 IPBN-MS (Supplementary Fig. S2a, b). Thus, the group B2 complex can be further split into Noelin1-TARP γ8-AMPAR complex (Supplementary Fig. S2a, b) and the Shisa6-Prrt-TARP γ8-AMPAR complex (Fig. 4e, f, g). Based on the IPBN-MS of Prrt1, Cnih2 and Frrs1l, a small population of TARP γ8 is present in between the size classes of the major Group-A and -B complexes, which has a peak at slice –5 with the composition of Cnih2-Frrs1l-TARPγ8-Prrt1-AMPAR (Supplementary Fig. S3a-d). Since it partially overlaps with the Group-A and -B complex, the Cnih2-Frrs1l-TARPγ8-Prrt1-AMPAR complex is obscured in the IPBN-MS of TARP γ8 (Supplementary Fig. S3e).

We performed co-expression studies to examine the possible interactions of proteins in the TARP γ8-GluA complexes. We found that: (1) When TARP γ8 and Shisa6 were co-expressed in HEK293T cells, IP of TARP γ8 pulls down Shisa6, at low levels, indicating a weak direct interaction of Shisa6 and TARP γ8 under these conditions (Supplementary Fig. S4a). (2) Prrt1 can be in complex with GluA2 and TARP γ8, without the need of additional neuronal accessory proteins (Supplementary
Fig. S4b, c). (3) Noelin1 directly interacts with GluA2, but not with TARP γ8 (Supplementary Fig. S4d, e).
Figure 6: Characterization of high MW Group-B TARPγ8 containing complexes. a) Pearson correlation clustering heat map of normalized elution profiles of all proteins identified in TARP γ8 IPBN/MS reveals two groups (group B1 centered at slice –2 and group B2 centered at slice –7) of complexes. Scale bar: rowsum normalized peak intensity from 0 (0%) to 1 (100%). b,c,d) Normalized peak intensities for the TARP γ8-Cnih2-AMPAR (Group B1) complex from three different IPBN-MS experiments for TARP γ8, Cnih2 and GluA2/3, shows that the TARP γ8-Cnih2-AMPAR complex elutes consistently at slice –2 with respect to the 720 kDa marker (dotted line). e,f,g) Normalized peak intensities for Shisa6-Prrt1-TARPγ8-AMPAR (Group B2) complex as observed in individual TARP γ8, Prrt1 and Shisa6 IPBN-MS experiments at slice –7 with respect to the 720 kDa marker. Scale x axis: Slice number relative to 720 kDa

**Resolving the Group-C high MW Cpt1c-Sacm1l complex**

Based on the high density IP data (Fig. 1), Cpt1c forms a distinct complex lacking TARP γ8 and Cnih2. Indeed, IPBN-MS of Cpt1c revealed co-migration with GluA1-3 in the high MW range (Fig. 7a). Frrs1l also co-eluted with this complex. Accordingly, the Frrs1l IPBN-MS revealed the co-migration of Cpt1c and Sacm1l at high MW, a complex that is clearly distinct from the low MW Cnih2-Frrs1l-AMPAR complex (Fig. 7b; cf. Fig. 4.). Thus, a distinct high-MW Frrs1l-Cpt1c-Sacm1l-AMPAR complex is likely to exist. Since Cpt1c forms a distinct TARP γ8-lacking complex, we hypothesized that Cpt1c has a different subcellular localization than TARP γ8. To this end, we performed immunoblotting on biochemically isolated subfractions (Fig. 7c) for Cpt1c, Frrs1l and Sacm1l and observed that indeed Cpt1c, Frrs1l and Sacm1l are enriched in the microsomal fraction, whereas TARP γ8 and TARP γ2 are enriched in the synapse and PSD fractions (Supplementary Fig. S5).

We next determined whether Cpt1c and Sacm1l can interact directly with the GluA2 subunit when co-expressed in HEK293T cells (Fig. 7e, f), and found this indeed was the case. When reconstituting Cpt1c-Frrs1l-GluA2 in HEK293T cells we found that Cpt1c and Frrs1l show a weak direct interaction. Frrs1l pull-down from this co-expression indicates GluA2 and Cpt1c as partners (Fig. 7g). Thus, Cpt1c-Frrs1l-GluA2 forms a stable complex in heterologous systems without the requirement of Sacm1l.
Figure 7: Characterization of the high molecular weight GluA-Cpt1c-Frrs1l-Sacm1l complex. a) Normalized intensity profiles for GluA1-3, Cpt1c, Sacm1l and Frrs1l in the Cpt1c IPBN-MS shows these proteins co-eluting at high molecular weight (slice –9 with respect to 720 kDa). b) Normalized intensity profile for the same proteins in Frrs1l IPBN-MS shows Cpt1c-Frrs1l-Sacm1l-AMPAR complex at the same molecular weight. c) Immunoblotting for the known PSD-enriched Grin2b, Dlg4 and the presynaptic synaptophsyn (Syp) in different biochemical fractions of the mouse hippocampus. Homo, homogenate; P2, pellet; M, microsome; SyS, synaptosome; SyM, synaptic membrane, PSD: postsynaptic density. Note the co-occurrence of the Frrs1l, CPT1c, and Sacm1 in the microsome fraction. d) Interaction of Cpt1c and GluA2, and e) Sacm1l and GluA2 in HEK293T cells. f) Triple expression of Cpt1c, Frrs1l and GluA2 HEK293T cell shows interactionFrrs1l, Cpt1c and GluA2 can form a stable complex in HEK293 cells. Scale x axis: Slice number relative to 720 kDa.

High-resolution microscopy

To validate interactions indicated by IPBN-MS the subcellular localization of some of the AMPAR interacting proteins was investigated by using structured illumination microscopy (SIM) on primary cultured hippocampal neurons. Co-staining of TARP γ8 along with GluA2 and Homer1 revealed 81.3 ± 2.1% (n = 19) of Homer1 puncta positive for TARP γ8 and 84.7 ± 3.4% (n = 16) homer puncta positive for GluA2 (Supplementary Fig. S5a). Furthermore, we found that 49.3% of TARP γ8 puncta were positive for AMPAR and within 500 nm from the center of homer spots, whereas 50.7% of TARP γ8 puncta were non-synaptic (>500 nm from homer spots) (Supplementary Fig. S5b). Thus TARP γ8 forms complexes both at the PSD shown by its localization with Homer1 puncta and outside the PSD as well, which is in accordance with the PSD and extra-synaptic localization of TARP γ8 containing complexes.

To determine the subcellular localization of the Shisa6-TARP γ8-AMPAR complex, we performed double staining for Shisa6-GluA2 and Shisa6-TARP γ8. Shisa6 staining was highly punctate and 50.1 ± 3.4% and 63.6 ± 5.5% of the Shisa6 puncta were positive for GluA2 and TARP γ8, respectively (Supplementary Fig. S6a, b). We then determined the subcellular localization of the GluA2-Noelin1-TARP γ8 complex by performing a triple immunostaining for GluA2, Noelin1, Homer1, and for Noelin1, TARP γ8, Homer1 (Supplementary Fig. S7a, b). Noelin1 overlapped with TARP γ8 in Homer positive puncta. We found that 54.3% of Noelin1 puncta positive for TARP γ8 co-localized with Homer1, whereas 45.0% were not co-localized (Supplementary Fig. S7c). We found that 44.0 ± 1.8% (Supplementary Fig. S7d) of Homer1 puncta were positive for Noelin1, thereby localizing the GluA-Noelin1-TARP γ8 complexes to both the PSD and extra-synaptic regions.
We then assessed the localization of the Cpt1c containing complex. Consistent with its occurrence in the microsomal fraction, we found that Cpt1c primarily co-localized with the AMPAR in the cell body and dendrites (Supplementary Fig. S8a). Co-staining of Cpt1c and Homer1 revealed that Cpt1c does not overlap with Homer1 and that Cpt1c is detected in the dendrite (Supplementary Fig. S8b). Thus, the Cpt1c-containing AMPAR complex is primarily localized outside of the synapse.
Discussion

Based on the high-density immuno-precipitation experiments we hypothesized that there are multiple AMPAR subcomplexes in the hippocampus. Using high-density interaction proteomics to identify complexes with distinct protein composition, only partial success in identifying AMPAR complexes was achieved due to the extensive overlap in proteins resident in different subcomplexes. To separate these subcomplexes, a multi-dimensional IPBN-MS strategy was devised to expand on the limited resolution of individual separation steps. This led to the identification of distinct AMPAR subcomplexes. Subsequent co-expression and co-IP experiments confirmed protein interactions and imaging by super-resolution microscopy demonstrated co-localization of some of the proteins from these complexes in specific sub-cellular domains.

In the high-density IPs, bait proteins were identified with high IBAQ values, indicating excellent sensitivity of the IP-MS experiments. The presence of AMPAR subunits in all IPs confirms the bait proteins as constituents of the AMPAR complex. Some AMPAR interactors reported previously were recovered at low level. Neuritin was detected only in the Noelin1 IP at the detection limit of the MS measurement. Rap2b was found in many IPs at low level and in subsequent IPBN-MS Rap2b was found in many BN fractions without a clear elution pattern typical for co-association with other proteins. Together, we were not able to confirm Rap2b as a specific AMPAR interactor.

Most prominently, IP-MS revealed the high complexity of auxiliary proteins of the AMPARs. For example, IP for TARP \( \gamma 8 \) identified GluAs, Cnih2, Shisa6, Noelin1–3, Dlg4 and Prrt1, and four other members of TARP \( \gamma \) family. Similarly, TARP \( \gamma 8 \) was present in IPs of Cnih2, Shisa6, Noelin1, and Prrt1 and at a lower level in the IP of Frrs1l. Thus, two different scenarios are possible; either these proteins all interact and are embedded in a single AMPAR complex, or they represent the collection of protein constituents originating from independent subcomplexes sharing subsets of protein species. As the classical high-density interaction proteomics cannot distinguish overlapping protein complexes, we explored the feasibility of using BN after IP (IPBN-MS) to resolve these potential complexes.

Prerequisite for IPBN-MS is that protein complexes have sufficient mass difference and that the protein constituents of the complex have the necessary affinity to retain the complex. The IPBN-MS using the antibody against GluA2/3 indeed resolved several distinct protein complexes. Importantly, TARP \( \gamma 8 \) and Cnih2 each had
a peak at a distinct molecular weight, in which the TARP γ8 lacking Cnih-Frrs1l forms a major subcomplex with AMPARs. There are apparently multiple complexes with overlapping masses extending > 1 MDa, most of which contain TARP γ8. Clearly, IPBN-MS using a single antibody has the resolving power to characterize the protein composition of subcomplexes.

To further gain insight into AMPAR subcomplexes, we carried out IPBN-MS on several AMPAR interactors. We reasoned that (1) each IPBN-MS would cover a subset of AMPAR protein complexes which favours the identification of individual AMPAR-containing complexes, and that, (2) integration of IPBN-MS data would allow reconstruction of full profiles of AMPAR subcomplexes, the masses of which should be in agreement with those revealed by AMPAR IPBN-MS. To calibrate each BN experiment, internal molecular weight standards were spiked into every sample prior to BN electrophoresis. This turned out a crucial step for the subsequent accurate alignment of protein masses across the different IPBN-MS experiments.

In the IPBN-MS experiment for GluA2/3 we observed a low molecular weight (slice +3 with respect to the 720 kDa) complex containing the majority of extractable AMPA receptors along with Cnih2 and Frrs1l, whereas the TARPs and others clearly eluted at higher molecular weights. Interestingly, in hippocampus a native receptor without associated proteins was not detected. Neither, did we find individual GluA subunits or AMPAR-associated proteins as single entities in the low MW range. This indicates that complexes are stable, survive BN separation and are a reflection of those immuno-precipitated. Obviously, we cannot exclude that less stable interactions might have been lost during IP. Reverse IPBN-MS for Cnih2 revealed that AMPAR, Frrs1l associated with Cnih2 also eluted at the low molecular weight. This was further validated by Frrs1l IPBN-MS, in which, all three of these components eluted at the same position as in the IPBN-MS of Cnih2 and GluA2/3. Thus, we confirmed the existence of AMPAR-Cnih2-Frrs1l complex from three independent experiments.

Furthermore, we demonstrated that the higher molecular weight complexes containing TARP γ8 could be subdivided into three subcomplexes, the known TARPγ8-Cnih2-AMPAR complex, Shisa6-Prrt1-TARPγ8-AMPAR complex, as well as the TARPγ8-Noelin-AMPAR complex. TARP γ8 and Cnih2 have been shown involved in trafficking of synaptic AMPARs, the membrane expression of AMPARs, and modulation of the biophysical properties of the AMPAR101,126–128,131,132. The present study reveals that all AMPAR subcomplexes contain multiple interactors, indicating that AMPAR properties and/or their trafficking/subcellular localization are most likely
regulated in a combinatorial manner depending on multiple interactors. Non-TARP containing complexes were identified to contain Cpt1c, Frrs1l and Sacm1l and from IPBN-MS for Cpt1c and Frrs1l, the high molecular weight complex containing Cpt1c-Frrs1l-Sacm1l-AMPAR was identified (summarized in Table 1).

Using co-expression and co-IP experiments of AMPAR subunits and their interactors in HEK293 cells we tested their mutual direct interaction. We determined that Cnih2, Frrs1l, Prrt1, Shisa6 and Noelin1, all have the ability to directly interact with the GluA2 subunit of the AMPA receptor. Furthermore, we validated that in HEK293 cells, a native AMPAR-Cnih2-Frrs1l complex can be reconstituted and we found that Cnih2 and Frrs1l themselves have a weak affinity for each other. In complexes containing TARP γ8, the presence of large number of AMPAR-associated proteins prevents the reconstitution of all components in HEK293 cells due to the limited number of constructs that can be transfected successfully. Therefore, we assessed direct interaction in a pairwise manner and observed that Shisa6 and TARP γ8 have a weak affinity, whereas Prrt1 and TARP γ8 can directly interact. Furthermore, no direct interaction between Noelin1 and TARP γ8 was observed. Since it is well documented that TARP γ8 and the AMPAR can directly interact\(^{101,127}\), in the case of the TARP γ8-Noelin1-AMPAR complex, the presence of AMPAR is critical for the complex formation. Similarly, for the Shisa6-TARPγ8-Prrt1-AMPAR complex, because of the weak affinity between TARP γ8 and Shisa6, we conclude that the presence of the AMPAR is critical for complex formation. Based on our data, TARP γ8 and Prrt1 can potentially occur in a pre-associated form. In case of the Cpt1c-Frrs1l-Sacm1l-AMPAR complex, we demonstrated that both Cpt1c and Sacm1l can directly interact with the AMPAR, and Cpt1c-Frrs1l-AMPAR complex can be reconstituted in HEK293 cells. Taken together, the HEK293 expression study validates the existence of the subcomplexes we described using IPBN-MS in heterologous systems in which in these components are not expressed endogenously.

Based on immunoblotting of AMPAR interacting proteins in different biochemical preparations, we observed primarily three groups of proteins. Those that are enriched in the PSD fraction (TARP γ8, -γ2, Shisa6 and Noelin1), those enriched in the microsome fraction (Cpt1c, Frrs1l and Sacm1l) and those that do not show particular enrichment in any fraction (Cnih2, Prrt1). To investigate PSD enriched sets of proteins, we performed structured illumination microscopy (SIM). Unlike most studies using confocal microscopy, SIM provides the resolution to classify a protein as present in the PSD\(^{194}\). Furthermore, we performed quantification of the data to determine
number of PSDs positive for a protein of interest and determined their distribution around synaptic spots to get an estimate of co-localization as well as the distribution of these co-localized spots. The unique components of the TARP γ8 containing complexes were Shisa6 and Noelin1. Using SIM, we determined that Noelin1 is distributed into extra-synaptic and synaptic pools with the AMPA receptor (Supplementary Fig. S8), consistent with the distribution of TARP γ8 at synaptic and extrasynaptic sites193. Thus, the AMPAR-Noelin1-TARPγ8 complex is distributed between extra-synaptic and synaptic pools. Shisa6, a highly PSD enriched protein54, showed high co-localization with TARP γ8. It has been shown that Shisa6 affects AMPAR lateral diffusion and as well promotes slower decay and channel properties54. It will therefore be interesting to determine the functional role of Prrt1, Shisa6 and TARP γ8 as part of the same AMPAR complex. The Cpt1c immunoreactivity is restricted to the cell body and the dendrites, but is never found in the PSD. This along with the microsomal enrichment of Cpt1c, Frrs1l and Sacm1l localizes the Cpt1c-Frrs1l-Sacm1l-AMPAR complex to intracellular compartments, suggesting that this represents a trafficking complex for delivery to the synapse103,140.

