Shisa proteins and AMPA-receptor function

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About the cover:
The Picture is called “The Elephant in the Room” by Nhazlam Enal, 2015.
Shisa proteins and AMPA-receptor function
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At first, it’s no more than a haze on the horizon, so you watch, you watch. Then it’s a smudge, a shadow on the far water. For a day, for another day, the stain slowly spreads along the horizon taking form until on the third day, you let yourself believe. You dare to whisper the word. Land.

Land.

Elisabeth, The golden Age, 2007
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Chapter 1

General Introduction
General Introduction

1.1 In short

The brain is without doubt the most complex organ of vertebrates. Anatomical studies indicate that the brain consists of billions of specialized cells, i.e., different types of glial cells and neurons, also called nerve cells. Neurons are highly interconnected through synapses, which are specialized anatomical junctions. In general synapses are divided into excitatory and inhibitory synapses, thus those facilitating and those inhibiting propagation of electrical signals between two neurons. The principal neurotransmitter at excitatory synapses is glutamate [1, 2].

Triggered by electrical activity at glutamatergic synapses, glutamate containing vesicles fuse with the presynaptic membrane and release glutamate into the synaptic cleft. This released glutamate then diffuses and binds to specific glutamate-receptors that are located in the postsynaptic membrane. Here, classically three types of glutamate receptors are present: NMDA-, AMPA- and kainate- receptors (for review see [3]). When binding to glutamate, AMPA receptors (AMPARs) undergo conformational changes, which lead to the rapid opening of their ion-channel pore, resulting in a postsynaptic cation influx. The rapid activation properties of AMPARs account for the vast majority of fast excitatory transmission and mediate the initial depolarization of the postsynaptic membrane [4, 5]. When in their open state the majority of synaptic AMPARs in the hippocampus, the brain area in focus of this thesis, will be permeable to potassium and sodium ions but impermeable to calcium, due to their subunit composition [6-8]. However, synaptic signalling and plasticity have been reported to be directly dependent on calcium influx into the postsynapse, which is mediated by the second major type of glutamate receptors, the NMDA-receptors (NMDARs). These receptors remain inactive upon initial glutamate binding until the AMPAR-mediated postsynaptic depolarization removes a magnesium ion that blocks their channel pore in a voltage dependent manner [9, 10]. Only when this depolarization occurs in a timely manner, such that the blockade is removed fast enough for glutamate to still be present at NMDARs, these receptors can then change into their activated state and mediate calcium influx into the postsynapse [11, 12]. Calcium influx in turn acts as a major second messenger for postsynaptic processes along with assisting further depolarization of the postsynaptic neuron (see figure 1 [13]). Even though the importance of NMDAR activity for synaptic plasticity is commonly accepted, little is known about the downstream signalling pathways that follow NMDAR activation [14]. However, it is without controversy that the activation of NMDAR is followed by the activation of various Ca2+ activated downstream signalling cascades that act as feedback loop regulating glutamate receptors among other things.
Chapter I

Figure 1. Schematic drawing of a glutamatergic synapse. Upon electrical activity in the presynapse, glutamate containing vesicles fuse with the presynaptic membrane and release glutamate into the synaptic cleft (1). Glutamate then diffuses onto AMPA- and NMDA- receptors at the postsynaptic site. AMPAR start conducting cations over the postsynaptic membrane (2). NMDA- receptors remain closed due to an intrinsic Mg2+ block. After the initial postsynaptic depolarization through AMPARs the Mg2+ block is removed and NMDA receptors start conducting Ca2+ into the postsynapse (3). The converted electrical signal can propagate towards the soma (4).

With regard to this tight interplay between AMPA and NMDA receptors, it is feasible that the number of AMPARs opening upon glutamate release at the presynapse directly determines the likelihood of NMDAR activity and therefore the likelihood of postsynaptic calcium influx [15]. These findings gave rise to widely accepted hypothesis that the number of AMPARs present at postsynaptic membranes directly correlates with synaptic strength and that changes in the number of these receptors directly underlie the phenomenon of synaptic plasticity, a term describing the usage-dependent strengthening and weakening of synapses over time [14, 16-18]. Synaptic plasticity is seen as the basis for all major brain-related processes including learning and memory formation [15].

In general, plasticity may lead to strengthening or weakening of synapses. The repeated activation of a synaptic connection will result in the strengthening of this particular synapse also called synaptic potentiation [19]. The expression of Long-term potentiation (LTP) reflects plasticity changes that can be long lasting. The opposite, namely the process of synaptic long – term weakening due to low synaptic activity is termed long-term depression or LTD.

Based on recent findings in AMPAR research, a three step model for AMPAR-mediated synaptic plasticity has been proposed [20]. In order to strengthen a synaptic connection, AMPARs are first inserted into synaptic membranes in a process involving exocytosis of AMPAR containing vesicles. Secondly, after membrane insertion, AMPARs diffuse laterally into the synaptic membrane where, in a third step, they are trapped/ anchored to the synaptic scaffold by protein interactions [21-24]. The synaptic weakening process and thus the removal
of AMPARs from synaptic membranes may either occur at synaptic sites directly, or after diffusion out of the synapse to a membrane area called the endocytic zone [25]. Each of these steps in the process of synaptic plasticity has been subject of intense AMPAR research over the past decade.

Although this step-wise model of synaptic plasticity and its formation is generally accepted, there are certain limitations. For instance, in this model, only the number of AMPARs at synapses is important to predict synaptic strength, all AMPARs are simplified conductance entities, disregarding the differences in subunit composition, splice variants and specific protein interactions that may influence their conductance properties and behaviour. Furthermore, though AMPARs were known to interact with a variety of intracellular proteins, none of these interactions fully accounted for the observed immobilization and stabilization of AMPARs at synapses during synaptic plasticity.

An important breakthrough in the understanding of AMPAR mediated processes came with the first description of AMPAR auxiliary proteins [26, 27]. These transmembrane proteins were shown to form stable complexes with AMPARs and to influence the behaviour of AMPARs on various levels, including receptor trafficking, surface expression and channel conductance [28]. Not only did these proteins provide the missing link between AMPARs and the synaptic scaffold, they also helped to understand AMPAR regulation and fine-tuning as well as they refined our view of glutamatergic transmission. The discovery of these proteins was an important step in understanding AMPARs, and many new questions arose concerning the precise working mechanism, binding distributions, structures etc., of these proteins, among which are investigated as described in this thesis.