Taken together, we found evidence for the existence of multiple AMPAR subcomplexes with enrichment in subcellular domains (Table 1). Given the differential biochemical enrichment and different localization based on SIM data, some AMPAR subcomplexes are likely present within a single neuron. This study cannot exclude that some AMPAR complexes might occur in different neurons. In recent years AMPAR-associated proteins have been shown to confer specific properties to the receptor, in particular regarding membrane mobility and biophysical properties of the receptor. Thus, it is likely to hypothesize that different subcomplexes of AMPARs, as shown here, reflect different functional units. Considering the complex regulation of the AMPARs involves multiple steps87, starting with biosynthesis in the ER to the delivery in the synapse, the observation of AMPAR subcomplex diversity is not surprising. For instance, Cpt1c, Cninh2 and TARP γ8 have been shown to regulate AMPAR biosynthesis and surface expression131, whereas others, such as Shisa6 and TARP γ8, have been shown to regulate AMPAR lateral mobility in the membrane in addition to AMPAR conductance-modifying properties54. The current study brings to notion that there is a need of understanding the interplay of the interacting proteins with the AMPAR. The focus on interactions of interactors and AMPAR on a one to one basis seem less relevant given that we find AMPARs only in assemblies that contain two other auxiliary proteins minimally. Another intriguing question is how in the route of
AMPARs, from site of synthesis to synaptic function, the subsequent AMPAR functional units might change composition and how these processes are regulated.
Table 1: Summary of AMPAR subcomplexes detected in the hippocampus using IPBN-MS. The table shows multiple experiments and the location on the gel in which the subcomplexes are observed along with information on biochemical enrichment and super-resolution microscopy.

<table>
<thead>
<tr>
<th>Subcomplex composition</th>
<th>Experiment in which it is observed</th>
<th>Slice number with respect to 720 kDa marker</th>
<th>Biochemical enrichment/ SIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 GluA-Cnih2-Frrs1l</td>
<td>1. GluA2/3 2. Cnih2 3. Frrs1l</td>
<td>1</td>
<td>Microsomal</td>
</tr>
<tr>
<td>2 GluA-Cnih2-TARP γ8</td>
<td>1. GluA23 2. Cnih2 3. TARP γ8</td>
<td>-3</td>
<td>Synaptic and ER/Golgi</td>
</tr>
<tr>
<td>4 GluA-Noelin1-TARPγ8</td>
<td>1. GluA2/3 2. TARP γ8 3. Noelin1 - IP</td>
<td>-8</td>
<td>Synaptic and extrasynaptic</td>
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Materials and Methods

Preparation of Synaptic P2 + Microsome (P2+M) Fractions

Adult C57Bl6 mice were sacrificed by cervical dislocation and the brain regions corresponding to cortex, hippocampus and cerebellum were dissected and stored at -80 °C until further use. Frozen brains were homogenized in ice-cold homogenization buffer (0.32 M Sucrose, 5 mM HEPES, pH 7.4) with protease inhibitor cocktail (Roche) using a glass homogenizer (PotterS from B. Braun) set at 900 rpm for 12 strokes. The homogenate was collected, centrifuged at 1000 x g for 10 min. to get rid of cell debris and nucleus. The supernatant was then centrifuged at 100,000 x g for 2 h at 4°C to obtain the P2+M pellet, which was suspended in sample suspension buffer (25 mM HEPES, 150 mM NaCl and protease inhibitor cocktail and stored at -80 °C until further use.

Affinity Purification of Protein Complexes by Immunoprecipitation (IP)

For a single IP experiment, 5 mg P2+M fraction was extracted twice in 1 ml extraction buffer (final concentration 1% n-Dodecyl β-D-maltoside (DDM) (Sigma), 150 mM NaCl, 25 mM HEPES pH 7.4 and Protease Inhibitor cocktail) for 1 h each at 4 °C on a rotor at 10 rpm. After each extraction, the samples were centrifuged at 20,000 x g for 20 minutes. The supernatant was pooled after each extraction. 10 µg of each antibody was added to the pooled supernatant and incubated overnight at 4 °C on a rotor at 10 rpm. 50 µl of protein A/G beads (Santacruz) were added per IP and incubated for 1 h at 4 °C. After incubation with the beads, the samples were centrifuged at 1000 x g for 1 minute and supernatant was discarded. The beads were washed four times with 1 ml washing buffer (0.1% DDM, 150 mM NaCl, 250 mM HEPES, pH 7.4), supernatants discarded and the final pellet was eluted in SDS sample buffer (SDS and DTT and bromophenol blue) for separation on SDS PAGE. As negative controls, we used empty beads (without the addition of antibody) and peptide blocking control. For peptide blocking controls, 10 µg antibody was incubated with 50 µg peptide for 30 minutes on ice, prior to addition to the supernatant and samples were treated similarly thereafter. Each IP was done at least twice with at least 5-fold enrichment versus respective controls.
IP-Blue native Electrophoresis (IP-BN) separation of Protein Complex Isoforms

For a single IP-BN experiment, 30 mg P2+M fraction was extracted twice in DDM containing extraction buffer. The supernatant was incubated with 100 µg antibody and incubated overnight at 4 °C. Post incubation, 1000 µl of Protein A/G beads were added to the supernatant with the antibody and incubated for 1 h at 4 °C. The immunopurified complexes were then eluted twice from the beads using 500 µg peptide dissolved in 1 ml washing buffer for 1 h. The eluted complexes were concentrated using a 30 kDa filter (Biorad) for 30 min. The samples were mixed with 8x BN-PAGE loading buffer (Invitrogen), 0.5 µl Molecular weight marker (Invitrogen), 1 µl Coomassie G-250 mix (Invitrogen) and then loaded on a 3-12% polyacrylamide precast BN-PAGE gel (Invitrogen). The gel was run at 1 mA constant current for 1 h and then switched to 2 mA constant current for 16 hs.

In gel digestion and recovery of peptides

For IP experiments, samples containing SDS loading buffer were heated at 98 °C. Cysteines were derivatized for using 30% acrylamide for 30 minutes at room temperature with shaking, after which they were loaded on 10% poly acrylamide gels run at 100 V. Post run, the gels were fixed overnight in fixation solution (Phosphoric acid and Ethanol) and later stained with colloidal coomassie. Each sample lane was cut into 5 pieces, and then further cut into approx. 1 mm by 1 mm pieces, washed and destained with a mixture of 50 mM ammonium bicarbonate in acetonitrile. Finally, the dried gel pieces were digested with trypsin (Promega) and dissolved in 50 mM ammonium bicarbonate overnight. The peptides were three times extracted in 0.1 % Trifluoroacetic Acid (TFA) and 50% acetonitrile, dried in a speed vac and stored at -20 °C until mass spectrometry analysis.

For IPBN experiments, the gels were fixed overnight in fixation solution (phosphoric acid and ethanol) and later stained with colloidal coomassie. The gels were scanned using a Biorad scanner. Post scanning, each lane of 7.2 cm was cut using a grid cutter (The Gel Company) into 72 slices of 1 mm each. The cysteines were derivatized using 1 mM TCEP in 50 mM ammonium bicarbonate for 30 minutes followed by blocking using 4 mM MMTS. Post derivatization, the gel pieces were destained as described previously, trypsin digested and the corresponding peptides were eluted using 50% acetonitrile and 0.1% TFA. The samples were dried in a speed-vac and stored at -20 °C before analysis on the mass spectrometer.
LC-MS/MS analysis

Peptides were analyzed by nano-LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific) coupled to the TripleTOF 5600 mass spectrometer (Sciex). Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 μm i.d., 5μm particle size, from Dionex) and fractionated on a 200 mm Alltima C18 column (100 μm i.d., 3 μm particle size). The acetonitrile concentration in the mobile phase was increased from 5 to 30% in 90 min, to 40% in 5 min, and to 90% in another 5 min, at a flow rate of 500 nL/min. The eluted peptides were electro-sprayed into the TripleTOF MS. The nano-spray needle voltage was set to 2500V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (m/z 350−1200, 250 ms) followed by a top 25 MS/MS (85 ms per MS/MS, precursor ion > 90 counts/s, charge state from +2 to +5) with an exclusion time of 16 s once the peptide was fragmented. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 10eVMS/MS spectra were searched against the Uniprot proteomics database (version 2013-01-06) with the MaxQuant software (version 1.3.0.5). The search parameters were set to cysteine alkylation with acrylamide from SDS-PAGE gels or with MMTS from BN-PAGE gels, and digestion with trypsin or trypsin+LysC.

Data Analysis

We apply a set of filters to the raw IP data in order to discriminate true- and false-positive bait-prey interactions. The large set of experiments was performed in several batches over time and in order to negate batch-effects, we pair IPs with their respective peptide-blocking and empty-bead controls within the same experiment batch. At the antibody level, preys of interest must be detected in at least half the replicates and be 10-fold enriched compared to available control experiments. Finally, we defined true-positive interactors for a bait as non-contaminant proteins (contaminants are proteins in the MaxQuant contaminant database or from trypsin, keratin, immunoglobulin protein families) that pass above criteria for each respective antibody in each experiment batch. Using prey proteins in the dataset that were previously reported as high confident AMPAR interactors\textsuperscript{115,116}, we plot a heatmap in which true-positive bait-prey interactions are shown as their average log10 abundance over all respective IPs and false-positive bait-prey interactions are set to zero.
Data Analysis, IP-BN peptide and protein quantification

Many proteins of interest are low abundant in the blue native gel slices and therefore hard to accurately quantify by peak detection algorithms. So we performed manual peak picking of each peptide of interest over all blue native gel slices using Skyline to quantify their abundance. For each IP-BN, we selected the corresponding MS1 peak area for the m/z and retention time at which a peptide was identified by MaxQuant (in any slice of a given experiment). For the same peptide, we performed peak-picking of the same precursor over all slices with consistency in m/z and retention time.

Peptide data was exported from Skyline and processed with the R language for statistics. The protein (abundance) profile over slices was computed in two stages. First, peptide abundances were divided by their total intensity over all slices in which all selected peptides from the same protein were detected (making peptide abundances/intensities comparable within the same protein, we refer to this as the ‘rowsum’ scaled abundances). The protein profile is then computed as the mean value at each slice. In typical figures, we scaled the protein profile between zero and the maximum value over all slices, which resulted in 0-100% relative intensities that enables intuitive interpretation of similarities and differences between protein elution profiles over the blue native gel. Alternatively, a protein profile can be scaled by the highest value of any of its peptides in any slice.

Preparation of Subcellular fractions

The subcellular fractions were prepared from adult C57Bl6 mouse hippocampus as described in von Engelhardt et al., 2010 with some modifications as in Chapter 3. Hippocampi were homogenized in homogenization buffer (0.32 M Sucrose, 5 mM HEPES (pH 7.4) with protease inhibitors) to obtain the homogenate fraction. The homogenate was centrifuged at 1,000 x g to obtain the supernatant, which was further centrifuged at 18,000 x g for 20 minutes to obtain the P2 fraction. The supernatant was further centrifuged at 100,000 x g for 20 minutes to obtain the microsomal fraction. The supernatant from the 1,000 x g step was separately loaded on to 0.85 M/1.2 M sucrose gradient, centrifuged for 2 hs at 100,000 x g to obtain the synaptosome fraction at the 0.85/1.2 M interface. The synaptosome fraction was either given an osmotic shock in 5 mM HEPES pH 7.4 for 30 minutes and then loaded again on 0.85/1.2 M sucrose gradient to obtain the synaptic membrane fraction or extracted twice with 1% Triton X-100 for 30 minutes, then loaded on 1.5/2 M sucrose gradient to obtain the postsynaptic density fraction. 5 µg of each subfraction was mixed with 5x SDS containing loading
buffer loaded on a SDS-PAGE gel, transferred overnight on PVDF membrane and stained for the protein of interest.

**Immunoblot Analysis:**

Samples containing SDS loading buffer were run on 5–12% Criterion™ TGX Stain-Free™ precast gels from Bio-Rad, transferred onto a PVDF membrane overnight. The membranes were then blocked using 5% non-fat milk in Tris-buffered saline (pH 7.4) with Tween-20 (TBST) followed by an overnight incubation with the primary antibody at 4 °C. The blots were washed three times in TBST after the primary antibody incubation followed by a 1 h incubation of HRP-conjugated secondary antibody in 3% non-fat milk. The blots were washed three times, incubated with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and scanned on an Odyssey® Fc scanner (Licor Biosciences).

**Immunostainings and Imaging**

Immunostainings and SIM microscopy were performed as described in chapter 2.

**SIM data analysis**

Reconstructed 3D-SIM images were analyzed with imageJ extended in the FIJI framework. Analysis was performed on maximum projections of the z-stack. A threshold, determined by the moments algorithm, was applied on PSD and interactor protein signals. Particles larger than 25 pixels were detected and marked as region of interest (ROI) center of mass of all ROIs and integrated AMPA signal inside the interactor ROIs were measured. Centers of mass were used to determine the distance between an interactor ROI and the closest PSD ROI.
Supplementary Figure S1. a) Immunoblot characterization of antibodies used for immunoprecipitation and IPBN-MS experiments. Arrows indicate the correct molecular weight of the protein. ‘+pep’ indicates experiments where peptide antigens were used to block the antibody prior to incubation with the blot. b) 1D Immunoblot of P2 + M fraction extracted in 2% DDM separated on blue native page (BN-PAGE) followed by immuno-detection for GluA2/3 and TARP γ8 reveals AMPAR-IP complexes near 720 kDa and TARP γ8 -IP complexes at higher molecular weights.
Supplementary Figure S2: a) Normalized intensity profile for GluA1-3, TARP γ8 and Noelin1 after GluA2/3 IPBN-MS shows co-elution at peak size at slice –8 with respect to the 720 kDa marker supporting the Noelin1-TARP γ8-AMPAR complex. b) Normalized intensity profile for GluA1-3, TARP γ8 and Noelin1 after TARP γ8 IPBN-MS shows co-elution at peak size also at slice –8 with respect to the 720 kDa marker confirming the Noelin-TARP γ8-AMPAR complex.
Supplementary Figure S3. IPBN-MS shows co-elution size at slice –5 with respect to the 720 kDa marker supporting the Cnih2-Frrs1l-TARPγ8-Prrt1-AMPAR complex as revealed by co-eluting peaks for GluA1–3, Prrt1, -2, TARP γ8, Cnih2 and Frrs1l after (a) GluA2/3 IP-BNMS, (b) Prrt1 IP-BNMS, (c) Frrs1l IP-BNMS, (d) Cnih2 IP-BNMS and (e) TARP γ8 IP-BNMS
Supplementary Figure S4. Direct interaction analysis of TARP γ8 and Shisa6 (a), Prrt1 and GluA2 (b), Prrt1 and TARP γ8 (c), Noelin1 and GluA2 (d), and Noelin1 and TARP γ8 (e) in HEK293T cells. Shisa6 and TARP γ8 show weak binding, whereas Prrt1 binds directly with GluA2 and TARP γ8. Noelin1 binds directly with GluA2, but not with TARP γ8.
Supplementary Figure S5. a) Triple staining of TARP γ8 (green), GluA2 (red) and Homer1 (blue) of primary cultured rat neurons at DIV17 reveals signal overlap of the three channels at synaptic puncta (Homer1 positive) as well as non-synaptic puncta. White lines demarcate zoomed in areas. b) Density plot from TARP γ8-GluA2-Homer1 staining showing TARP γ8 puncta positive for GluA2 as a function of distance from the nearest Homer1 puncta reveals 49.3% TARP γ8 co-localized with GluA2 are at a distance < 0.5 µm from the nearest Homer1 puncta (n = 16) (red line represents 0.5 µm chosen as cutoff for the size of the PSD). c) Bar plot of percentage of Homer1 puncta positive for TARP γ8 (green, n = 19) and GluA2 (red, n = 16) obtained from stainings shown in panels a and b. d) Immunoblotting for TARP γ8 and TARP γ2 in different biochemical fractions of the mouse hippocampus shows enrichment of TARP γ8 in the synaptic fractions and TARP γ2 only in the PSD fraction. (Homo, homogenate; P2, pellet 2, M, microsome; SyS, synaptosome; SyM, synaptic membrane; PSD, postsynaptic density).
Supplementary Figure S6. Primary cultured rat neurons at DIV17 reveal overlap between Shisa6 and GluA2 and Shisa6 and TARP γ8 immunoreactivity. a) Double staining of Shisa6-FLAG (green) and GluA2 (red). b) Shisa6-FLAG (green) and TARP γ8 (red). Zoomed in areas in the white boxes. Scale bar: 5 µm for images on left and 2 µm for zoomed images on right c) Bar plot of percentage of Shisa6 puncta positive for TARP γ8 (Green, n = 9) and GluA2 (red, n = 9) obtained from staining shown in panels a and b.
Supplementary Figure S7. a) Triple staining of Noelin1 (green), GluA2 (red) and Homer1 (blue) of primary cultured rat neurons at DIV17 reveals overlap between of three channels at synaptic puncta (Homer1 positive) as well as non-synaptic puncta. b) Triple staining of Noelin1 (green), TARP γ8 (red) and Homer1 (blue) of primary cultured rat neurons at DIV17 reveals overlap of the three channels at synaptic puncta (Homer1 positive) as well as non-synaptic puncta. White line demarcates zoomed in areas. Scale bar: 5 µm for images on left and 2 µm for zoomed images on right. c) Density plot from Noelin1-GluA2-Homer1 staining showing Noelin1 puncta positive for GluA2 as a function of distance from the nearest Homer1 puncta reveals that 54.3% Noelin1 puncta co-localized with GluA2 are at a distance below 0.5 µm from the nearest Homer1 puncta (n = 7) (red line represents 0.5 µm chosen as cutoff for the size of PSD). d) Bar plot of percentage of Homer1 puncta positive for Noelin1 (Green, n = 17) and TARP γ8 (red, n = 19) and GluA2 (blue, n = 16) obtained from staining in panels a and b.

Supplementary Figure S8. a,b) Double staining of Cpt1c-myc (green) and GluA2 (red)(a), and Cpt1c- myc and Homer1 (b) in primary cultured rat neurons at DIV17 reveals overlap between Cpt1c and GluA2 in the cell body and dendrites, whereas Cpt1c does not co-localize with Homer1 at synaptic spots. White line represents areas that have been zoomed in. Scale bar: 5 µm for images on left and 2 µm for zoomed images on right.
Chapter 5

Noelin1 affects lateral mobility of synaptic AMPA receptors

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#Corresponding authors
Abstract

Lateral diffusion of the AMPA-type glutamate receptor (AMPAR) in the neuronal plasma membrane serves an important role in synaptic plasticity. Here, we investigated the role of Noelin1 in AMPAR mobility and its dependency on the extracellular matrix (ECM). We found that Noelin1 interacts with the AMPAR with high affinity, however, it does not affect AMPAR channel conductance and desensitization properties. Noelin1 co-localizes with synaptic and extra-synaptic AMPARs. Using single particle tracking we show that, whereas the ECM does not constrain the synaptic pool of AMPARs, the interaction of Noelin1 with the AMPAR reduces lateral mobility of both synaptic and extra-synaptic AMPARs. Thus, both ECM and Noelin1 mediate the constraint on AMPAR lateral mobility, however in different pools of receptors. Finally, we show that Noelin1 expression at the synapse is regulated in an activity-dependent manner. This is the first evidence for the role of a secreted AMPAR-interacting protein that acts specifically on receptor mobility leaving the AMPAR channel properties unaltered.
Introduction

Excitatory synaptic transmission in the brain largely depends on glutamate signaling involving AMPA-type glutamate receptors (AMPARs). Regulation of synaptic strength is a crucial element in signal processing, in particular in plasticity-dependent processes, such as learning and memory\(^{195}\). Synaptic plasticity can originate pre- and postsynaptically. Postsynaptic plasticity mechanisms most importantly include the alteration of the number of synaptic AMPARs and/or the modulation of their biophysical properties. In recent years, several AMPAR-interacting proteins have been identified and functionally characterized. Some of these have been shown to fulfill the criteria of an auxiliary subunit. In particular, transmembrane AMPA regulatory proteins (TARPs)\(^{118,196}\), cornichons\(^{128}\), Shisa6\(^{54}\) and Shisa9\(^{134}\) (CKAMP44) were shown to modify AMPAR biophysical properties and/or affecting receptor trafficking and/or trapping these receptors at the postsynaptic density (PSD).

Several auxiliary subunits critically affect aspects of lateral diffusion of the AMPAR. For instance, the C-terminal tail of TARP-γ2 and Shisa6 can bind to PSD95, and this interaction was shown to trap AMPARs at the postsynaptic density\(^{53,54,92,93}\). Elevated Ca\(^{2+}\) levels inside spines after synaptic potentiation are known to alter the affinity of AMPAR bound TARP to postsynaptic scaffolds. These processes might determine the numbers of surface synaptic receptors, can restrict the localization of receptors to synaptic nanodomains\(^{188,189}\), affect desensitization and alter short- and long-term synaptic plasticity. Spines themselves may also act as a diffusion barrier. Membrane lipid composition and architecture are other factors that affect AMPAR lateral diffusion\(^{109}\). Apart from these factors, the extracellular matrix (ECM) was shown to alter membrane receptor lateral diffusion and thereby affect short term synaptic plasticity\(^{157}\), as it might act as a local diffusion barrier for AMPARs on dendrites and into synapses.

Here, we focused on assessing the function of the secreted glycoprotein Noelin1. This protein was initially identified as an AMPAR-interacting protein by subunit GluA1 immunoprecipitation of synaptosomal preparations using GluA1 gene deletion as control\(^{134}\) (Engelhardt et al., 2010), and this was recently validate\(^{115,116,133}\). The Noelin1 gene gives rise to four alternatively spliced transcripts (Noelin1-1, 1-2, 1-3, 1-4)\(^{197}\). The Noelin1 transcript generates protein isoforms sharing the M-region, with isoforms 1-1 and 1-3 specifically containing the Olfactomedin domain (Supplementary Fig. 1a). The Noelin1 gene is highly expressed throughout the brain, predominantly in
the hippocampus and cortex (Supplementary Fig. 1b,c). Noelin1 is a highly interspecies-conserved protein. Western blots of Noelin1 in brain tissue and heterologous expression the protein in HEK293T cells shows that it forms covalently bound tetramers with a molecular weight > 250 kDa. Noelin1 was shown to interact with the Nogo receptor and is implicated in the modulation of axonal outgrowth. Also, it has been shown to interact with the amyloid precursor protein and to modulate cortical cell migration. These and other studies have pointed to a role of Noelin1 as a regulatory extracellular signaling molecule. However, the role that Noelin1 plays in AMPAR regulation has remained largely unexplored. Here, using biochemical, electrophysiological and cellular imaging approaches, we identify a role of Noelin1 in AMPAR mobility, justifying its role as a putative AMPAR auxiliary subunit with modulatory function.
Results

Noelin1 is part of a stable complex with the AMPA receptor and TARP-γ8

AMPAR immunoprecipitations ( IPs) have repeatedly identified Noelin1 as an AMPAR-interacting protein\textsuperscript{115,134,200}. In agreement with this, we find Noelin1 present in synaptosomal and synaptic membrane fractions and particularly enriched in the Triton X-100 insoluble postsynaptic density fraction (Fig. 1a). To establish the molecular composition of the Noelin1-AMPAR complex, we first determined whether the Noelin1-AMPAR complex contained other AMPAR-interacting proteins. First, we used a specific Noelin1 antibody and performed immunoprecipitation followed by LC-MS-MS analysis of hippocampal P2+Microsome fractions. Examining the list of potential interacting proteins of Noelin1 for currently known AMPAR-interacting proteins\textsuperscript{189} reveals that apart from AMPAR subunits GluA1, -2, -3 the Noelin1 IP also contains TARP-γ8 and Neuritin (Table 1).

Inspection of the peptides obtained for Noelin1 in the AMPAR IPs in the hippocampus revealed that these were not unique to a specific Noelin1 splice variant (Supplementary Fig. 1a). Therefore, to determine which specific isoform of Noelin1 interacts with the AMPAR, we performed GluA2/3 IPs in absence and presence of a peptide epitope block followed by Western blotting. This revealed the specific interaction of Noelin1-1, 1-3 and 1-4 with GluA2/3 (Fig. 1b), with enrichment of Noelin1-3 and Noelin1-4. Noelin1-2 has been proposed to be a part of the WAVE1 complex in mitochondria\textsuperscript{202}, which might explain that Noelin1-2 has no specific interaction with the AMPAR. Reverse IP using the Noelin1 antibody also revealed enrichment of the Noelin1-1, 1-3 and 1-4 isoforms in the IP along with GluA2/3 compared to a peptide epitope blocking control (Fig. 1b). Thus, these experiments demonstrate that most Noelin1 isoforms predominantly form a stable complex with TARP-γ8-containing AMPARs, with Noelin1-3 being most abundant.

Having established that Noelin1 and the AMPAR are part of one protein complex, we determined whether the interaction between the AMPAR and Noelin1 is direct or enabled by other neuronal accessory proteins. GluA1 and GluA2 AMPAR subunits were co-expressed with an EGFP-fused form of Noelin1-1, and untagged Noelin1-3 and 1-4 in HEK293T cells, and GluA1 and GluA2 IPs were performed. SDS-PAGE and Western blotting for Noelin1 (Fig. 1c, Supplementary Fig. 2a) show protein bands corresponding to Noelin1-1, 1-3 and 1-4 in both GluA2 and GluA1 IPs that were
absent in the controls. This indicates that Noelin1 directly interacts with the AMPAR, without the necessity of additional neuronal expressed proteins.

**Figure 1. Noelin1 is enriched in the PSD and interacts with AMPA receptor subunits.**

a) Biochemical fractions (homogenate (H), microsomes (M), crude synaptic membranes (P2), synaptosomes (SS), synaptic membranes (SM), postsynaptic density fraction (PSD; Triton X-100 insoluble fraction)) of mature mouse hippocampus reveals an enrichment of Noelin1 isoforms in the PSD together with GluA2/3, and distinct from the presynaptic marker Synaptophysin (Syp).

b) Western blot (WB) analysis of native hippocampal immunoprecipitated GluA2/3 complexes reveals the co-precipitation of Noelin1 isoforms (Noelin1-1, 1-3, 1-4) enriched in the GluA2/3 IP vs. the peptide blocking (PB) control (left panel). The size of the Noelin1-2 isoform corresponds to antibody light chain and hence shows lesser enrichment. Western blot analysis of immunoprecipitated Noelin1 complexes in the Noelin1 IP vs. the PB control confirms the specific interaction with GluA2/3 (right panel).

c) Heterologous expression (HEK293T cells) of individual Noelin1 isoforms (Noelin1-1, left; Noelin1-3, middle; Noelin1-4, right) with the GluA2 subunit shows specific enrichment of each isoform in cell lysates co-precipitated with GluA2. The molecular weight (kDa) is indicated.
Table 1: Mass spectrometric analysis of native hippocampal Noelin1 complexes reveals association with AMPAR and established AMPAR interactors. Analysis of native Noelin1 complexes, immunoprecipitated from the hippocampi of WT animals (crude synaptic membranes; n=4 IPs), identified AMPAR subunits and Neuritin, an established AMPAR interactor, as most prominent parts of the Noelin1 complex compared with the IP using an IgG antibody (negative control). For reference, 2 IPs are listed for GluA2/3 and GluA2, respectively. IP-ed proteins: Shisa6 (dark blue), AMPAR subunits (light blue), established AMPAR interacting proteins (yellow). Values (iBAQ) obtained from MaxQuant search of IP experiments are indicated for each IP.

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Surface plasmon resonance biosensor analysis reveals a Noelin1 oligomerization-dependent interaction with the AMPAR

Surface plasmon resonance (SPR) biosensor technology was used to characterize the interaction between Noelin1-3 and the AMPAR, and to determine the kinetic rate constants. A C-terminal GluA2 antibody was covalently immobilized on a biosensor surface, and an n-Dodecyl β-D-maltoside solubilized GluA2 AMPAR was subsequently captured to the surface. After preparing the surface, Noelin1-3, obtained from transfected HEK293T cells after ultrafiltration (Supplementary Fig. 2b,c), was injected in 2-fold dilution series (7.2 nM – 0.45 nM) and a clear interaction with GluA2 AMPARs was observed (Fig. 2a). The apparent association and dissociation rate constants were determined by using a reversible 1-step interaction model to \( k_{\text{on}} = 2 \times 10^6 \text{Ms}^{-1} \) (\( p_k_{\text{on}} = -6.3 \pm 0.1, n = 5 \)) and \( k_{\text{off}} = 2 \times 10^{-3} \text{s}^{-1} \) (\( p_k_{\text{off}} = 2.6 \pm 0.1, n = 5 \)), which corresponds to an affinity of \( K_D = 1 \times 10^{-9} \text{M} \). Control experiments with samples from non-transfected HEK293T cells were performed under the same conditions as for Noelin1-3 and no interaction was detected (Fig. 2b, grey traces). These results confirm that Noelin1-3 interacts directly, and with high affinity, with the GluA2 AMPAR.

As Noelin1 is known to form oligomers via disulfide bridges present in the common M-region of Noelin1 (Ando et al., 2005) (Supplementary Fig. 1a), we investigated whether Noelin1 oligomerization (Supplementary Fig. 2d) has an important role in the interaction with the AMPAR. Noelin1-3 was injected over the GluA2 AMPAR-coated surface in the presence and absence of 1 mM dithiothreitol (DTT), otherwise under the same conditions. Noelin1-3 was not seen to interact with the GluA2 AMPAR under reducing conditions (Fig. 2b, red traces), whereas the interaction was detected under non-reducing conditions (Fig. 2b, blue traces). To control for potential loss of GluA2 AMPAR functionality due to the injection of DTT, Noelin1-3 was injected alternatively in the presence and absence of DTT. The experiments demonstrated that the immobilized GluA2 AMPAR was not affected by the injection of DTT over the sensor surface, and that the observed loss of binding under reducing conditions was specific for Noelin1-3 (Fig. 2b,c). These data support the observation that Noelin1 can form disulfide-linked homo-tetramers. We then established whether Noelin1 is capable of forming high molecular weight complexes with the AMPAR in vivo. Indeed, a particularly high molecular weight complex (ranging from 400 – 1200 kDa) is observed in Noelin1 protein complexes from the hippocampus when separated by Blue-Native PAGE (Fig. 2d).
Figure 2. Noelin1-3 interacts directly with the GluA2 subunit of the AMPA receptor. a) Sensorgrams from interaction of Noelin1-3 with immobilized GluA2 (black lines) and from samples of non-transfected HEK293T cells that served as a negative control (gray lines). A reversible 1-step model was fitted to the double referenced experimental data (green lines). b) Left: Double referenced sensorgrams from the interaction of Noelin1-3 with GluA2 under non-reducing (blue, cycles 10 and 28) and reducing (red, cycles 19 and 37) conditions. Right: Bar chart illustrating the binding levels upon the injections of 7 nM Noelin1-3 over the GluA2 AMPAR surfaces in the absence (blue) and presence of 1 mM DTT (red). The signals were extracted from the sensorgrams displayed in panel b at the indicated time points (black dots). The numbers in parenthesis represent the respective cycles within the analysis. c) 1-D BN-PAGE followed by Western blot (WB) for Noelin1 on the P2 + M fraction of the hippocampus, extracted by 1% and 2% DDM respectively, reveals high molecular weight native complexes of Noelin1.
Noelin1 does not affect AMPAR conductance and desensitization properties

Since Noelin1 and AMPARs are partners of the same hippocampal protein complex and interact in vitro, we examined whether Noelin1 can affect the biophysical properties of AMPARs. AMPAR-mediated currents were measured in response to fast glutamate applications in the presence and absence of Noelin1 co-expressed in, or incubated with, HEK293T cells. Neither co-expression, nor addition of Noelin1 and GluA1 subunits altered homomeric GluA1 AMPAR currents (rise time, decay time, rectification) induced by a 1 ms glutamate application (Supplementary Fig. 2e-f). AMPAR current rise times remained unchanged in the presence of Noelin1. We next assessed whether Noelin1 altered the biophysical properties of GluA1/2 heteromeric AMPARs. Upon addition of Noelin1, we did not observe differences in rise time ($P = 0.943$), decay time ($P = 0.948$) or recovery from desensitization ($P = 0.944$) (Fig. 3c, inset). Also, Noelin1 did not alter the rectification properties of heteromeric AMPARs (Fig. 3c, inset). These data show that Noelin1 does not affect conductance or desensitization properties of AMPARs.