AMPAR auxiliary subunit proteins originate from three well-established families: Transmembrane AMPAR Regulatory Proteins (TARPs), Cornichons (CNIH) and Cysteine-Knot-AMPAR-modulatory proteins (CKAMPs), also known as the Shisa – family (for overview see [28, 29]). Until now, only one member of the multi-protein Shisa family, CKAMP44, has been described in terms of its function at the AMPAR [30]. The high evolutionary conservation of this protein family across vertebrate species indicates important functional implications of the Shisa family members. In the work at hand, I characterized a newly discovered AMPAR interacting protein from the Shisa family, Shisa6 (see Chapter 2 and 3), and I present insights into the working and regulatory mechanism of Shisa6 and Shisa9 (CKAMP44) through C-terminal interactions [31].

To do so, I will first give a detailed introduction on the structure and mechanisms of AMPAR function, their importance for synaptic transmission and how AMPAR movement, trafficking and interaction underlie common models of synaptic plasticity. Furthermore, I will highlight the major findings in the field of AMPAR auxiliary proteins and how their discovery impacted on our understanding of AMPAR regulation and also postsynaptic function.
1.2 The AMPA receptor

The AMPA receptor belongs to the class of ionotropic glutamate receptors and is the most ubiquitously expressed receptor in the central nervous system. Its name derived from its ability to be activated by the artificial glutamate analogue AMPA, α-amino-3 hydroxy-5-methyl-4-isooxazole-propionic acid [32]. Located at the postsynaptic site of synapses, AMPARs rapidly open their channel pore upon glutamate binding and mediate the vast majority of fast excitatory synaptic transmission [4, 5, 33]. The importance of AMPAR for synaptic function is underlined by multiple studies investigating deletion of AMPAR subunits, which either result in major impairments in learning and memory formation or in lethality [34-37].

The AMPAR was the first ionotropic glutamate receptor to be crystallized [38] enabling to understand their structure in detail. Structurally, AMPARs are composed as tetrameric assemblies of highly homologous subunits GluA1-4, which can assemble in various combinations [3, 4, 39, 40]. Each subunit contains four hydrophobic domains that constitute three membrane-spanning domains and one re-entrant loop into the cytoplasm that forms the channel pore (see Figure 2) [38, 40]. As for most transmembrane proteins, AMPARs can be structurally divided into an N-terminal, a transmembrane and a C-terminal domain. The N-terminal domain is located on the extracellular side and harbours the ligand-binding site, which is formed by two parts split by a transmembrane domain [41]. In the membrane region, four hydrophobic domains span the membrane a total of three times. The fourth hydrophobic sequence forms a re-entrant loop, which reaches into the membrane and leaves the plasma membrane on its entry side [42]. Upon tetramerization, this domain constitutes the channel pore. The C-terminal domain reaching into the intracellular side has been implicated in the regulation of AMPARs through posttranslational modifications and by mediating protein interactions [17, 43-47].

![Figure 2. AMPAR structure](image)

**Figure 2. AMPAR structure**, A. Cartoon depicting the domain structure of an individual AMPAR subunit. B. Cartoon showing the tetrameric assembly of four AMPAR subunits, which form the functional receptor. Note the RNA-edited Q/R site in the GluA2 subunit (figure taken from Fleming & England, Nature Chemistry, 2010 [48], with permission of the publisher).
The assembly of functional tetrameric AMPARs was shown to take place in two steps. First, two subunits dimerize, followed by the dimerization of the two pre-existing dimers. The newly formed tetramer is then processed through the trans-Golgi network and is subject to several posttranslational modifications [49]. The initial dimerization was shown to take place in parallel to translation of AMPAR subunits in the ER and it seems to be mediated by interactions of two N-terminal domains [50, 51]. The extracellular S2 loop and the membrane region (including the pore) play a crucial role in the formation of the tetramer [52]. How the specific subunit stoichiometry of AMPARs is determined remains elusive. However, it is known that AMPARs assemble as preferential or obligatory heteromers when incorporating the GluA2 subunit [51, 53-55].

AMPARs are generally permeable to calcium and monovalent cations. However, the GluA2 subunit is subject to RNA editing on its pore-forming domain. Here, the DNA originally encodes a glutamine (Q) residue, which is exchanged for a positively charged arginine (R) [8]. This modification renders AMPARs incorporating this subunit calcium impermeable and insensitive to polyamine blockade at positive membrane potentials, resulting in a linear current-voltage relationship as opposed to an inward rectification of all other AMPAR types [8, 56-59]. Since about 95% of hippocampal AMPARs incorporate the GluA2 subunit (and this work focusses on hippocampal neurons) [6, 7], hippocampal AMPARs can be widely regarded as calcium impermeable, making calcium influx upon synaptic activity largely NMDAR dependent.

The intracellular C-terminal domain (CTD) of AMPARs plays an important role in the regulation of AMPARs by being a target for numerous posttranslational modifications including phosphorylation, palmitoylation and protein-interactions through multiple protein interaction domains including a PDZ-interaction motif [49]. The most important function however, seems to be regulation of AMPAR trafficking as well as synaptic targeting and trapping.

AMPARs were found to be highly mobile within the membrane and were found trafficking into and out of synapses [60, 61]. Among other factors, AMPAR mobility is influenced directly by the structure of the C-terminal tails of receptor subunits incorporated into the tetramer. Subunits GluA1, 4 and alternatively spliced subunit GluA2 have long cytoplasmic tails, subunits GluA3, the predominant splice variant of GluA2 and an alternatively spliced version of GluA4, contain relatively short homologous C-terminal tails. Those receptors with relatively short cytoplasmic tails (GluA2/3) continuously cycle in and out of the synapse, whereas receptors with a long tail enter the synapse in an activity-dependent manner [17, 62]. A more recent study questions the importance of the C-terminal tail in the regulation of AMPAR trafficking [63]. However, it is clear that at the PSD, AMPAR movement seems to be more restricted than outside of synapses, indicating an immobilization at these sites, resulting in postsynaptic high-density clusters of glutamate receptors. The nature of this immobilization has been an intense field of research over the past decade and will be reviewed later in this
text. It has been postulated that due to their relatively low affinity for glutamate, AMPARs need to be positioned in direct opposition to the presynaptic release site in order to ensure optimal synaptic transmission efficacy [64]. Today, it is well described that trafficking and membrane insertion are heavily influenced by C-terminal phosphorylation of AMPARs and protein-protein interactions at this site [61].