Localizing Noelin1 using high-resolution imaging

As a next step, to assess whether Noelin1 is a potential auxiliary AMPAR subunit that alters mobility of the AMPAR, we determined whether Noelin1 is co-localized with AMPARs. Thereto, we performed stimulated emission depletion (STED) microscopy for Noelin1, GluA2 and Homer1 on hippocampal primary neurons (Fig. 4a). Detailed quantification was obtained by making line scans across different segments on dendrites and synapses (Fig. 4b,c, respectively). First, along the dendrites we find that Noelin1 overlaps with AMPAR and Homer1 spots, inside and outside Homer1 positive puncta (Fig. 4b). Line scans through dendritic spines show that Noelin1 and GluA2 co-localize with Homer1 positive puncta (Fig. 4c). Thus, the interaction of GluA2 and Noelin1 is largely synaptic, and to some extent extrasynaptic. This is in accordance with biochemical fractionation (Fig. 1a), and the distribution of TARP-γ8 over synaptic as well as extrasynaptic sites (Fukaya et al., 2006). Furthermore, we observe Noelin1 outside of GluA2 and Homer1 puncta and this extrasynaptic Noelin1 frequently co-localizes with the ECM marker Brevican (Supplementary Fig. 3).

It has been shown that upon treatment with hyaluronidase, that disrupts the ECM, the dendritic Brevican signal is drastically reduced (Frischknecht et al., 2009). We hypothesized that if Noelin1 is associated with the ECM, Noelin1 immunoreactivity
should be similarly reduced after hyaluronidase treatment. To test this we performed triple staining for Noelin1, Brevican and Homer1 in control and hyaluronidase treated cells, followed by quantification of the total dendritic signal and the perisynaptic intensity for Brevican and Noelin1 (Supplementary Fig. 4a). Indeed, upon hyaluronidase treatment, total Brevican immunoreactivity at dendrites was reduced to 27.25 ± 2.697 (P < 0.0001) and likewise, a reduction to 67.74 ± 6.18 (P = 0.003) in dendritic Noelin1 immunoreactivity was measured (Supplementary Fig. 4b). Perisynaptic Brevican and synaptic Noelin1 immunoreactivity were reduced to 57.68 ± 10.21% and 71.53 ± 5.48% after hyaluronidase treatment, whereas the number of synaptic puncta positive for Noelin1 remained unchanged (Supplementary Fig. 4c).

**Figure 3. Noelin1 has no effect on AMPAR channel properties.** a,b) Bar graphs (mean±SEM) summarize the absence of effect of addition of Noelin1-3 on rise time (a), and decay time (b) of AMPAR currents mediated by heteromeric AMPARs in HEK293T cells shows no significant effect of addition of Noelin1 (red) (rise time: GluA1/2 vs. GluA1/2 & Noelin1-3, P = 0.943 (unpaired t-test); decay time: GluA1/2 vs. GluA1/2 & Noelin1-3, P = 0.945 (unpaired t-test). Currents were evoked by direct application of 1 mM glutamate during 1 ms. c) Recovery of desensitization (two consecutive 1-ms glutamate applications with an inter-pulse interval of 20, 50, 100, 200, 300, 400, 500, 750, 1000 ms) from HEK293T cells expressing a heteromeric AMPAR channel in the absence (black) or presence (red) of Noelin1-3. Insets show normal rectification, not influenced by the presence of Noelin1 (left), and no effect on recovery of desensitization (τrecovery; right; GluA1/2 vs. GluA1/2 & Noelin1-3, P = 0.944 (unpaired t-test)).
Figure 4. Noelin1 is enriched at extrasynaptic and postsynaptic sites of hippocampal neurons, where it colocalizes with the AMPAR. a) STED imaging in cultured hippocampal neurons at DIV 21 showing an overview or zoom-ins of dendrites (right) or spines (right, inset). Noelin1 (green), GluA2 (red) and, Homer1 (blue, synaptic marker) are shown. Merge shows color-overlay images of the three channels. Scale bars are indicated. Inset shows a 2-fold enlargement. Line scans over dendrite (1, 2) and spines (3) are displayed (b,c). b,c) Numbered dashed white lines on the overlay image indicate locations of line scans across the three channels (x-axis: distance (µm); y-axis: intensity (arbitrary units)). Graphs illustrate co-enrichment of immunofluorescence intensities of Noelin1, GluA2 and Homer1 at dendrites and spines, with the latter showing both synaptic and extrasynaptic localization.
Noelin1 affects AMPAR mobility

Association of Noelin1 and AMPARs yields high molecular weight complexes as observed after heterologous expression (Fig 2d, Supplementary Fig. 2d). Therefore, Noelin1-AMPAR association can have a role in the mobility of AMPARs (Heine, 2012). To test this, first, AMPAR mobility depending on Noelin1 in HEK293T cells was measured, using GluA1::pHluorin (Supplementary Fig. 4a) or GluA2::pHluorin (Fig. 5a) expression and single particle tracking. The effect of intracellular expressed Noelin1, or Noelin1 applied extracellular as purified protein, on AMPAR mobility was measured (Fig. 5a). Upon co-expression with Noelin1, the instantaneous diffusion coefficient, a measure for surface mobility of AMPARs, was decreased (D_{inst} control: -2.358 (4.385 × 10^{-3}) μm²/s, IQR: (6.135 × 10^{-3}) 0.477; D_{inst} GluA2::Noelin1: -3.228 (5.916 × 10^{-4}) μm²/s, IQR: (2.00 × 10^{-3}) 0.975 (P < 0.001; Fig. 5b). Similarly, addition of purified Noelin1 significantly reduced the diffusion coefficient (D_{inst} control: -2.128 (7.447 × 10^{-3}) μm²/s, IQR: (7.527 × 10^{-3}) 0.463); D_{inst} GluA2: -2.660 (2.187 × 10^{-3}) μm²/s (IQR: (4.116 × 10^{-3}) 0.921; P = 0.002; Fig. 5b). Quantification of the immobile fraction showed a significant reduction of GluA2 AMPAR mobility upon both co-expression (GluA2-GFP: 20.5±1.8 % vs. GluA2-Noelin1: 41.01±4.1 %; P < 0.001) and addition of Noelin1 (GluA2+solvent: 15.7±1.7 %; GluA2+Noelin1: 32.50±3.23 %; P < 0.001) (Fig. 5c). The same effect was observed on GluA1-containing AMPARs in which a significant reduction on GluA1 mobility upon co-expression with Noelin1 (P = 0.034) was observed and a significant increase in the percentage of immobile receptors after addition of Noelin1 (P = 0.024) was found (Supplementary Fig. 4). Thus, Noelin1 independent of its route of delivery, i.e., co-expression or external application, has the ability to reduce AMPAR mobility in HEK293T cells.

We subsequently tested the effect of addition of Noelin1 on AMPAR mobility in primary neuronal cell culture. First, young primary cultured neurons (DIV 11 – 13) were tested, which still lack a well-defined ECM (Frischknecht et al., 2009). GluA1::pHluorin and GluA2::pHluorin were expressed in primary neurons along with Homer1::dsRed to identify synapses (Fig. 5a). We observed a reduction in the mean square displacement (MSD) of extra-synaptic GluA2-containing AMPA receptors (Fig. 5d,e). The distribution of the diffusion coefficient for extra-synaptic GluA2-containing AMPARs (D_{inst}) was shifted to smaller values upon treatment of primary neurons with Noelin1 (GluA2+Noelin1) vs. control (GluA2 + control) treated cells (Fig. 5e). Quantification of the diffusion coefficient of mobile AMPARs showed a significant reduction (D_{inst} control: -
1.878 (1.324 \times 10^{-2}) \mu m^2/s, IQR: 0.094 (2.643 \times 10^{-3}); D_{inst} \text{ GluA2 } -2.036 (9.204 \times 10^{-3}) \mu m^2/s, IQR: 0.136 (3.086 \times 10^{-3})) \text{ upon treatment with Noelin1 } (P = 0.001) \text{ (Fig. 5f). The percentage of low-mobility extra-synaptic GluA2-containing AMPARs also increased significantly after Noelin1 treatment (control: } 16.9 \pm 0.9 \% \text{; Noelin1: } 22.9 \pm 1.1 \% \text{; } P < 0.001 \text{; Unpaired t test) (Fig. 5g). The same effect was observed for mobility of extra-synaptic GluA1-containing AMPA receptors } (P = 0.059) \text{ and a significant reduction in percentage of immobile GluA1 AMPARs was observed upon addition of Noelin1 } (P = 0.020); \text{ Supplementary Fig. 5a–c). Analysis of the synaptic fraction of GluA2-containing AMPARs showed no significant difference in the diffusion coefficients } (P = 0.304) \text{ or in the percentage of the immobile fraction } (P = 0.111) \text{ (Supplementary Fig. 5d–f). In contrast, synaptic GluA1-containing AMPAR diffusion was significantly reduced upon Noelin1 treatment } (P = 0.0163) \text{ and the percentage of immobile synaptic GluA1 containing receptors showed an increase upon Noelin1 addition } (P = 0.055); \text{ Supplementary Fig. 5g–i). Thus, alike the ECM-free HEK293T cell environment, in a non-mature neuronal culture without a well-developed ECM, Noelin1 can reduce AMPAR mobility.
Figure 5. Noelin1 regulates AMPA receptor mobility in HEK293T cells and young neurons. a) Representative traces of sep::GluA2 receptors expressed in HEK293T cells (upper panel) or in young (DIV 11–13) neurons at extrasynaptic and synaptic sites (lower panel) without/control (left) and with (right) (addition of) Noelin1-3. In the lower panel, the Homer1::dsRED signal is indicated. Scale bars indicate 2 µm. b) Box plots show the diffusion coefficient ($D_{\text{inst}}$) for HEK293T cell expression of single particles of GluA2 (black), GluA2 co-expressed with Noelin1-3 (GluA2 Noelin1, red), GluA2 plus control medium (GluA2 + control, gray) and GluA2 plus medium containing Noelin1-3 (GluA2 + Noelin1, orange). In both cases, Noelin1 reduces the AMPAR diffusion (GluA2 vs. GluA2 Noelin1, $P < 0.001$ (unpaired t-test with Welch's correction); GluA2 + control vs. GluA2 + Noelin1, $P = 0.002$ (unpaired t-test). c) Bar graphs (mean±SEM) show the proportion of immobile sep::GluA2 particles for GluA2 vs. GluA2 Noelin1, $P < 0.001$ (unpaired t-test with Welch's correction), GluA2 + control vs. GluA2 + Noelin1, $P < 0.001$ (unpaired t-test with Welch's correction). d) Temporal dynamics of mobility (area covered) of GluA2::pHluorin receptors in cultured hippocampal neurons (DIV 11–13) shows a significant reduction upon incubation with Noelin1. e) Distribution of instantaneous diffusion coefficients ($D_{\text{inst}}$) for extrasynaptic mobile GluA2::pHluorin AMPARs without and with Noelin1 incubation. f) Box plots indicate a significant effect on $D_{\text{inst}}$ for extrasynaptic sep::GluA2 AMPARs without vs. with Noelin1 incubation (GluA2 + control vs. GluA2 + Noelin1, $P = 0.001$; Mann Whitney U-test). g) Quantification of the immobile fraction obtained from panel f; GluA2 + control vs. GluA2 + Noelin1, $P < 0.001$ (unpaired t-test). All n-numbers indicated represent number of cells from at least two independent biological replicates.
ECM-dependent Noelin1 rescues the effect of hyaluronidase treatment on AMPAR mobility

It has been shown previously that for mature primary neurons (DIV 21 – 23), the ECM exerts an inhibitory constraint on AMPAR mobility (Frischknecht et al., 2009). Because Noelin1 is affecting AMPAR mobility, we tested whether addition of Noelin1 altered AMPAR mobility in primary neurons in which the ECM is fully formed. To do so we treated primary neurons at DIV 21 – 23 after transfection with GluA1::pHluorin and Homer1::dsRed with Noelin1 or control medium. Single particle tracking analysis revealed no change for extrasynaptic ($P = 0.627$) and synaptic ($P = 0.895$) AMPAR mobility after Noelin1 application (Fig. 6). Thus, Noelin1 is not able to change AMPAR mobility in presence of a fully maturated ECM.

To test whether the ECM overrules the effect of Noelin1, we used hyaluronidase treatment to remove the ECM and measured AMPAR lateral diffusion. Hyaluronidase (Hya) treatment caused a significant increase in extra-synaptic diffusion coefficient in neurons ($D_{\text{inst}}$ control: $-2.050 (8.913 \times 10^{-3}) \, \mu m^2/s$, IQR: $0.360 (7.743 \times 10^{-3})$; $D_{\text{inst}}$ Hya -1.879 (1.321 x 10^{-2}) \, \mu m^2/s, IQR: 0.285 (9.667 x 10^{-3})) (Hya + control vs. Con + control; $P = 0.009$) (Fig. 6b,c). Addition of Noelin1 after hyaluronidase treatment strongly reduced the extra-synaptic diffusion coefficient of the AMPARs ($D_{\text{inst}}$ Hya+Noelin1: $-2.142 (7.211 x 10^{-3}) \, \mu m^2/s$, IQR: 0.536 (9.153 x 10^{-3})) (Fig. 6b,c).

Furthermore, the observed decrease on the percentage of the immobile fraction after hyaluronidase treatment was brought to control level upon application of Noelin1 (Con + control: 29.3 ± 1.1 %; Hya + control: 22.5 ± 1.6 %; Hya + Noelin1: 31.7 ± 2.4 %) (Con + control vs. Hya + control: $P = 0.002$; Hya + control vs. Hya + Noelin1: $P = 0.005$) (Fig. 6d). Thus, Noelin1 and the ECM are non-additive in their effects. The ECM seems to cause a maximum effect on limiting AMPAR mobility that cannot be further enhanced by addition of Noelin1.

Previously it was shown that the diffusion coefficient of the synaptic AMPA receptors was not significantly altered by hyaluronidase treatment (Frischknecht et al., 2009) (Fig. 6d,e). Interestingly, neurons treated with Noelin1 after hyaluronidase treatment caused a significant decrease in diffusion of synaptic AMPARs in neurons (Fig. 6d,e) ($D_{\text{inst}}$ Hya + control: $-1.92 (1.210 \times 10^{-2}) \, \mu m^2/s$, IQR: 0.496 (1.422 x 10^{-2}); $D_{\text{inst}}$ Hya + Noelin1: $-2.28 (5.224 \times 10^{-3}) \, \mu m^2/s$, IQR: 0.509 (5.971 x 10^{-3}); $P = 0.003$). Correspondingly, Noelin1 application introduced more synaptic immobile particles upon hyaluronidase treatment (Hya + control: 24.6 ± 1.8 %; Hya + Noelin1: 31.50 ± 1.84 %;
$P = 0.038$) (Fig. 6f). Taken together, synaptic AMPAR receptors do not have saturated amounts of Noelin1 bound. This opens the possibility that Noelin1 secretion might act on synaptic receptors to regulate these.

Finally, to assess whether Noelin1 expression in primary cultured neurons is modulated in activity-dependent manner, we induced chemical long-term potentiation (LTP) using a 15-minute treatment with 4-Aminopyridine (4-AP) and Bicuculline (4-AP/BIC) in DIV 21-old primary neurons. Indeed, Noelin1 surface staining was increased at dendrites (32.6% increase, $P = 0.048$; Fig. 7a–c) and at synaptic sites within 1 h after LTP induction (Noelin1: 24.5% increase, $P = 0.031$; Fig. 7a,d,e). Interestingly, surface GluA2 levels did not significantly increase at dendrites 1 h after LTP induction (23.7% increase, $P = 0.144$) but was clearly elevated at synapses (GluA2: 32.5% increase, $P = 0.010$). Thus, Noelin1 surface expression is regulated in an activity-dependent manner and may contribute to accumulation and stabilization of synaptic GluA2-containing AMPA receptors at the PSD.
Chapter 5: Noelin1 affects AMPA receptor lateral mobility
Figure 6. Noelin1 rescues the effect of hyaluronidase treatment on AMPA receptor mobility in older neurons. a) Representative traces of GluA1::pHluorin receptors expressed in primary neurons at DIV 21 at extrasynaptic and synaptic locations after control (left) or hyaluronidase (right) treatment and subsequent incubation without (control) or with Noelin1-3 in the medium. Scale bars indicate 4 µm (left) and 2 µm (right; zoom). b–f) Distribution of instantaneous diffusion coefficients ($D_{inst}$) and immobile fraction for extrasynaptic (b–d) and synaptic (e–g) sep::GluA1 receptors. c) Box plot shows $D_{inst}$ for extrasynaptic particles. Hyaluronidase treatment induced a significant increase in mobility in control cells (Con + control vs. Hya + control, $P = 0.009$ (unpaired t-test)). Whereas addition of Noelin1 was unable to alter AMPAR mobility in these older neurons (Con + control vs. Con + Noelin1, $P = 0.627$ (unpaired t-test)), Noelin1 in combination with hyaluronidase treatment was able to reduce AMPAR mobility (Hya + control vs. Hya + Noelin1; $P = 0.002$ (unpaired t-test with Welch’s correction)). d) Bar graphs (mean±SEM) show the proportion of immobile AMPARs, showing a similar effect of hyaluronidase treatment and Noelin1 incubation (Con + control vs. Hya + control; $P = 0.002$ (unpaired t-test:); Con + control vs. Con + Noelin1; $P = 0.529$ (unpaired t-test:); Hya + control vs. Hya + Noelin1; $P = 0.005$ (Mann Whitney test); Con + Noelin1 vs. Hya + Noelin1; $P = 0.380$ (Mann Whitney test). f) Box plot shows $D_{inst}$ for synaptic particles. Hyaluronidase treatment induced a non-significant increase in mobility in control cells (Con + control vs. Hya + control; $P = 0.269$ (unpaired t-test)). Incubation with Noelin1 after hyaluronidase treatment significantly reduced AMPAR mobility (Hya + control vs. Hya + Noelin1; $P = 0.003$ (unpaired t-test)), without affecting other parameters. g) Incubation with Noelin1 after hyaluronidase treatment significantly increased the fraction of synaptic immobile AMPARs (Hya + control vs. Hya + Noelin1; $P = 0.038$ (unpaired t-test)), without affecting other parameters. N-numbers are indicated.
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(a) Images showing control and chemical LTP 1 h conditions for Noelin1, GluA2, Homer1, and Merge.

(b) Graph showing Noelin1 total dendritic intensity with normalized bars for Control 15 min, Control 1 h, 4-AP/BIC 15 min, and 4-AP/BIC 1 h.

(c) Graph showing GluA2 total dendritic intensity with normalized bars for Control 15 min, Control 1 h, 4-AP/BIC 15 min, and 4-AP/BIC 1 h.

(d) Graph showing Noelin1 synaptic intensity with normalized bars for Control 15 min, Control 1 h, 4-AP/BIC 15 min, and 4-AP/BIC 1 h.

(e) Graph showing GluA2 synaptic intensity with normalized bars for Control 15 min, Control 1 h, 4-AP/BIC 15 min, and 4-AP/BIC 1 h.

P-values: 0.326, 0.048, 0.027, 0.144, 0.031, 0.010.
Figure 7. Chemical LTP recruits AMPAR and Noelin1 to the synapse. a) Representative images for immunostaining for Noelin1, GluA2 and Homer1 under basal conditions and 1 h after stimulation with 4-AP and Bicuculline (4-AP/BIC) shows increased immunoreactivity for Noelin1 and GluA2. b,c) Bar graphs (mean±SEM) summarizing the normalized total dendritic Noelin1 and GluA2 under control conditions and after a 15-minute 4-AP/BIC stimulation, either taken directly (4-AP/BIC 15 min), or incubated for another h (4-AP/BIC 1 h). Noelin1: Control 15 min (1.00 ± 0.08) vs. 4-AP/BIC 15 min (0.89 ± 0.07), \( P = 0.326 \) (unpaired t-test); Control 1 h (1.00 ± 0.10) vs. 4-AP/BIC 1 h (1.33 ± 0.12), \( P = 0.048 \) (unpaired t-test)). GluA2: Control 15 min (1.00 ± 0.06) vs. 4-AP/BIC 15 min (0.84 ± 0.07), \( P = 0.027 \) (Mann Whitney U-test); Control 1 h (1.00 ± 0.09) vs. 4-AP/BIC 1 h (1.24 ± 0.11), \( P = 0.144 \) (Mann Whitney U-test). d,e) Bar graphs (mean±SEM) summarizing the synaptic Noelin and GluA2 as indicated in panels b,c. Noelin1: Control 1 h (1.00 ± 0.08) vs. 4-AP/BIC 1 h (1.25 ± 0.07), \( P = 0.031 \) (unpaired t-test). GluA2: Control 1 h (1.00 ± 0.09) vs. 4-AP/BIC 1 h (1.33 ± 0.06), \( P = 0.010 \) (Mann Whitney U-test). N-numbers are indicated.
Discussion

We identified Noelin1 as a true high affinity AMPAR-interacting protein using several independent methods, i.e., immunoprecipitation of detergent-solubilized hippocampal AMPAR complexes, direct interaction after co-expression in HEK293T cells and by SPR biosensor technology. In order to qualify as a bona fide functional auxiliary subunit of the AMPAR according to criteria as outlined previously\textsuperscript{117}, we furthermore showed that Noelin1 limits AMPAR lateral mobility in the absence of a well-formed extracellular matrix both in HEK293T cells and in primary cultured neurons. However, Noelin1 did not alter channel properties of the AMPAR. Finally, we showed that Noelin1 is enriched in synapses, and the expression of Noelin1 is increased upon ex vivo generated LTP. Together, this indicates that Noelin1 is an intrinsic auxiliary subunit of AMPAR complexes in the brain\textsuperscript{117}.

Noelin1 is expressed in cortex, cerebellum and hippocampus, consistent with existing in situ hybridization data from the Allen Brain Atlas\textsuperscript{203}. Immunoprecipitation experiments on dodecyl maltoside (DDM)-extracted AMPAR complexes combined with mass spectrometry demonstrated that Noelin1 is part of the AMPAR complex in all three brain regions\textsuperscript{133}. Hence, Noelin1 might have a widely conserved role with respect to its interaction with the AMPAR across all three brain regions, in contrast with several other AMPAR interactors, e.g., TARP- γ2 and γ8, which show brain region-specific interactions due to their differential expression across the brain\textsuperscript{116,133}. We find that AMPARs containing Noelin1 also comprise TARP- γ8 in the hippocampus. In addition, Neuritin is abundantly present in Noelin1 IPs in both hippocampus and cerebellum, whereas this protein is normally observed with low abundance values in GluA2/3 IPs\textsuperscript{116}. Furthermore, Noelin1 IPs also reveal Noelin2 and Noelin3, the paralogs of Noelin1. These paralogs have a similar olfactomedin domain as Noelin1 and are also present in AMPAR IPs\textsuperscript{204}. In this study, we focused on Noelin1 and its role in regulation of the AMPAR. Whether Noelin2 and Noelin3 might have similar roles remains to be investigated.

Using co-expression in HEK293T cells we used all three Noelin1 isoforms, demonstrating that Noelin1-1, 1-3 and 1-4 interact directly with the GluA2 subunit of the AMPAR. In GluA2/3 IPs, followed by Western blot experiments, we observed a specific enrichment for the isoform Noelin1-3, and this isoform was also the most abundantly present in the postsynaptic density fraction.
Because *bona fide* AMPAR auxiliary subunits have been shown to affect AMPAR kinetics and/or mobility, we tested both possibilities for Noelin1. Using a fast glutamate application system, we performed measurements on GluA1 homomers and GluA1-2 heteromers in the presence of Noelin1. In contrast to other AMPAR-associated proteins, e.g., TARPs, Cornichon-2, Shisa6, Shisa9, we did not observe an effect on AMPAR channel properties in the presence of Noelin1. We therefore tested whether Noelin1 has a structural role in affecting AMPAR lateral diffusion without altering channel conductance properties.

We employed SPR biosensor technology for real-time interaction analysis of the AMPAR, which showed the direct *in vitro* interaction between Noelin1-3 and GluA2 AMPA receptors. We have previously used a similar approach for characterizing small ligand interactions with the homo-oligomeric GABA_A β3 receptor\textsuperscript{205}, demonstrating the suitability of SPR methodology for real-time interaction studies of ligand-gated in channels. Using SPR, we characterized the kinetics of the interaction by employing a 1-step interaction model for determining the kinetic rate constants. This analysis indicated that Noelin1 associates relatively fast with immobilized GluA2 AMPARs on the SPR biosensor surface and forms a stable complex with low nanomolar affinity. Quantification of the kinetic rate constants for the Noelin1-GluA2 AMPAR interaction is challenging. This is due to the difficulty in determining the protein concentration in the final analysis as some protein may be lost during the sample preparation, potentially leading to an underestimation of the Noelin1 concentration. Thus, the kinetic rate constants were determined within the accuracy of the concentration determination relevant for the calculation of the kinetic analysis. Due to the tetrameric structure of the GluA2 AMPAR and tetrameric Noelin1\textsuperscript{205}, their interactions are potentially more complex than is described by a 1-step interaction model. Therefore the kinetic rate constants need to be regarded as approximations. Nevertheless, the SPR-based approach demonstrates that kinetic information can be obtained and that Noelin1 can associate with low nanomolar affinity with the AMPA receptor.

It has been shown that Noelin1 forms oligomers via disulfide bridges\textsuperscript{197}. Our data shows that Noelin1 does not interact with the GluA2 AMPAR under reducing conditions (1 mM DTT), whereas the interaction is clearly occurring under non-reducing conditions. The interaction of Noelin1 with immobilized GluA2 AMPARs is not affected by prior injections of DTT. This demonstrates that the formation of Noelin1 oligomers is a prerequisite for the interaction with GluA2 AMPARs and may argue for a role in receptor clustering (Pronker et al., 2015). Because Noelin1 can form high molecular
weight oligomers, we tested whether it had a role in receptor clustering in HEK293T cells. Indeed we found reduced mobility of both GluA1- and GluA2-containing AMPARs and increase of the immobile pool of AMPARs, both upon co-expression with Noelin1 and upon addition of Noelin1 in the culture medium. These experiments show that Noelin1 can bring down AMPAR mobility upon acute delivery of Noelin1. This reduction in mobility is not likely caused by the increase in mass, as previous experiments with antibody bound beads to the AMPAR have shown, that membrane diffusion is determined predominantly by the size of the complex in the membrane and the membrane lipid fluidity (Heine, 2012), or by extracellular matrix molecules (ECM)\textsuperscript{157}. Most likely, the oligomeric Noelin1 clusters AMPARs into larger entities with lower membrane mobility. In agreement, when measuring the effect of Noelin1 on AMPAR mobility in young neurons (DIV 11 – 13) without a well-formed ECM, Noelin1 reduced the mobility of extrasynaptic GluA1- and GluA2-containing AMPARs. For synaptic receptors the addition of Noelin1 led to a significant reduction in mobility, in particular in the pool of synaptic AMPARs. Although only Noelin1-3 was used for these experiments, it has been shown that Noelin1 can interact with its paralog proteins, Noelin2 and Noelin3, which are also known to be secreted glycoproteins and have a similar Olfactomedin domain\textsuperscript{204}. Thus in vivo, the combined effect of different (iso)forms of Noelin1 on AMPAR lateral mobility might be even stronger.

When assessing the effect of Noelin1 on AMPA receptor mobility in mature neurons, where the ECM is well formed (Frischknecht et al., 2009), we found that addition of Noelin1 protein has no significant effect on AMPAR lateral diffusion on dendrites. This is most likely due to the saturating effect of ECM. However, Noelin1 altered the mobility of synaptic receptors, in contrast to the hyaluronidase sensitive ECM, which does not affect synaptic receptors (Frischknecht et al., 2009). In particular, it was shown that ECM removal does not significantly increase synaptic GluA1 mobility. These mobile synaptic AMPARs can be slowed down by the further addition of Noelin1, indicating that they are in principle prone to Noelin1 regulation. Mobility of extrasynaptic receptors was increased after hyaluronidase treatment, confirming previous results\textsuperscript{157} and brought to control levels after bath application of Noelin1. Together with the notion that Noelin1 co-localizes and associates with ECM this may indicate that part of the barrier function of the ECM may emerge from Noelin1.

Using STED, we observed that Noelin1 co-localizes with GluA2 at synaptic Homer1 puncta and extra-synaptically with the ECM protein Brevican. Since ECM expression is largely absent on synapses (Frischknecht et al., 2009), we propose that
in mature neurons, which have a well formed ECM, there might be at least two pools of Noelin1; the extrasynaptic pool, associated largely with the ECM, and a variable synaptic pool bound to AMPAR. One might predict that under conditions of synaptic potentiation the fraction of immobile synaptic receptors increases. When testing this, we found that induction of chemical LTP increases the level of the synaptic Noelin1, indicative for a role in limiting synaptic mobility of AMPARs under these conditions. Thereby Noelin1 may contribute to the consolidation of LTP by limiting the exchange between synaptic and extrasynaptic pool. We previously observed that Noelin1 shows a down regulation with ECM components in the dorsal hippocampus during consolidation of a contextual fear memory, a moment when plasticity in the preparation is high. Given that Noelin1 was found colocalized with the ECM, in principle, activity-dependent remodeling of the ECM might lead to increase or decrease in the presentation of Noelin1 to the AMPARs on extrasynaptic sites, affecting their mobility and regulating the availability of extrasynaptic AMPARs to be exchanged with synaptic AMPARs. Noelin1 is the first example of a secreted AMPAR auxiliary protein that tunes the receptor’s membrane mobility without influencing its conductance properties.
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Materials and Methods

Antibodies

Anti GluA2/3 (antibody was raised against QNFATYKEGNYVYGIESVKI in rabbit (Genscript)) and Anti GluA2 (anti-mouse; Neuromab, clone L21/32) was used for IP (10 µg/IP) or Western blotting (1 in 1,000). For Western blotting and IPs the following antibodies were used: Anti-GluA2 (anti-mouse; Neuromab, 1 in 1,000), anti-Noelin1 (anti-mouse; Neuromab, clone K96/7, 1 in 500), anti-PSD-95 (anti-mouse; Neuromab, Clone K28/43, 1 in 5,000), anti-Synaptophysin (anti-mouse; Santacruz, sc-9116, 1 in 500) was used. For immunocytochemistry, the same antibodies as indicated above were used (GluA2 (1 in 250); Noelin1 (1 in 250)), in addition to those against Homer1 (anti-rabbit, Synaptic Systems, cat no.160 004, 1 in 1,000), and Brevican (anti-rabbit; a generous gift from Contanze Seidenbecher and Renato Frishknect’s group, 1 in 1,000) antibodies were used. For SPR analysis GluA2 (Neuromab) antibody was used. For single particle tracking using quantum dots, anti-GFP mouse monoclonal antibody (Roche Product Nr. 11814460001) was used. Secondary antibodies used for immunocytochemistry using confocal or wide field microscopy, were anti-mouse Alexa 488 (1 in 1,000), anti-rabbit Alexa 568 (1 in 1,000), and anti-guinea pig Alexa 647 (1 in 1,000) (all from Molecular Probes). For STED microscopy, anti-rabbit Abberior STAR 580 (1 in 250, from Abberior GmbH, and Anti-Mouse Atto 647N (1:250) from Atto-Tec GmbH was used.