The number of posttranslational events that modify AMPARs is vast and the precise function of singular events remains elusive. For instance, AMPAR subunits were shown to be phosphorylated on over 20 different sites by a multitude of kinases including CaMKII, PKA, PKC and JNK [17, 49]. Furthermore, phosphorylation events regulate AMPAR responses and influence synaptic targeting and trapping along with influencing conductance properties of AMPARs [17, 49]. In addition, multiple of interacting proteins have been identified that influence an AMPARs behaviour during its lifespan. These modifications can range from folding to degradation, including membrane insertion, synaptic targeting and diffusional trapping [61]. Along these lines, much more research is needed in order to understand and disentangle the influence of these modification events, analyse their time-course and investigate their impact on synaptic transmission.

AMPAR research over the past years has focussed on receptor trafficking and synaptic targeting. It is widely accepted that the number of AMPAR present at the postsynapse directly correlates with the strength of a particular synapse [14, 16-18]. Processes such as AMPAR membrane insertion, synaptic trafficking and retention are the key processes underlying the activity dependent strengthening or weakening of synapses.

1.3 Downstream cascade

Little is known about the downstream cascade following AMPAR activation and the precise feedback mechanisms on AMPAR trafficking and synaptic insertion or removal. It is widely accepted that the cation-influx through AMPAR channels leads to the activation of NMDA-receptors, which are non-covalently blocked by magnesium at resting membrane potential [9, 10, 14]. NMDA activation then leads to a significant calcium influx into the postsynapse [11, 12], which acts as second messenger for a multitude of processes and signalling pathways that ultimately lead to the regulation of AMPARs at synapses [14, 65]. This regulation may include direct posttranslational modifications of AMPAR channels as well as the regulation of AMPAR synaptic insertion/removal or anchoring [17, 45, 62, 66-69].

Despite the efforts made to shed light onto these processes, synaptic signalling downstream of ion-channels remains complex. Following NMDAR activation, multiple kinases were shown to be active including calcium/calmodulin dependent protein kinase II (CaMKII), phosphoinositide 3-kinase (PI3K), Protein kinase A, Protein kinase C and several others [70-75]. A com-
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A complicating factor is that not all of these kinases gain activity simply by calcium influx through NMDAR channels, but rather depend on the paradigm used to activate the particular synapse [70-73, 76, 77], underscoring the difficulties of solving the precise mechanisms downstream of AMPAR activation.

However, there is consensus in the field of AMPAR and plasticity research, that CaMKII plays a key role in the downstream signalling of hippocampal glutamate synapses. CaMKII activation is directly involved in the formation of synaptic plasticity and has been shown to facilitate AMPA receptor immobilization at synapses by direct phosphorylation [78-83]. Along these lines, knock-in mice carrying a phosphor-resistant version of the CaMKII phosphorylation site showed both impaired LTP and LTD [84]. CaMKII is recruited to the postsynaptic density (PSD) following calcium influx through NMDARs where it binds to the GluN2B subunit of NMDARs. Disrupting the interaction between CaMKII and NMDARs was shown to disrupt synaptic potentiation [85-87]. Accordingly, inhibition of CaMKII alone is sufficient to block NMDAR dependent synaptic potentiation.

Even though these studies indicate the involvement of multiple kinases and signalling molecules in the LTP formation process, further studies will be needed to disentangle the mechanisms following AMPAR/NMDAR activation upon synaptic activity.

1.4 AMPAR trafficking and plasticity

The discovery and demonstration of activity dependent activation of “morphological silent synapses” led to the idea that AMPARs could be mobile within the plasma-membrane and move into synapses in an activity-dependent manner [88-91]. These findings provided an explanation why synaptic failure rates dropped after LTP-induction which can be explained by AMPAR delivery to the synaptic surface upon LTP-induction and the insertion of AMPARs into synaptic sites paralleling synaptic strengthening [90, 92, 93]. Vice versa, following Long-Term Depression (LTD) induction, AMPARs were shown to be internalized and thus removed from the synaptic surface, which parallels synaptic weakening [94-96]. Taken together, these findings indicated that changes in the number of AMPARs at synaptic sites could directly underlie synaptic function and hence synaptic plasticity. Today, the relationship between the number of AMPARs and synaptic strength has been well documented [45, 46, 65, 68].

As described above, a three-step model describing synaptic trafficking and trapping at of AMPAR at synapses during synaptic potentiation has been proposed [20]. It involves the delivery and membrane insertion of AMPAR containing vesicles near synaptic sites, the movement of extra-synaptic AMPARs into the synapse by lateral diffusion, and the synaptic trapping of AMPARs by interactions with scaffolding proteins in the postsynaptic density (PSD) [21, 97]. The reverse course of action is believed to underlie synaptic depression. Here
AMPARs are removed from the PSD and incorporated into AMPAR containing vesicles via endocytosis. These vesicles can then fuse with the membrane for subsequent rounds of synaptic potentiation or labelled for proteosomal/lysosomal degradation. A cartoon of these processes is shown in figure 3. Intense research has led to many new insights on each of those processes of the past decade.

Figure 3. AMPAR movement into- and out of the synapse. AMPAR get inserted into the dendritic shafts or the spine lateral to the PSD via exocytosis (1). Membrane bound AMPAR (newly inserted or pre-existing) diffuse along the dendritic spine into synaptic sites (2). At synapses, AMPARs can bind to activity regulated binding sites at/in the PSD (3). During LTD, AMPARs detach from PSD proteins and diffuse out of the synapse (4). At the endocytic zone, AMPARs get internalized through endocytosis (5). Endocytosed AMPARs either get recycled for subsequent rounds of potentiation or sorted for lysosomal degradation (6). Modified from Opazo, Choquet 2012 [98].
1.4.1 AMPAR synaptic trafficking

The number of AMPARs at synapses depends on the relative rates of exocytosis and endocytosis at postsynaptic membranes, where enhanced exocytosis results in synaptic potentiation and vice versa [99, 100]. It was shown that following synaptic potentiation AMPAR containing vesicles were recruited to synaptic sites, whereas AMPARs got removed during synaptic weakening through endocytosis [94-96]. Strikingly, disruption of exocytosis by using exocytosis inhibitors suppresses the induction of LTP, underlining the importance of exocytosis for LTP expression [101, 102]. Accordingly, use of endocytosis inhibitors blocks LTD formation [102].