Preparation of subcellular fractions

Subcellular fractions were prepared from adult C57/Bl6J mouse (Charles River) hippocampus as described previously (Klaassen et al., 2016) with some modifications. Tissue was homogenized in homogenization buffer (0.32 M Sucrose, 5 mM HEPES, pH 7.4; protease inhibitor cocktail (Roche)), to obtain the homogenate fraction. The homogenate was then centrifuged at 1000x g for 10 minutes to obtain the supernatant. For P2+microsome (P2+M) pellets, the supernatant was then centrifuged at 100,000x g for 2 h at 4 °C, which were resuspended in sample suspension buffer (25 mM HEPES, 150 mM NaCl, pH 7.4 and a protease inhibitor cocktail) and stored at –80 °C. For subcellular fractionation of the separate P2 and microsome fractions, the supernatant was centrifuged at 18,000x g for 20 minutes to obtain the P2 fraction. The microsomal fraction was subsequently obtained by centrifuging the supernatant at 100,000x g for 20 minutes. For subcellular fractionation of synaptosomes up to the
postsynaptic density, the supernatant was separately loaded onto a 0.85 M/1.2 M sucrose gradient, centrifuged for 2 h at 100,000x g to obtain the synaptosome fraction at the 0.85/1.2 M interface. The synaptosome fraction was either given an osmotic shock in 5 mM HEPES, pH 7.4 for 30 minutes and then loaded on a 0.85/1.2 M sucrose gradient to obtain the synaptic membrane fraction, or extracted twice with 1% Triton X-100 for 30 minutes, then loaded on a 1.5/2.0 M sucrose gradient to obtain the postsynaptic density fraction. Of each sub-fraction 5 µg was mixed with 5x SDS-containing loading buffer loaded on a SDS-PAGE gel, transferred overnight on PVDF membranes and stained for the protein of interest.

**Affinity purification of protein complexes by immunoprecipitation**

Methods for affinity purification was as previously described (Chen et al., 2014). In short, for a single IP experiment, 2 mg of Whole homogenate fractions was extracted twice in 1 mL extraction buffer (25 mM HEPES, 150 mM NaCl, 1% DDM (Sigma Aldrich), pH 7.4 and a protease Inhibitor cocktail) for 1 h each at 4 °C on a rotor at 10 rpm. After each extraction, the samples were centrifuged at 20,000x g for 20 minutes. The supernatant was pooled after each extraction. Of each antibody 10 µg was added to the pooled supernatant and incubated overnight at 4 °C on a rotor at 10 rpm. Per IP, 50 µL of protein A/G beads (Santa Cruz) were added, and incubated for 1 h at 4 °C. After incubation with the beads, the samples were centrifuged at 1000x g for 1 minute and the supernatant was discarded. The beads were washed four times with 1 mL washing buffer (25 mM HEPES, 150 mM NaCl, 1% DDM, pH 7.4), supernatants were discarded and the final pellet was dissolved in 2% SDS buffer (pH 8.8) for separation on SDS-PAGE for Western blotting experiments or trypsinized peptides were extracted using the filter aided sample preparation protocol (Wiśniewski et al., 2009). As negative controls, IgG or peptide blocking control (Li et al., 2012). For peptide blocking controls, 10 µg antibody was incubated with 50 µg peptide for 30 minutes on ice, prior to addition to the supernatant; samples were treated similarly thereafter.

**LC-MS/MS analysis**

Peptides were analyzed by nano-LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific) coupled to the TripleTOF 5600 mass spectrometer (Sciex).

Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 µm i.d., 5µm particlesize, from Dionex) and fractionated on a 200 mm Alltima C18 column (100 µm
i.d., 3 μm particle size). The acetonitrile concentration in the mobile phase was increased from 5 to 30% in 90 min, to 40% in 5 min, and to 90% in another 5 min, at a flow rate of 500 nL/min. The eluted peptides were electro-sprayed into the TripleTOF MS. The nano-spray needle voltage was set to 2500V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (m/z 350–1200, 250 ms) followed by a top 25 MS/MS (85 ms per MS/MS, precursor ion > 90 counts/s, charge state from +2 to +5) with an exclusion time of 16 s once the peptide was fragmented. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 10eV.

The MS raw data were imported into MaxQuant (Cox and Mann, 2008) (version 1.5.2.8, and searched against the UniProt mouse proteome (SwissProt December 2015 release) with MMTS as the fixed modification and Methionine oxidation and N-terminal acetylation as variable modifications. Match between runs was activated. All other parameters were set to default.

**Western blot analysis**

Samples containing SDS loading buffer were run on 5–12% Criterion TGX Stain-Free Precast Gels (Bio-Rad) and transferred on a PVDF membrane overnight. The membranes were then blocked using 5% non-fat milk in TBS with Tween-20 followed by an overnight incubation with the primary antibody at 4 °C. The blots were washed three times in TBS with Tween-20 after the primary antibody incubation followed by a 1-h incubation with a HRP conjugated secondary antibody in 3% non-fat milk. After three more washes, the blots were incubated with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and scanned on an Odyssey Fc scanner (Licor Biosciences).

**DNA constructs**

Full length cDNA constructs for Noelin1-1 (pN1_Noelin1-1-GFP-Kanamycin) [NM_019498.2], Noelin1-4 (pcDNA 3.1_Noelin1-4_Ampicillin_CMV) [NM_001038614.1] and Noelin1-3 (pDEST_Noelin1-3_IRES2_mCherry_Ampicillin_CMV) [NM_001038613.1] were used for expression in HEK293T cells. For GluA2, full length GluA2 (flip isoform) [NM_001039195] was inserted in pTRCGW construct to give pTRCGW_GluA2_Ampicillin_CMV. Sep::GluA1 and sep::GluA2 constructs were used as described previously (Klueva et al., 2014).
HEK293T cell transfections

HEK293T cells were transfected with either Noelin1 constructs alone or in combination with GluA2 constructs using polyethylenimine (PEI 25000). Briefly, 2.2 x 10^6 cells were grown in 10 cm dishes in Dulbecco's modified eagle medium + 10% fetal calf serum until 70% confluence was reached. The cells were transfected with 2.5 µg of DNA + 35 µL PEI + 250 µL PBS mix. The cells were harvested 48 h post transfection for IP experiments.

For preparation of HEK293T cell pellets for SPR biosensor analysis, 1.8 x 10^7 cells were plated on a 245 mm by 245 mm plate (Corning) and allowed to reach 70% confluence. The cells were then transfected with 50 µg of GluA2 or Noelin1-3 DNA or control GFP DNA along with 350 µL PEI and 2500 µL PBS. Cells were washed 48 h post-transfection for three times with ice-cold PBS and scraped cells were collected in falcon tubes and stored at –80 °C.

For collection of Noelin1-3 medium or control medium, HEK293T cells grown on 245 mm by 245 mm plates were transfected at 70% confluence with Noelin1-3 construct or empty vector respectively. The medium from transfected cells was replaced with OptiMEM (Gibco, Life Technologies) 24 h post-transfection. The cell medium was collected 48 h after cell medium change, centrifuged at 800 rpm for 5 minutes to remove cell debris and concentrated 100 times using a 10 kDa cutoff filter (Vivaspin 10, Sartorius) and stored at –80 °C.

Determination of Noelin1-3 concentration in medium

Medium (10 µL) was mixed with 5x SDS sample buffer and run on an SDS-PAGE gel. Gel slices at approximately 75 kDa, corresponding to Noelin1-3 bands, were cut out followed by in gel digestion. Half of the samples were analyzed in triplicates as described before on Orbitrap mass spectrometer. In order to determine the amount of protein, Peptide LTGISDPVTVK, which is a tryptic product of Noelin1-3, was run in a concentration of 100, 200, 1000 and 2000 fmol to make a standard curve using the total peak area of the peptide (m/z = 565.38) using skyline v2.5(Bateman et al., 2014). The Noelin1-3 concentration in the sample was calculated 720 fmol/µL^-1.

Co-precipitation from HEK293T cells

For extraction of proteins, HEK293T cells were washed in ice-cold PBS, scraped and resuspended in lysis buffer (25 mM HEPES, 150 mM NaCl, 1% DDM, Protease inhibitor cocktail) and incubated for 1 h at 4 °C while gently shaking at 10 rpm. The
lysates were centrifuged at 20,000x g for 30 minutes and the supernatants were incubated with GluA2 antibody for 1 h while gently shaking at 10 rpm. Post-antibody incubation, the lysates were incubated with 50 µL protein A/G beads (Santa Cruz) for 1 h, washed three times in wash buffer (0.1% DDM) and the bound proteins were eluted off with Laemmli buffer followed by SDS-PAGE Western blot analysis.

**Immunocytochemistry and image acquisition.**

Primary hippocampal neurons were obtained from E18 pups as described previously (Frischknecht et al., 2009). Briefly, 18,000 cells were grown on a glial feeder layer in neurobasal medium supplemented with B27 on poly D-Lysine coated coverslips. Coverslips were fixed with ice-cold methanol for 10 minutes, followed by three washes in sterile H2O and PBS. After blocking and permeabilization (1% FCS, 0.1% Triton X-100 in PBS) for 1 h, neurons were incubated with primary antibodies in blocking buffer overnight at 4 °C, and with respective secondary antibodies. After washing and fixing on glass slides (Superfrost Plus, Thermo) using Moviol, confocal images were taken using a LSM Meta 510 confocal microscope for Fig S4 using 63x oil immersion lens (N.A. 1.4) and analyzed using ImageJ.

Immunostainings of for fluorescence microscopy were acquired on a Zeiss Axio Imager A2 microscope using a 63x oil immersion lens (N.A. 1.4) with Cool Snap EZ camera (Visitron Systems) controlled by VisiView (Visitron Systems GmbH) software. All coverslips compared in one experiments were processed in parallel using identical antibodies solutions and other reagents.

Dual color STED data was obtained (Ivanova et al., 2015) and line scan analysis was performed (Frischknecht et al., 2009) as previously described.

Quantification of synaptic Noelin1 immunoreactivity after hyaluronidase treatment and 4-AP/BIC treatment was done using OpenView software (Tsuriel et al., 2006) using Homer1 staining to identify synaptic puncta. In **Fig. 7c–f** Noelin1 and GluA2 immunoreactivity was measured in a 0.36-µm box around the Homer1 puncta.

**In situ hybridization**


**Surface plasmon resonance biosensor based interaction analysis**

GluA2-containing AMPA receptors were extracted from HEK293T cell pellets using 25 mM HEPES, 150 mM NaCl, 1% DDM (w/v) (Affymetrix), pH 7.4 and one protease
inhibitor tablet per 50 mL buffer (buffer S). Buffers were chilled prior to usage and solubilization was performed on ice or at 4 °C. Buffer S (2.5 mL, GluA2; 5 mL, control) was added to the frozen cell pellets. Thawed pellets were homogenized by 10 strokes using a glass homogenizer (Kontes Glass Co). Solubilization was performed on a rocker shaker for 1 h, followed by centrifugation (20,000 x g, 30 minutes) in a Beckman L7-55 ultracentrifuge (Beckman Coulter) using a Ti-50 rotor. Aliquots were stored at – 80 °C.

SPR-based biosensor studies were performed using a Biacore S51 instrument and CM5 sensor chips (GE Healthcare). The GluA2 antibody was covalently immobilized on two spots of the sensor surface by standard amine coupling chemistry at a temperature of 25 °C. The antibody, in 10 mM Na-acetate, pH 4.5, was injected for 2 min (10 µLmin⁻¹) at a concentration of 50 µgµL⁻¹. The running buffer consisted of 10 mM HEPES, 150 mM NaCl, and 0.05% Tween-20 (v/v), pH 7.4. After amine coupling, the temperature was reduced to 10 °C. Solubilized membranes, containing the GluA2 AMPAR were injected for 75 minutes at a flow rate of 2 µLminute⁻¹, generating GluA2 receptor surfaces. Control surfaces were generated by injecting solubilized membranes from non-transfected cells. The running buffer consisted of 25 mM HEPES, 150 mM NaCl, 0.1% DDM (w/v), pH 7.4 (buffer A).

Interaction analyses were performed at 10 °C using buffer A as the running buffer. Noelin1-3, diluted into buffer A, was injected in 2-fold dilution series (7.2 nM – 0.45 nM) in ascending order for 120 s (30 µLmin⁻¹) and allowed to dissociate for 180 s. Buffer injections served as blank samples. Analyses were repeated on freshly prepared biosensor surfaces. For experiments under reducing conditions, Noelin1-3 was diluted into Buffer A supplemented with 1 mM DTT and incubated for 30 minutes at room temperature on a rocker shaker (10 rpm).

Biacore BIAevaluation Software 4.1 (GE Healthcare) was used for data analysis. Sensorgrams from GluA2 receptor surfaces were double-referenced by subtracting the signals from control surfaces and the average signals from the blank injections. The kinetic rate constants were (k_on and k_off) was determined by global non-linear regression analysis using a reversible 1-step interaction model (Fig. 2b).

**Single nanoparticle tracking (QD) for surface diffusion of AMPAR**

For AMPAR-QD tracking, hippocampal neurons transfected with GluA1::pHluorin or GluA2::pHluorin along with Homer1::dsRed as previously described (Klueva et al., 2014). Briefly, neurons were incubated with a monoclonal antibody directed to
GluA1::pHluorin or GluA2::pHluorin subunit for 10 minutes followed by a 5-minute incubation with QDs 655 Goat F(ab')2 anti-mouse IgG (Invitrogen). QDs were detected by using a mercury lamp and appropriate excitation/emission filters. Images were obtained with an interval of 30 ms and up to 1000 consecutive frames. QD recording sessions were processed with the Metamorph software (Universal Imaging Corp.). The instantaneous diffusion coefficient (D\text{inst}) was calculated for each trajectory, from linear fits of the first 4 points of the mean square displacement versus time function using MSD(t) = \langle r^2 \rangle (t) = 4Dt. The two dimensional trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images using a Vogel algorithm. Average QD traces and Homer1::dsRed co-localizing spots were used to define synaptic and extra-synaptic structures (Frischknecht et al., 2009). Immobile receptors were defined as having D\text{inst} < 0.001 \mu m^2 s^{-1}.

For assessment of Noelin1 effect on mobility after hyaluronidase treatment, cells were treated with Hyaluronidase (250 units) for 1 h prior to measurement.

**Electrophysiology**

All electrophysiological recordings were made as previously described (Klaassen et al., 2016) with minor modifications. HEK293T cells were perfused with standard artificial cerebrospinal fluid (room temperature). Control cells were bathed in either control medium or Noelin1-expressing medium for at least 1 h prior to measurement and as well during the course of the measurements.