Even though exocytosis is important for synaptic strengthening, it was shown that AMPAR exocytosis does not per se lead to an increase in synaptic AMPAR mediated currents [22, 103]. Also the induction of exocytosis takes longer than the actual formation of synaptic potentiation and can therefore not be its underlying mechanism [104, 105]. Supporting these findings, knockdown studies of 4.1N, a protein known to promote the insertion of AMPAR containing vesicles into the plasma membrane upon LTP induction, only showed a delayed LTP impairment [106]. Interestingly, exocytosis events were observed prior to synaptic facilitation and the existence of an extra-synaptic pool of AMPARs was reported [21, 97, 105-111]. From here, AMPARs diffuse laterally into synapse upon synaptic stimulation, without the necessity of exocytosis to take place [60, 106, 109]. Accordingly, depletion of this pre-existing AMPAR pool abolishes synaptic potentiation [63, 112, 113]. Exocytosis might thus play a crucial role in maintaining and restoring a pool of extra-synaptic AMPARs for synaptic potentiation rather than mediating it directly [21, 97, 111]. Supporting data come from studies indicating that newly inserted AMPARs do not contribute to LTP expression [105] and that chemically induced LTP results in the trapping of pre-existing AMPARs exclusively [108].

Although there is consensus about the extra-synaptic insertion of AMPARs, the exact subcellular site of AMPAR exocytosis is still under debate. Data indicate membrane fusion of AMPAR containing vesicles in the dendritic shaft or at perisynaptic sites at syntaxin rich domains [104, 105, 107, 109, 114-117]. It is believed that the extra-synaptic insertion of AMPAR containing vesicles might help to preserve the integrity of the postsynaptic density.

Although there is no doubt for exocytosis to contribute to LTP, little is known about the exact mechanism underlying AMPA receptor exocytosis. It is known that Kinesin/Dynein motors as well as calcium sensitive motor proteins MyosinVa/Vb play a role in the delivery of AMPAR containing vesicles to the plasma membrane [116, 118]. Here, membrane fusion is SNARE complex mediated with SNAP-23 and syntaxin as postsynaptic v-SNARE and t-SNAREs [115, 119]. In addition, AMPAR subunits were shown to interact with protein NSF that is an essential part of the SNARE complex [43, 120, 121]. However, more research is needed to elucidate the precise triggers and molecules involved in the activity dependent exocytosis and delivery of vesicles to synaptic membranes.
As described above, following their extra-synaptic insertion into the plasma-membrane, AMPARs are mobile structures and diffuse laterally along the plane of the membrane, probably by Brownian motion [21, 97, 105, 111, 126-130]. With most AMPAR movement data being acquired in cultured neurons, a recent study confirmed AMPAR movements along the dendrite “from synapse to synapse” in vivo [131]. If not retained, AMPARs can enter, scan and leave synapses, with an average dwell time of around 2 seconds. However, experimental data indicates that the surface mobility of AMPARs decreases at synaptic locations [21, 97, 111, 127]. This argues for either changes in the composition of the plasma membrane which alter Brownian movement or the presence of AMPAR-specific binding sites at synapses which trap diffusing receptors [128, 129, 132, 133]. Specifically the high density of AMPA and NMDA receptors at synaptic sites implies an interaction of these receptors with the cytoskeleton or synaptic structures such as the PSD [134].
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Strong experimental evidence for an interaction between AMPARs and PSD proteins came from studies showing that overexpression and knockdown of key PSD family members, e.g., PSD-95, results in the increase or decrease of synaptic AMPARs respectively [22, 24, 139-141]. Convincingly, the synaptic content of PSD-95 also directly correlates with the mobility of AMPARs at synapses, providing further evidence for PSD-95 being able to trap AMPARs and retain them at synaptic sites [111]. Interestingly, the synaptic content of NMDARs is not altered in PSD-95 overexpression studies, arguing for the addition of AMPAR specific binding sites through PSD-95 overexpression rather than the synaptic PSD-95 content to directly correlate with overall synaptic receptor levels [139, 142]. How this specificity comes to play is not yet understood. However, it is known that posttranslational modifications like palmitoylation and phosphorylation both affect synaptic PSD-95 content and regulate the amount of AMPARs present at synapses [143, 144], providing a potential mechanism for receptor specific binding site regulation.

Indicating a high level of redundancy among MAKUG family members, synaptic AMPAR currents through PSD-95/93 knockdown can be fully rescued by SAP97 overexpression [145, 146]. Interestingly, overexpression and down regulation of MAGUK family members PSD-93, SAP97 or SAP102 alone does not greatly affect synaptic transmission, highlighting the predominant role for PSD-95 in synaptic retention of AMPARs or argues for their redundancy [141, 145]. Studies focussing on PDZ domain mediated interactions of AMPARs identified several interactors, among which GRIP, PICK1 and SAP97. Surprisingly, none of these interactors affected AMPAR currents and synaptic content to a similar extend as PSD-95, leaving synaptic AMPARs widely intact (for overview see [100, 138].

Strikingly, deletion studies where the PDZ-domain of GluA1 and GluA2 was deleted, displayed normal AMPAR synaptic targeting and trafficking, convincingly calling for a different mechanism that regulates the synaptic trafficking of AMPARs [72, 111, 147, 148].

1.5 AMPAR auxiliary proteins

Ion flow across membranes at excitatory synapses is mediated by two distinct types of ion-channels: Voltage-gated channels and ligand-gated channels. Voltage-gated channels open their channel pore at specific membrane potentials, whereas ligand-gated channels start conducting ions upon binding to their ligands. With respect to the limited number of receptors present at synapses and in order to ensure maximal spatial and temporal resolution, fine-tuning of receptor responses becomes a necessity. Along these lines, expression of ionotropic glutamate receptors alone in heterologous tissue is sufficient to express functional
glutamate receptors. However, AMPAR currents from heterologous cells or recombinant receptors seem to have aberrant properties to those measured from native tissue [149-152]. One of the reasons for this observation might be that AMPARs are known to be part of multi-protein complexes when expressed in native tissue and the potential absence of native interactors in heterologous cells [153]. In this scenario, AMPARs' behaviour/properties would be influenced strongly by their interacting proteins.