**Statistics**

Data are expressed as mean±SEM or as median and inter quartile range (IQR, 25%/75%). Statistical significances were tested using Graph Pad Prism (GraphPad Software v6.0, USA). D'Agostino & Pearson omnibus normality test was performed to test for data normality followed by F test to compare variances (P-value cutoff > 0.05) and subsequently unpaired t-tests or unpaired t-tests with Welch's correction was applied. If data was not normally distributed, Mann Whitney U-test was used.
Supplementary Figure S1. Noelin1 isoforms are expressed throughout the brain. a) Schematic representation of the four Noelin1 (Olfactomedin1) protein isoform that arise from alternative splicing. All forms share the common M-region (yellow). In this region the antigen is indicated to which the Noelin1 antibody was generated (orange box). Cysteine residues (black lines) in the M-region are involved in oligomerization (C73, C75, C85). Noelin1-1 and -2 isoforms contain the A1-region (also called B-region; dark red), and Noelin1-3 and -4 isoforms contain the A2-region (also called A-region; dark blue) at their N-terminus. Noelin1-1 and -3 isoforms contain the C1-region (also called Z-region; green) at the C-terminus, which contains the Olfactomedin domain. The M-region of Noelin1-2 and 1-4 only has a short C-terminal region (C2- or Y-region; brown). Red empty boxes show the peptides for Noelin1 obtained by LC-MS/MS analysis of GluA2/3 immunoprecipitation from hippocampus. b) Allen brain atlas in situ hybridization of Noelin1 (Olfm1; probe RP_040324_01_F03) in adult mouse brain shows Noelin1 expression in hippocampus, cortex and cerebellum. c) Immunoblotting for Noelin1 on P2+M isolated from hippocampus (HC), cortex (Cx), cerebellum (Cb) shows that the antibody recognizes all four isoforms. The expression in cerebellum is lower than in hippocampus and cortex.
Supplementary Figure S2. Noelin1 interact with GluA1 AMPARs and form tetramers upon expression in HEK293T cells but does not affect homomeric GluA1 AMPAR currents. a) Noelin1-1, Noelin1-3 and Noelin1-4 individually expressed with GluA1 subunit shows immunoreactivity in lysate. Each Noelin1 isoform is specifically enriched in GluA1 IP. b) Standard curve for the peptide (LTGISDPVTVK) to estimate Noelin1-3 levels in medium of HEK293T cells. Red circle shows the peak area for the Noelin1-3 sample used for SPR measurements. c,d) Cell medium from Noelin1-3 expressing HEK293T cells shows immunoreactivity at 75 kDa, but not in control medium (c). When HEK293T cell medium was run under non-reducing (without DTT) conditions, additional immunoreactivity is observed above 250 kDa (d). e,f) Bar graphs (mean±SEM) summarize the absence of effect of Noelin1-3 on rise time (e), and decay time (f) of homomeric GluA1 AMPAR currents in HEK293T cells either after co-expression (GluA1_Noelin1-3; light red) or addition of Noelin1 (GluA1 + Noelin1-3, dark red). P-values for rise time were GluA1 (n = 24) vs. GluA1_Noelin1-3 (n = 9) $P = 0.970$ (unpaired t-test), GluA1 vs. GluA1 + Noelin1-3 (n = 9) $P = 0.861$ (Unpaired t-test), and P-values for decay time were GluA1 vs. GluA1_Noelin1-3, $P = 0.961$ (unpaired t-test), GluA1 vs. GluA1 + Noelin1-3, $P = 0.207$ (Mann Whitney U-test). Currents were evoked by direct application of 1 mM glutamate during 1 ms. Number of cells used are indicated. g) Both co-expression and addition of Noelin1 shows normal rectification of homomeric GluA1 AMPARs.
Supplementary Figure S3. Noelin1 is enriched at extrasynaptic and postsynaptic sites of hippocampal neurons, where it colocalizes with Brevican. a) STED imaging in cultured hippocampal neurons at DIV 21 showing an overview or zoom-ins of dendrites (right) or spines (right, inset). Noelin1 (green), Brevican (red) and Homer1 (blue, synaptic marker) are shown. Merged picture shows color-overlay images of the three channels. Scale bars are indicated. Inset (right) shows a 2-fold enlargement. Line scans over dendrite (1, 2) are displayed in panel b. b) Numbered dashed white lines on the overlay image indicate locations of line scans across the three channels (x-axis: distance (µm); y-axis: intensity (arbitrary units)). Graphs illustrate the co-enrichment of immunofluorescence intensities of Noelin1, Brevican and Homer1 at dendrites and spines, with the latter showing extrasynaptic and synaptic sites.
Supplementary Figure S4. Noelin1 regulates GluA1 AMPAR mobility in HEK293T cells. a) Frequency distribution histogram of all particles for sep::GluA2 receptors in HEK293T cells with either coexpression of GFP or Noelin1 (GluA2_GFP; GluA2_Noelin1 respectively), and with either addition of control medium or Noelin1-containing medium (GluA2 + control, GluA2 + Noelin1, respectively) shows that the sep::GluA2 AMPARs move slower. b) Frequency distribution histogram of all particles for sep::GluA1 receptors in HEK293T cells with either coexpression of GFP or Noelin1 (GluA1_GFP, GluA1_Noelin1, respectively) and with addition of Noelin1-containing medium (GluA1 + Noelin1) shows that the sep::GluA1 AMPARs move slower. c) Box plots indicate a significant effect on the diffusion coefficient ($D_{\text{inst}}$) for sep::GluA1 AMPARs upon coexpression of Noelin1 (GluA1 (n = 31) vs. GluA1 Noelin1 (n = 26); $P = 0.034$ (Unpaired t-test), and a trend for addition of Noelin1 (GluA1 vs. GluA1 + Noelin1 (n = 24); $P = 0.100$ (unpaired t-test). d) Quantification of the immobile fraction obtained from panel b shows that immobility of GluA1 AMPARs is significantly increased upon addition of Noelin1 (GluA1 vs. GluA1+Noelin1, $P = 0.0242$ (unpaired t-test)), and shows a trend for co-expression of Noelin1 (GluA1 vs. GluA1 Noelin1; $P = 0.094$ (Mann-Whitney U-test))
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![Graphs showing diffusion and immobile particles for different conditions with Noelin1.](image)

- **b** Diffusion (log; μm² s⁻¹) with sample sizes: 15 (control), 11 (GluA1 + Noelin1).
- **c** Immobile particles with P = 0.020.
- **d** Synaptic GluA2: GluA2 + control vs. GluA2 + Noelin1.
- **e** Diffusion (log; μm² s⁻¹) with sample sizes: 17 (control), 17 (GluA2 + Noelin1).
- **f** Immobile particles with P = 0.111.
- **g** Synaptic GluA1: GluA1 + control vs. GluA1 + Noelin1.
- **h** Diffusion (log; μm² s⁻¹) with sample sizes: 15 (control), 11 (GluA1 + Noelin1).
- **i** Immobile particles with P = 0.055.
Supplementary Figure S5. Noelin1 regulates AMPAR mobility in neurons. a-c) Mobility of extrasynaptic sep::GluA1 AMPARs in young neurons (DIV11-13). a) Frequency distribution histogram of GluA1 AMPAR after 1-h incubation of control medium or Noelin1-containing medium. b) Box plots indicate a trend for the diffusion coefficient of sep::GluA1 AMPARs without and with Noelin1 incubation (GluA1 + control (n = 15) vs. GluA1+Noelin1 (n = 11); P = 0.059 (Mann Whitney U-test)). c) Quantification of the immobile fraction obtained from panel a shows a significant increase in the immobile fraction (P = 0.020; Mann Whitney U-test). d-f) Mobility of synaptic sep::GluA2 AMPARs in young neurons (DIV11-13). d) Frequency distribution histogram of GluA2 AMPARs after 1-h incubation of control medium or Noelin1-containing medium. e) Box plots indicate no effect for the diffusion coefficient of sep::GluA2 AMPARs without and with Noelin1 incubation (GluA2 + control (n = 17) vs. GluA2 + Noelin1 (n = 17); P = 0.304 (unpaired t-test)). f) Quantification of the immobile fraction obtained from panel d shows a trend (P = 0.111; unpaired t-test). g-i) Mobility of synaptic sep::GluA1 AMPARs in young neurons (DIV11-13). g) Frequency distribution of GluA1 AMPAR after 1-h incubation of control medium or Noelin1-containing medium. h) Box plots indicate a significant effect for the diffusion coefficient of sep::GluA1 AMPARs without and with Noelin1 incubation (GluA1 + control (n = 15) vs. GluA1 + Noelin1 (n = 11); P = 0.016 (unpaired t-test with Welch's correction)). i) Quantification of the immobile fraction obtained from panel g shows a trend (P = 0.055; unpaired t-test with Welch's correction).
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Additional Information

Smit holds shares in Alea Biotech BV. The author(s) declare no competing financial interests
Chapter-6

General discussion
General discussion

In this thesis, I describe several approaches to dissect the function of proteins important for glutamatergic synaptic transmission in the brain. In particular, my work covers aspects of protein isolation, separation of protein complexes and identification of their constituents using new combinations of technologies, and illustrating these technologies using glutamate receptors and their interactions. In more detail, I used different biochemical, electrophysiological and imaging methods to characterize a newly identified AMPAR interacting protein, Noelin1.

With the aim of understanding the underlying mechanisms that drive complex neuronal functions, over the last decades research has focused on elucidating proteins and protein complex composition residing in neuronal sub-domains. Various methods have been used to obtain insight in protein-protein interaction. Longstanding methods, such as yeast two hybrid screening and affinity purification, have been the gold standard tools for elucidating protein-protein interactions. In yeast two hybrid screening, usually the direct interaction between a pair of proteins is assessed by checking the ability of two fusion proteins to bind inside yeast cells. In recent years, this conventional yeast two hybrid method has undergone improvements, as in Membrane Yeast Two Hybrid (MYTH) and Mammalian Membrane Two Hybrid (MaMTH) Luminescence-based Mammalian Interactome Mapping (LUMIER). However, these techniques have in common that they rely on the expression of non-native proteins in heterologous cells. Alternatively, affinity purification followed by LC-MS/MS (AP-MS) analysis is an excellent method for identifying true protein complexes from native tissue.

Since AP-MS generally will identify several hundred proteins, the use of proper negative controls is key to distinguish true from false-positive interacting partners. Having identified protein interaction partners, the next step usually involves validation and characterization of the interaction in primarily three steps. 1) Validating the interaction using reverse IP experiments. 2) Establishing whether the interaction is direct or not. 3) Characterization of the subcellular localization of the interaction. In this thesis, I used interaction proteomics to characterize glutamate receptor interacting proteins and validate those using different independent methodologies. First, I will discuss experiments towards identification of proteins relevant to the synapse designed to provide hints of specific protein interactions by a multidimensional biochemical purification approach.
Determining protein co-isolation using multidimensional biochemical fractionation.

A route often taken to the first characterization of a novel protein involves determining the sub-cellular enrichment of the protein. Since neurons are highly polarized cells they are in principle amenable to analysis by enrichment of different subcellular organelles, such as postsynaptic density and microsomal compartments. Such an approach in principle can be quite effective. However, due to the inherent impurities in the biochemical fractionation protocols, analysis of single biochemical fractionation can lead easily to misinterpretation of the data. In this thesis, I used the separation of proteins over a series of biochemical fractions as a way to determine co-purification. Next, the correlation of purification over different dimensions, enables to detect proteins that are in the same biophysical environment, or are associated through interaction. We used high resolution mass spectrometry to identify and quantify approximately 2000 proteins in each fraction. Correlation profiling was helpful in identifying proteins that are truly enriched in the PSD, thereby allowing us to identify true PSD proteins. Since several PSD proteomes have been published before, a comparative analysis with other methods is of interest (Table 1). In particular, the study of Bayes et al \cite{Bayes2008} and Distler et al \cite{Distler2008}, are two of the largest PSD protein datasets. In Bayes et al, extensive fractionation was used to profile the PSD proteome and identify approximately 1500 proteins. However, the study lacked analysis of a corresponding synaptosome fraction and thereby the PSD dataset defined, although extensive, lacks essential information about enrichment and contaminants, which limits to distinguish PSD proteins. The study of Distler et al used correlation profiling to determine true PSD enriched proteins, however, the enrichment factors computed in this study for canonical PSD proteins such as NMDAR were around 1.5–2-fold, which is much lower than reported previously \cite{44,50}. This can be attributed to two aspects. 1) They used young animals (3 weeks) and thus their dataset might represent not fully developed or mature synapses. 2) They used MS\(^E\), a data independent method to identify and quantify proteins, which however, inherently suffers from ion suppression because of the vast number of product ion spectra. In our study, we found over 1900 proteins in the synaptosome and synaptic membrane fractions and 880 in the PSD fraction across all biological replicates of the hippocampus. Due to extensive coverage of the PSD fraction, we hypothesized that we might identify previously unreported PSD-enriched proteins. By performing correlation profiling with well-known PSD enriched proteins, we observed many proteins that potentially could be PSD enriched. Upon inspection of the PSD
enriched proteins, we found two candidates, Adgra1 and Plekha5 as potential PSD enriched proteins and their PSD localization was validated using super-resolution imaging.

Using the same methodology, we evaluated known datasets of AMPA receptor interacting proteins, and observed differences in their enrichment, with proteins Cpt1c, Frrs1l and Sacm1l, enriched in the microsomal fraction, as opposed to synaptosome or PSD fraction for other well characterized AMPAR interacting proteins, e.g., TARP γ2, −8. As Cpt1c has already been described to affect AMPAR surface expression and localization\textsuperscript{103,140}, we hypothesized that Frrs1l and Sacm1l may have similar roles. Thus, this dataset can be further mined to understand localization and hypothesis formulation on the function of hitherto functionally uncharacterized proteins.

With the advent of more sensitive mass spectrometers, it is likely that similar studies will yield more comprehensive datasets with higher coverage of brain biochemical fractions. With greater sensitivity, future studies might even describe enrichment for proteins in specific sets of neurons isolated using laser capture microdissection and FACS. Several challenges remain to be addressed. For instance, the miniaturization of protocols for pure PSD isolation needs to be developed. In combination with super-resolution imaging, in particular nanoscopy methods, such as STED and PALM, and the more tedious EM, will allow assessment of a more detailed sub-cellular localization of proteins in the synapse and neurons in general. Furthermore, label-free quantification of synapse preparations under different stimulated and/or disease conditions has been widely performed to understand the synaptic changes in neurons in different states. However, based on this study, such approaches would need to be to applied to multiple fractions beyond the frequently used 'single-synaptosome preparation' to provide insight in regulation and in co-regulation of proteins. This might aid the understanding of stimulus dependent regulation of sub-proteomes and biological processes in health and disease.
Table 1: Summary of PSD proteomics studies performed on PSD fraction. In most studies, no co-relation or enrichment of PSD proteins is studied leading to large list of contaminant proteins in the PSD list.

<table>
<thead>
<tr>
<th>Study</th>
<th>Source</th>
<th>Fractions analyzed</th>
<th>Fractionation method</th>
<th>Mass spectrometer</th>
<th>Number of proteins in PSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li <em>et al.</em>, 2004</td>
<td>Rat Forebrain, 30 day old</td>
<td>PSD</td>
<td>2D Gel electrophoresis</td>
<td>Maldi TOF/TOF (Sciex)</td>
<td>250 (No enrichment)</td>
</tr>
<tr>
<td>Li <em>et al.</em>, 2004</td>
<td>Rat Forebrain, 30 day old</td>
<td>Synaptic membrane, PSD</td>
<td>ICAT labelling, 2D gel electrophoresis, Co-relation profiling</td>
<td>Maldi TOF/TOF (Sciex)</td>
<td>60 ICAT pairs enriched in PSD (Enrichment by ICAT)</td>
</tr>
<tr>
<td>Yoshimura <em>et al.</em>, 2004</td>
<td>Rat Forebrain</td>
<td>PSD</td>
<td>Isoelectric focusing, SDS PAGE</td>
<td>Q-TOF (Micromass)</td>
<td>492 (No enrichment)</td>
</tr>
<tr>
<td>Jordan <em>et al.</em>, 2004</td>
<td>Rats and Mice</td>
<td>PSD</td>
<td>SDS PAGE, 25/50 bands</td>
<td>Q-TOF (Micromass)</td>
<td>750 (No enrichment)</td>
</tr>
<tr>
<td>Cheng <em>et al.</em>, 2006</td>
<td>Rat, Forebrain and Cerebellum</td>
<td>PSD</td>
<td>ICAT labelling, Absolute quantification for 32 proteins</td>
<td>LCQ-DecXP-Plus ion trap (Thermo)</td>
<td>296, 43 different between forebrain and cerebellum (No enrichment)</td>
</tr>
<tr>
<td>Collins <em>et al.</em>, 2006</td>
<td>Mouse, Forebrain</td>
<td>PSD, IP for GluA2, NR2, Arc</td>
<td>SDS PAGE</td>
<td>Q-TOF (Micromass)</td>
<td>620 proteins from PSD, 9 in GluA2 IP, 100 in NR1 IP, 170 in NR2B IP.1124 proteins present in PSD list.</td>
</tr>
<tr>
<td>Distler <em>et al.</em>, 2014</td>
<td>Mouse, 19 Day old Hippocampus</td>
<td>P2, Synaptosome, PSDI, PSDII</td>
<td>Single fraction, FASP</td>
<td>DIA, Synapt G2-S HDMS Mass spectrometer (Waters)</td>
<td>2102 proteins in PSDI and PSDII (Enrichment by co-relation profiling)</td>
</tr>
<tr>
<td>Our study</td>
<td>Mouse, Adult Hippocampus</td>
<td>P2, Microsome, Synaptosome, Synaptic membrane, PSDII</td>
<td>SDS PAGE, Single fraction</td>
<td>Triple TOF, Sciex</td>
<td>1412, 300 enriched in Hippocampus (Enrichment by Co-relation profiling)</td>
</tr>
</tbody>
</table>
Understanding protein complexes of glutamate receptors

Glutamate receptors play a critical role in synaptic transmission with ionotropic receptors (GluNs, GluKs and GluAs) and metabotropic glutamate receptors (mGluRs). With the understanding that the activity and localization of neurotransmitter receptors are modulated by auxiliary proteins, the hunt for those regulating glutamate receptor function has intensified.