The concept of proteins assisting in fine-tuning ion-channel function, by forming stable binding partners, is not new. Voltage-gated channels are associated with smaller auxiliary subunits, which can affect when, where and how an ion channel gets activated. They are known to stably bind to voltage-gated channels and influence the channel complex on different levels including trafficking, surface expression and gating kinetics. However, for some time glutamate receptors were believed to be solitary proteins and the discovery of stable binding partners revolutionized the view of glutamatergic synaptic transmission.

To date, three major families of transmembrane proteins are widely accepted as AMPAR auxiliary subunits: Transmembrane AMPAR regulatory proteins (TARPs). Cornichons (CNIH) and Cystein-knot AMPAR modulatory proteins (CKAMPs). Although the present work will focus on these three major families, the field of AMPAR interacting protein is a particularly active field on research. In the past years, many more proteins have been discovered, that potentially fulfil a role as AMPAR modulatory protein.

1.5.1 TARPs

First evidence for the existence of AMPAR auxiliary subunits came with the discovery of Stargazer mice. These mice show a severe behavioural phenotype including abnormal head movement, cerebellar ataxia and frequent spike-wave seizures [154]. Analysis revealed that stargazer mice carry a mutation in the Stargazin gene, which shows sequence homology to γ-subunits of voltage-gated calcium channels [155]. Physiologically, stargazer mice show no AMPAR activity at cerebellar mossy-fiber to granule cell synapses and lack miniature EPSCs in primary cerebellar granule cell cultures [27, 156, 157]. Further analysis revealed alterations in AMPAR gating properties and surface trafficking in the absence of Stargazin. Interestingly, NMDAR currents at stargazer synapses seem to be intact, arguing for an AMPAR-specific synaptic dysfunction.

The transmembrane nature of Stargazin and its specific effect on AMPAR currents made Stargazin the founding member of the TARP (Transmembrane AMPAR Regulatory Protein) family [158]. Today more members of the TARP family have been identified to functionally assist and tune AMPAR responses. They can be found in all parts of the brain and are conserved across species [159-163]. Reintroduction of Stargazin or several TARP family mem-
bers rescued AMPAR surface expression in the stargazer background, indicating functional redundancy among certain family members [158].

Among the most striking effects in Stargazer mice is the reduction of surface expression of AMPARs with no observed alterations in the rates of endocytosis [164]. Moreover, the co-expression of TARPs results in enhanced AMPARs surface expression in heterologous cells [165-168]. Data from stargazer mice indicates a large portion of AMPARs to be retained in the ER with immature posttranslational modifications, implying that AMPARs are unable to leave the ER and fully mature in the absence of Stargazin [164, 169]. Stargazin contains sites that promote ER-exit and direct receptors to specific membrane compartments [170]. Accordingly, it has been hypothesized that TARPs have to be incorporated into nascent AMPAR complexes at some point between tetramerization and ER export, thus at a very early stage in the life of AMPARs [171].

Proteins of the PSD and especially PSD-95 were/was shown to have dramatic impact on synaptic AMPAR function and plasticity (for overview see [138]). However, AMPARs do not or only sparsely bind to proteins of the PSD and do not bind to PSD-95. Interestingly, TARP proteins contain one or more PDZ-ligand motifs which are located on their C-terminal domain. Deletion of a single or multiple motif(s) results in defects in AMPAR- synaptic trapping while leaving AMPARs surface expression unchanged, indicating that the PDZ-domain of Stargazin is directly involved in the synaptic trapping of AMPARs [27]. Later a PDZ-domain mediated interaction between Stargazin and PSD-95 was confirmed, highlighting the importance of such adapter proteins for AMPAR function. Accordingly, disruption of Stargazin – PSD-95 binding prevents trapping of freely diffusing AMPARs at synaptic sites [111]. These data were later confirmed by competition assays of Stargazin's PDZ-domain mediated interactions, which resulted in enhanced AMPAR surface mobility [172].

Posttranslational modifications were shown to contribute to the synaptic trapping of AMPARs and play a major role in the regulation of AMPAR specific binding sites at the PSD. In particular, phosphorylation of AMPARs and PSD proteins were both shown to influence synaptic trafficking/targeting of AMPARs [144, 173] Interestingly, the C-terminal domain of Stargazin is subject to phosphorylation events by a similar set of kinases including PKA, PKC and CaMKII that has been associated with AMPAR- phosphorylation [75, 174, 175]. These kinases were shown activated following calcium influx through NMDARs after LTP induction and are seemingly involved in the activity-dependent trafficking and trapping of AMPARs. Since AMPARs are tightly bound to TARPs (which heavily influence their synaptic trafficking) phosphorylation of TARPs could be a major regulator of AMPAR synaptic trafficking/targeting. Accordingly, Stargazin phosphorylation by CaMKII was shown to trigger the diffusional trapping and enhance synaptic accumulation of AMPARs [97, 175]. Furthermore, experiments using phosphomimic versions of stargazin, where all C-terminal serine residues are phosphorylated, enhances synaptic delivery of AMPARs and prevents LTD [75, 174], whereas
phospho-null mutations of Stargazin were shown to prevent the expression of LTP [75]. Recent work revealed that Stargazin is able to form phosphorylation-dependent complexes which regulate endocytosis and activity dependent LTD [176].

A model on how phosphorylation of Stargazin influences AMPAR trafficking has been proposed [177]. In this model Stargazin interacts with the cell membrane through its positively charged amino acids located on its C-terminal tail, resulting in immobilized AMPARs at the plasma membrane outside of synapses. Activity-dependent phosphorylation events reduce this positive charge, disrupt these interactions with the membrane and mobilize AMPAR for the recruitment to PSD proteins, which in turn immobilize AMPAR through anchoring them at synaptic locations. Along these lines, phosphomimic-Stargazin enhances cerebellar mossy-fiber/granule cell AMPAR EPSCs whereas phopho-null constructs reduce, but not eliminate, EPSCs [177].

**Figure 4. TARPs modulate kinetics of decorated AMPARs in HEK293T cells.** A and B. Average responses of outside-out patches to 1ms (A) or 100 ms (B) applications of 1mM glutamate are normalized and aligned to the peak. Superimposed responses of individual patches are displayed in gray, and averages across experiments are shown in colour. Weighted time constant values calculated from the area under the peak-normalized response are displayed as mean ± SEM. C, D, E. Bar graphs summarize changes in time constants of deactivation, desensitization and rise kinetics. Asterisks indicate significant changes compared to control (con) conditions (GluA1, flip isoform). Figure taken from Milstein et al., Neuron 2007, with the permission of the publisher [180].
Apart from influencing AMPAR trafficking, TARPs were shown to affect the pharmacology and gating properties of AMPARs. Early studies already indicated that AMPAR currents measured from heterologous cells generally showed faster kinetics than AMPAR currents measured from neurons [149, 150]. Along these lines, heterologous cells show slower AMPARs deactivation, desensitization, recovery from desensitization and smaller amplitude, when co-expressed with Stargazin and other TARP family members (for overview see Figure 4) [167, 169, 178-180]. This effect on AMPAR gating properties however, seems to be independent of the role of TARPs to influence AMPAR trafficking and is TARP-subunit dependent [167]. For instance, it has been shown that γ-4 and γ-8 both slow glutamate evoked currents to a greater extent than γ-2 and γ-3 [180, 181]. Another good example of subtype specific regulation was the discovery that only some TARPs (γ-4, γ-7, γ-8) introduce a phenomenon called “resensitization”, where fully desensitized AMPARs slowly recover in the sustained presence of the agonist [182, 183]. These TARP subtype-dependent effects were first observed in heterologous cells and later largely confirmed in neurons, where the presence and absence of TARPs leads to altered channel kinetics and amplitudes in AMPAR responses [180, 181]. Data indicate that the subtype specificity is for the most part mediated by the extracellular domains of TARP subunits [180, 181].

Along with changing the gating properties of AMPARs, the presence of TARPs was shown to alter AMPAR pharmacology. The presence of TARP-subunits at AMPARs turns partial agonist kainate into a full AMPAR agonist [167, 179] and competitive antagonist CNQX into a partial agonist [184, 185]. More recent findings, however, attribute this change from antagonist to (partial) agonist by the increase in glutamate affinity that is observed in the presence of TARPs. [167, 168, 178, 179, 186]. In addition, GluA2-lacking AMPARs are less susceptible to polyamine block at positive membrane potentials and show enhanced charge transfer and calcium entry when co-expressed with TARPs [187-189]. All these findings convincingly prove that the presence of TARPs at AMPARs changes the AMPAR conductance properties itself. The exact mechanism of these changes remains elusive. For instance, even though today it is widely accepted that TARP proteins assist the fine-tuning of AMPARs through direct and indirect mechanism, the stoichiometry of TARP-AMPAR complexes is still under debate. Also the association and assembly of AMPAR, TARP-proteins and potential other auxiliary subunits is not understood. Additional research is needed to provide insights into the TARP-proteins working mechanism to understand AMPAR function. In particular co-crystallization between AMPARs and TARP-proteins could help to clarify multiple important aspects of this interaction, for instance the binding site, changes in conformation/biophysical properties or the subunit stoichiometry.
1.5.2 Cornichons

Proteins of the Cornichons family (CNIH2/3) were identified as AMPAR interacting proteins by a proteomic approach [190]. Both proteins contain a transmembrane domain, and are expressed widely across the brain. The AMPAR population decorated by CNIH proteins was initially reported to be non-overlapping with the population decorated by TARP family members. However, it is now clear that CNIHs and TARPs readily appear at the same AMPAR complexes [191-193].

The effects of CNIH on AMPAR currents are tissue dependent. In heterologous cells, CNIH are potent facilitators of surface expression of AMPARs and were shown to slow deactivation and desensitization kinetics of agonist evoked currents [190, 191, 194, 195]. Like TARPs, CNIH can immunoprecipitate GluA1 and promote its forward trafficking in the ER and Golgi [196, 197]. Knockout of both CNIH2 and 3 results in the loss of synaptic and surface AMPARs in the hippocampus, probably due to selective loss of GluA1 containing receptors, leaving a small pool of GluA2/3 containing AMPARs at the neuronal surface [198]. The observed change in kinetics, however, can be attributed to the change in AMPAR composition at the synaptic membrane rather than to CNIH-induced altered conductance properties. Unlike TARPs, CNIHs do not contain an export signal, but alter the glycosylation pattern of AMPARs that in turn promotes ER and Golgi exit [197]. However, there is no evidence that CNIHs are involved in the extrasynaptic targeting of AMPARs [195]. Similar to Stargazin, CNIHs were shown to increase the main channel conductance of AMPARs with no change in open probability and to reduce spermine affinity for GluA2-lacking receptors [195].

Interestingly, studies in TARP deficient mice showed reduced CNIH2 surface expression arguing that surface expression of CNIH depends on TARPs [193]. Accordingly, when expressed in stargazer background, CNIH was unable to rescue AMPAR currents. However, when γ-8 was used to rescue stargazer mice, co-expression of CNIH was shown to slow down synaptic currents. Furthermore, in a study that co-expressed CNIH with AMPARs that were covalently linked to TARP γ-8 to ensure TARP saturation, CNIHs could introduce a further slowdown of AMPAR channel kinetics, suggesting the presence of non-overlapping binding sites for both γ-8 and CNIH. It appears plausible that CNIH and TARPs interact with common AMPAR complexes and thereby both influence AMPAR gating and pharmacology [192, 193]. However, more research is needed to disentangle the effects of TARPs and CNIH proteins in order to understand their individual function and importance for AMPAR transmission.
1.5.3 Shisa9/ CKAMP44

In the beginning of 2010, SHisa9/ CKAMP44 was described as a new AMPAR auxiliary protein [30]. This 44 kDa protein is the first member of the Shisa family described as AMPAR interacting protein. Interestingly, the N-terminal domain comprises a cysteine-rich motif that is believed to form a complex tertiary structure through cysteine bridges and is believed to fold into an elaborate tertiary structure like that of toxins. Shisa proteins are highly conserved throughout vertebrates with known homologs bilaterian invertebrates and prolifera, underlining the importance of this protein family [199].