Shedding light on type 1 mGluR complexes

Of the mGluRs, type 1 mGluRs, mGluR1 and mGluR5, have been implicated in a wide variety of neuropsychiatric disorders\(^{62,71}\). The role of the mGluR in mechanisms that can rescue fragile X syndrome has pinpointed mGluR modulators as potential therapeutic agents. With the lack of mGluR1/5 interacting proteins, the need for identifying mGluR1/5 modulating proteins has increased. In chapter 3, we used a knockout controlled interaction proteomics strategy to identify novel mGluR5 interacting proteins in cortex and hippocampus \textit{ex vivo}. Interestingly, using mGluR5 immunoprecipitation we revealed the presence of mGluR1. Since mGluR1 and mGluR5 seem to have largely complementary expression profiles in the brain, we validated this interaction by performing immunoprecipitations for mGluR1 and found mGluR5 to be enriched in these. In addition, we discovered that apart from mGluR1, detergent-extracted mGluR5 complexes contain members of the known homer family of interacting proteins, which show brain region specificity. We validated the mGluR1-mGluR5 interaction in both cortex and hippocampus by: 1) Performing reverse IP experiments using an mGluR1 antibody; 2) Validating a direct interaction between mGluR1 and 5 in a heterologous system; 3) Demonstrating co-localization between the mGluR1-mGluR5 complex in homer positive (PSD) and Homer1 negative extrasynaptic puncta. Detailed examination of the peptides obtained for mGluR1 in the mGluR5 IP experiments revealed isoform specificity of mGluR5; namely isoform b of mGluR1 interacts with mGluR5. The fact that the interaction is observed in the microsomal fraction as well as in P2 fraction, indicates that mGluR1-5 interaction might occur already in the endoplasmic reticulum or the Golgi apparatus and also at synapses.

Although typical IP experiments show over 200-300 proteins, with the use of knockout controls, we were able to limit the list of true mGluR interacting proteins to a few true positives, which were validated using reverse IP experiments. Since transient interactions are easily lost during IP experiments, this study likely revealed a highly stable mGluR1-5 heterodimer. In future studies it needs to be determined whether all of
mGluR1 and -5 interact, or whether this only represents a partial pool of these receptors. Furthermore, the cell types and dendrites in which this complex is particularly enriched needs to be determined. Also, it is of interest to determine whether this complex composition is altered in fragile X syndrome and other neuropsychiatric disorders, which might pave the way for the design of positive or negative allosteric modulators of mGluR1-5 interaction and function.

Teasing apart AMPAR subcomplexes

Unlike mGluR1, -5, AMPARs have been shown to interact stably with a large number of proteins of which many regulate AMPAR trafficking, lateral diffusion and/or channel properties. Previously, we showed that the AMPAR forms distinct local protein complexes in a brain region specific manner and thus potentially underlying the divergence in plasticity mechanisms in different brain regions.

In Chapter 4, we focused on the hippocampus to characterize AMPAR complexes, and performed high-density IP experiments using the interacting proteins as baits. Surprisingly, we observed that IP experiments for TARPs (TARP γ8), Cornichon (Cnih2) and Frrs1l presented multiple overlapping proteins. This let us to hypothesize that these proteins might be involved in multiple AMPAR complexes, which gave rise to the need of a method to identify them. To this end, we combined the power of immunoprecipitation and blue native page to develop IPBN-MS, a method to enrich and separate intact protein complexes. Although BN-PAGE has been used previously to separate protein complexes, the key step in IPBN-MS is the specific enrichment of protein complexes using IP, followed by eluting these with the antigen peptides used to generate the IP antibody, followed by BN-PAGE analysis and LC-MS/MS to identify protein complexes. AMPAR complexes were observed eluting over a wide range of molecular weights, between 700–1000 kDa, with the Cnih2-Frrs1l-AMPAR complex observed at the lowest molecular weight range. This indicated that native AMPARs hardly exist in the hippocampus, and secondly, that this complex is stable and survives separation by BN. Obviously the steps before BN, e.g. the IP itself may suffer from lost proteins. One of the key steps in deciphering AMPAR subcomplexes was to perform IPBN-MS for individual AMPAR interacting proteins. This approach allowed us to determine 7 classes of AMPAR complexes, the evidence for which was obtained from multiple IPBN-MS experiments. We validated whether these subcomplexes could really form using co-expression in HEK293 cells and using immunocytochemistry to determine their subcellular colocalization.
Another crucial step in the characterization of subcomplexes was the use of spiked-in molecular weight markers in the samples before the BN-PAGE separation. This along with precise cutting of the gel using a grid-cutter was important in aligning and thereby identifying different subcomplexes over multiple experiments.

Taking the analysis of IPBN-MS a step further would involve determining the AMPAR complex stoichiometry. This is challenging because the protein AMPAR we identify represents only the extractable pool of AMPAR complexes and lack the set of AMPARs located deep within the non-extractable postsynaptic density. Moreover, it will be hard to establish whether stoichiometry is not influenced already during IP. From a biochemical perspective, further studies will focus on characterization of AMPAR subcomplexes in other brain regions using the same methodology. In effect, it is important to define the core AMPAR machinery and AMPAR subcomplexes in different brain regions.

With the identification of AMPAR subcomplexes, a remaining step is the characterization of functions of these. Until recently, several studies have focused on determining the function in a one to one manner\textsuperscript{54,124,126,132,134}. However, with the understanding that different auxiliary proteins can decorate the AMPA receptor, a combinatorial approach will be necessary. One challenge in studying the physiology and stoichiometry of these multiprotein complexes lies in the ability to reconstruct them in cell lines. With having the difficulty to express more than 3 proteins in HEK cells in a reliable, ratio-metric manner, studying these in Xenopus oocytes is a requirement. To study their function \textit{in vivo} is the ultimate challenge. To this end, double or triple knockouts using the CRISPR-CAS system might help to shed light on the role auxiliary proteins contribute in these subcomplexes.

**Determining function of AMPAR interaction partners**

Chapter 5 of this thesis focused on the characterization of the function of an AMPAR interacting protein, Noelin1, a member of the olfactomedin family. Noelin1 may have unique properties as it is the only known secreted AMPAR interacting protein. In this study, we used a combination of biochemical, imaging and electrophysiological approaches to assess Noelin1 function on the AMPAR and show that it classifies as AMPAR auxiliary protein\textsuperscript{117}. According to these criteria, a candidate protein needs to be a non-pore forming subunit, modulate the properties of the receptor and is necessary \textit{in vivo}. We demonstrated that Noelin1 is a secreted glycoprotein that interacts directly with the AMPA receptor using expression in HEK cells and we provided evidence for
this by using surface plasmon resonance (SPR) measurements. In this process, we were able to set up the first SPR measurement of the whole AMPA receptor. Noelin1 co-localizes with the AMPAR in synaptic as well as in extra-synaptic locations. We demonstrated that Noelin1 can regulate AMPAR lateral mobility from the extracellular side and that its expression overlaps with the extracellular matrix and is regulated in an activity-dependent manner. Other members of the olfactomedin family of proteins, which also interact with the AMPAR (Noelin2 and Noelin3), are likely to have a similar function and may also belong to the same complex.

Thus, in this study, we showed a novel role of a secreted glycoprotein that binds directly to the AMPA receptor and regulates AMPAR membrane mobility. Since Noelin1 synaptic expression increases after chemical LTP treatment, it is likely responsible for regulating AMPAR movement at the synapse. It has been shown previously that the extracellular matrix is responsible for regulating AMPAR lateral diffusion at extrasynaptic localizations, but not at the synapse\textsuperscript{157}. With Noelin1, we identified an extracellular component, which is responsible for regulating AMPAR lateral diffusion at the synapse. Further studies might focus on assessing Noelin1 function in learning and memory and how it affects short-term plasticity at synapses. This study also serves as a template for determining functions of other yet uncharacterized AMPAR interacting proteins using a multilevel analysis.
Outlook

The idea that a single protein can be a part of different complexes based on its subcellular localization is not new, however until now, the tools to determine this in a single study have been lacking. The advent of IPBN-MS or a modified workflow of IP followed by size fractionation will allow us to determine protein subcomplexes in more detail. Distinct protein complexes might form through specific posttranslational modifications of their components. Thus, the next logical step in this process would be to identify posttranslational modifications associated with the formation or disruption of these subcomplexes and those that are stimulus dependent. Revealing the dynamics of protein complexes is a next frontier. With the added, dimension of subcomplexes, some method development will be necessary to down scale the amount of cells needed for such experiments. Other experiments would be the analysis of dynamics of protein subcomplexes and their associated post-translational modifications in models of human disease. Ultimately we should be able to understand by which triggers complexes form and dissociate, and how this is regulated in health and disease. The universal applicability of the IPBN-MS workflow will allow for answering these questions, not only in neurobiology, but in other fields as well.
Summary

Even with the evolved complexity of the human brain, the underlying processes of communication between neurons, the primary information processing cells, have remained conserved. Neuronal communication serves different functions, e.g., from regulating physical processes in the human body to generating thoughts, feelings, acquiring new concepts and memory. This fast neuronal mode of communication occurs via specialized intercellular contacts, called synapses. Synapses are structured to facilitate signal transmission. They are extremely large in number with over 100–1000 synapses per neuron, bringing neuronal communication to staggering numbers and complexity. Neuroscientists have observed several well-conserved underlying mechanisms of synaptic transmission and plasticity of transmission across different types of neurons in the brain. Compromising these mechanisms, for instance, due to mutation, can lead to impairments of synaptic function, referred to as a synaptopathies. These synaptic impairments can lead to brain disorders that impact on human health. Understanding molecular processes of communication between synapses, would allow us to comprehend healthy function but also facilitate the development of treatment strategies necessary to combat the disease states.

One of the ways to elucidate the complex processes that occur at synapses is to use a proteomics approach. In particular, this allows us to understand the protein composition and their interplay at synapses. This can best be done using modern mass spectrometry methods to identify and quantify proteins that are present in synapses, which are biochemically isolatable cellular compartments. In chapter 1, the introduction of my thesis, I describe the current knowledge of synaptic proteomes, focusing on the excitatory neurotransmission facilitated by glutamate receptors, namely metabotropic glutamate receptors, specifically mGluR1 and 5, and ionotropic glutamate receptors, specifically the AMPARs.

By now we have a good understanding of the composition of the average excitatory synapse, mostly based on a number of standalone studies on synaptic composition using proteomics approaches. However, the comprehensive analysis of synaptic, non-synaptic and postsynaptic density proteomes has been lacking in a single study. Thus in chapter 2, I performed a detailed synaptic, postsynaptic density proteome analysis from the hippocampus, enabling us to create a resource of proteins present at the synapse in biochemically defined sub-structures, and to identify and validate novel proteins present at the post synaptic density. Because of the use of highly sensitive mass spectrometry analysis, we achieved excellent depth and...
coverage of the synaptic proteome with over 4000 proteins identified and quantified. Further evaluation of the datasets is bound to reveal novel insights in proteins in different synaptic compartments.

Proteins regulate each other’s activities by being part of protein complexes. Thus, apart from using proteomics tools to identify proteins present in a particular sample, biochemical enrichments using specific antibodies by immunoprecipitation (IP), has the potential to identify protein complexes, a study commonly referred to as interaction proteomics. This thesis focuses on elucidating complexes of glutamate receptors in the brain. In particular, In chapter 3, I focused on mGluR5 complexes. mGluR5 is an important postsynaptic enriched metabotropic glutamate receptor, best characterized for its role in modulating long term synaptic depression (LTD). Additionally, mGluR5 modulators have important potential in therapy for fragile X mental retardation. Using the mGluR5 knockout mouse as a negative control for IP experiments, I observed a specific mGluR5 complex with mGluR1 in the hippocampus and the cortex. This complex was validated using reverse IP for mGluR1, co-expression in heterologous systems and imaging demonstrating the postsynaptic localization of the complex. This finding of mGluR1-5 dimers has implications for the design of positive and negative allosteric modulators on the mGluR1-5 complex for therapy for fragile X-syndrome and other neuropsychiatric disorders.

Fast excitatory neurotransmission in the brain is primarily mediated by AMPA receptors (AMPARs) that unlike mGluRs, have been demonstrated to stably interact with a myriad of proteins. The interacting proteins of the AMPAR affect several properties of AMPAR function, all the way from biosynthesis to their eventual localization in the postsynaptic density. The identity of many of these interacting proteins is now well established. However, the current model of AMPAR complexes assumes the same receptor composition throughout the cell, which is unlikely based on different enrichment of these interacting proteins in different cellular fractions. Current methods in protein complex analysis using classical IP experiments do not allow for identifying these probable receptor subcomplexes. In chapter 4, in addition to using a high density reverse IP strategy to identify AMPAR subcomplexes, we developed a novel strategy to identify protein complexes, by combining the specificity of IP experiments with the ability of blue native page (BN-PAGE) to separate native protein complexes, collectively termed as IP-BNMS. Using this approach, we for the first time demonstrate that AMPARs are indeed comprised of different subcomplexes. Further
functional experiments will be needed to elucidate the function of these individual protein complexes, rather than looking at these interactions in a one to one manner.

In addition to identifying proteins that stably interact with the AMPARs, we were interested in performing functional analysis of these interactions as well. To this end, in chapter 5, I focused on the AMPAR interacting protein called Noelin1. The role of Noelins in neuronal development and axonal migration has been reasonably well characterized, but its influence on the AMPAR remained poorly understood. Noelin1 is a secreted glycoprotein and forms a stable complex with AMPARs at synaptic and extrasynaptic sites. However, we found that, unlike many transmembrane AMPAR interacting proteins, Noelin1 does not affect the channel properties of AMPARs, but instead affects the lateral diffusion of AMPARs. In fact, to our surprise, we observed that Noelin1 immunoreactivity reduces after treatment with hyaluronidase, which indicates that it is a component of the extracellular matrix. Additionally, we also observed that Noelin1 reduces the lateral diffusion of AMPARs even in the absence of a well-formed ECM. This finding, apart from the fact that Noelin1 is regulated in learning and memory processes, demonstrates that Noelin1 is one of the important components of the AMPAR complex and regulates important AMPAR functional properties. This study also forms a framework for further studies of other Noelin1 family members.

Finally, in chapter 6, I discuss potential pitfalls and the future course of action for each chapter. In particular, the future of synapse proteomics in health and disease is an important discussion point. The use of modern mass spectrometers will allow us to do more in depth analysis of synaptic proteomes from different brain regions. I also discuss in detail the future of interaction proteomics with multidimensional approaches, such as BNPAGE and how to derive to subcomplexes and their stoichiometry. Finally, I discuss how future studies on AMPAR complexes will focus on various combinations of interacting partners as demonstrated by us.
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About the Author

Nikhil Janak Pandya was born on 29th December, 1986 in Mumbai, India. He started his training in life sciences with an integrated Masters in Biotechnology and Bioinformatics from the Institute of Bioinformatics and Biotechnology (IBB) from the University of Pune (now known as Savitribai-Phule University). During his masters training, his first stint in the lab was in a project with Dr. Sarayu Davawala in synthetic organic chemistry. Followed by projects on protease inhibitor screening with Dr. Bimba Joshi, Mathematical modeling with Dr. Chetan Gadgil (National Chemical Labs, Pune) and finally master’s thesis with Dr. Anjali Shiras in oncology at National Center for Cell Sciences, Pune. Further, he worked as a research associate in the lab of Dr. Kai Kaila at the University of Helsinki in electrophysiology after which he started his doctoral training in Neuroproteomics in the lab of Prof. Dr. August B Smit. Dr. Ka wan li and Prof. Dr. Sabine Spijker at the Department of Molecular and Cellular Neurosciences at the Center of Neurogenomics and Cognitive research at the Vrije University in Amsterdam. His doctoral project was on the Structural and functional characterization of Glutamate receptor complexes in the brain.
Publications


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None the less, as I sit at a railway station traveling to my beloved wife, I wonder this story would not have been possible without the union of two of the most amazing people in the world; my parents. It is hard to put in words my thanks to all the blood, sweat and tears that have been shed in the process of making me who I am. Mom dad, I know you are proud of me, but I am more proud of you. So far, but always a phone call away to act as my friends, mentors, confidantes and LIFE!. As all these memories of countless times I have relied on you come rushing back, neither the English language, nor these few pages I have, will do justice to the many thanks for the innumerable things I would like to say. Mom for invoking every god possible for my success, Dad, for being their numerous times with words of encouragement, Many thanks!

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