Shisa9 is widely expressed throughout the brain at very low levels with high expression in dentate gyrus granule cells. It was shown to interact specifically with all AMPARs subunits, forming no association with NMDA- or kainate- receptors. Interestingly, Shisa9 was shown to precipitate along with Stargazin, which argues for both proteins to appear in the same complexes. When expressed in heterologous cells it reduces currents through GluA1-3 containing AMPARs with no change in AMPAR surface expression. The presence of Shissa9 at AMPARs was shown to slow deactivation and accelerate desensitization kinetics and to prolong the time of recovery from desensitzation [30] in heterologous cells and neurons (see figure 5). These findings are in contrast to TARPs which prolong both deactivation and desensitization and show no effect on the recovery from desensitized states [200, 201]. Overexpression of Shisa9 in CA1 pyramidal cells, where Shisa9 expression is naturally low, results in slower mEPSCs and reduced paired-pulse ratio (see figure 5). A recent study indicated, that much like Cornichons proteins, Shisa9 is decorating overlapping AMPAR populations in the dentate gyrus with TARP γ-8 [202]. Interestingly, γ-8 and Shisa9 show opposing effects on short-term-plasticity and only γ-8 but not Shisa9 is necessary for LTP. How both proteins specifically bind to the AMPAR and the mutual modulation of AMPAR responses is regulated and tuned, remains elusive.
Figure 5. CKAMP44 changes the properties of decorated AMPARs. A. GluA1-, GluA2-, and GluA3- but not GluK2- or GluN1/2B-mediated steady-state currents (with co-application of CTZ) in oocytes are strongly reduced by co-expression of CKAMP44. Sample traces are shown for GluA1-mediated currents without and with co-expression of CKAMP44. Error bars represent the mean T ± SD. B. Overexpression of CKAMP44 increases the deactivation time constant (τ_{deact}) of AMPAR-mediated currents. Error bars represent the median T IQR (interquartile range). C. Overexpression of CKAMP44 leads to slower, and CKAMP44 KO to faster, recovery from desensitization (two 1-ms glutamate pulses with interpulse-intervals of 10, 30, 100, 300, 1000, and 3000 ms) in nucleated patches. Error bars represent the mean T ± SEM (left panel) or median T ± IQR (right panel). (figure taken/modified from von Engelhardt et al, Science, 2010 [30], with the permission of the publisher)
1.6 Synopsis of the thesis

CKAMP44 is part of a multi-protein family. Little is known of the function of other Shisa proteins, however, high sequence and structural homology indicates that other Shisa family members might serve functions as AMPAR auxiliary subunits. In Chapter 2 of this thesis, I will focus on CKAMP family member Shisa6. This protein showed high sequence homology to Shisa9 and was shown to be associated with AMPA receptor complexes by means of immunoprecipitation. Since Shisa9 was shown to function as an auxiliary subunit for AMPARs and based on the physiological association with this complex, we hypothesized a similar function for Shisa6. We show that it interacts with AMPARs at native complexes and is capable of changing both AMPAR currents in heterologous cells as well as changing transmission at hippocampal glutamate synapses. In addition, we present evidence that the C-terminal PDZ-interaction motif of Shisa6 mediates interactions with synaptic scaffolding complexes. These interactions prove to be of importance for the synaptic delivery and anchoring of these proteins and especially their immobilization at synaptic sites. These findings mark a further step in the understanding of the working mechanisms of AMPARs.

As described above, data from literature indicate that alterations in synaptic delivery and anchoring of AMPARs closely relates to changes in long-term plasticity. In Chapter 1 of this thesis we highlighted the importance of Shisa6 in the process of trapping AMPARs at synapses through interactions with PSD proteins. Disruption of PDZ-domain mediated interactions with the synaptic scaffold resulted in enhanced AMPAR movement at synapses. In Chapter 3 we follow the hypothesis that Shisa6 might itself influence the formation of synaptic plasticity through aberrant trapping of AMPARs at synapses. Accordingly, we find enhanced LTP in Shisa6 KO animals compared to wildtypes. Though the exact mechanism of this finding remains elusive, it provides strong evidence for an activity-driven interaction with PSD proteins.

Chapter 4 of this thesis focusses on how the presence of Shisa9 at AMPARs influences their function. Our study therefore focusses on the PDZ domain mediated interactions at the PDZ-binding motif present on the C-terminal tail of Shisa9/CKAMP44. These sites can classically be occupied by synaptic scaffolding complexes of the MAGUK family, which are known to determine synaptic localization of neurotransmitter receptors. The binding partner of Shisa9 at this site and the impact of such interaction on AMPAR function, however, remain elusive. Our study indicates, as hypothesized, this PDZ-motif is able to interact with several PDZ-domain containing proteins in vitro, including PSD-95 and PSD-93. We next designed a peptide competition assay to compete with C-terminal interactions of Shisa9 and tested the importance of those interactions in slice physiology experiments. Our study indicates an aberrant paired-pulse ratio and altered EPSP kinetics that may result from alterations in AMPAR trapping at synaptic sites. In addition, we found that diluting Shisa9 C-terminal PDZ-domain interactions results in sharper hippocampal oscillations.
Shisa6 traps AMPARs at postsynaptic sites and prevents their desensitization during high frequency synaptic activity

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Abstract

Trafficking and biophysical properties of AMPARs in the brain depend on interactions with associated proteins. Here we identify Shisa6, a single transmembrane protein, as a stable and directly interacting bona fide AMPAR auxiliary subunit. Shisa6 is enriched at hippocampal postsynaptic membranes and colocalizes with AMPARs. Shisa6 C-terminal harbors a PDZ domain ligand that binds to PSD-95/DLG4, constraining lateral membrane mobility of AMPARs and confining them to postsynaptic densities. Shisa6 expressed in HEK293 cells alters GluA1 and GluA2-mediated currents by prolonging decay times and decreasing the extent of AMPAR desensitization. Using gene deletion, we show that Shisa6 increases rise- and decay times of hippocampal CA1 mEPSCs. Furthermore, we show that Shisa6 decreases AMPAR desensitization during repeated synaptic activation. Shisa6-containing AMPARs show prominent sustained currents, thereby protecting synaptically trapped AMPARs from full desensitization and preventing synaptic depression at high stimulation frequencies.
Introduction

Fast excitatory synaptic transmission in the adult brain is predominantly mediated by AMPA-type glutamate receptors (AMPARs). The strength of glutamatergic transmission can be adjusted in an activity-dependent manner by different mechanisms in pre- and postsynaptic elements [203, 204], postsynaptic plasticity being largely determined by regulation of both the number and gating properties of AMPARs [17, 45, 205-209]. Post-translational modifications and protein interactions enable activity-dependent plasticity underlying learning, memory, and synapse turnover [16, 46, 68, 210]. Identification of additional components of native brain-derived AMPAR complexes has revealed a wide variety of mostly transmembrane proteins that directly interact with AMPARs14 [153]. These proteins can potentially act as auxiliary subunits of AMPARs and affect channel kinetics, trafficking, surface mobility, and activity-dependent regulation of these processes. Well-established AMPAR auxiliary subunits include the transmembrane AMPAR Regulatory Proteins (TARPs) [112, 158], the Cornichon homologs (CNIH-2 and CNIH3)17 [190], and the recently identified Cysteine-Knot AMPAR Modulating Protein (CKAMP44) [30, 31], also known as Shisa9 (20 [199]; Supplemental Fig. 1). Both TARPs and cornichons decrease deactivation and desensitization rates of the activated AMPAR, and promote synaptic targeting. Overexpression in CA1 of Shisa9 increases AMPAR decay; slows down recovery of desensitization, and decreases AMPA receptor short-term plasticity. In contrast to Shisa9, which is expressed most prominently in the hippocampus dentate gyrus (DG), Shisa6 is highly expressed throughout the hippocampus, in DG as well as CA regions. It is unknown whether Shisa6 interacts with AMPARs. Here we demonstrate that Shisa6 is an auxiliary subunit of the AMPAR, which traps them at postsynaptic sites through interaction with PSD-95/DLG4. By altering biophysical properties of AMPARs, Shisa6 keeps AMPARs in an activated state in the presence of glutamate, preventing full desensitization and synaptic depression.

Results

Shisa6 is expressed at hippocampal synapses

Shisa6 shares high sequence identity with the established AMPAR associated protein Shisa9 (Fig. 1a), and features the Shisa family's signature cysteine-rich motif, a single-pass transmembrane region, and a type-II PDZ ligand-motif (EVTV) at the C-terminal tail of the intracellular domain (Supplemental Fig. 1). Real-time PCR revealed abundant expression of the Shisa6 gene in the brain, with a transcript containing the alternatively spliced exon (exon 3) located in the intracellular C-terminal part (Supplemental Fig. 1). Gene expression analysis and immunoblotting with a Shisa6-specific antibody, validated on Shisa6 knockout (KO) mice, showed selective expression of Shisa6 in the hippocampus and cerebellum (Supplemental Fig. 2). Subcellular fractionation of the hippocampus revealed that Shisa6 is highly en-
riched in the Triton-X100-insoluble postsynaptic density (PSD) fraction, where it co-purified with PSD-95, GluA2 and GluN2A (Fig. 1b), and was absent in the synaptophysin-enriched (presynaptic) fraction.

The subcellular distribution of native Shisa6 was further explored by immunofluorescence staining of mature (DIV21) primary hippocampal cultures (Fig. 1c,d). Paraformaldehyde-fixed neurons were permeabilized with Triton-X100 and triple-stained for endogenous Shisa6 and two synaptic markers: Homer1, a scaffolding protein localized to the PSD, and an antibody directed against an intracellular epitope of GluA2 (Fig. 1c). A moderately high level of granular staining for Shisa6 was present along dendrites, often co-localizing with similar granular staining for GluA2. Staining for Homer1 was more punctate, reflecting its selective enrichment at postsynaptic sites [211]. The most intense staining for Shisa6 and GluA2 proteins occurred at these Homer1 puncta. Line scans drawn along spine-like dendritic protrusions containing Homer1 puncta showed that Shisa6 is highly enriched at these putative synaptic sites, along with GluA2 (Fig. 1d,e). Shisa6 enrichment in the PSD fraction and the immunofluorescence localization strongly indicate that Shisa6 is localized with AMPARs at postsynaptic sites.

Shisa6 interacts with AMPARs

Next, we addressed whether Shisa6 is an AMPAR-interacting protein. First, we investigated the presence of Shisa6 in native hippocampal AMPAR protein-complexes, by immunoprecipitation of DDM-extracted crude synaptic membranes using an antibody specific for AMPAR subunit GluA2. Indeed, Shisa6 is contained within GluA2-complexes, and absent in the IgG control (Fig. 1f). Reverse immunoprecipitation of native Shisa6 protein-complexes from hippocampus confirmed the stable association between Shisa6 and GluA2 (Fig. 1g). In addition, it identified GluA1 and GluA3 as part of the Shisa6 protein complex.

We then investigated whether Shisa6 binds directly to the AMPAR GluA1, GluA2, GluA3 subunits and kainate receptor subunit GluK2 by co-expression in HEK293 cells. GluA1, GluA2, GluA3 and GluK2 were each expressed individually as monomeric receptors in the presence or absence of Flag-Shisa6. Immunoblot analysis revealed that GluA1, GluA2, and GluA3 were co-immunoprecipitated with Flag-Shisa6 (Fig. 1h). GluK2 was not pulled-down with Flag-Shisa6. In conclusion, Shisa6 binds directly and non-preferably to AMPAR subunits GluA1-3, but not to kainate receptor subunits.
Characterization of Shisa6

Figure 1. Shisa6 is type-I transmembrane protein enriched at postsynaptic sites of hippocampal neurons, where it colocalizes with AMPARs. a) Shisa6 is closely related to AMPAR auxiliary subunit Shisa9, featuring a signal peptide (SP), extracellular domain with conserved cysteine-knot motif, single transmembrane region (TM), and intracellular domain with PDZ-ligand motif (EVT). Exon4 (Ex4) is an alternative-splice region in Shisa9, whereas this is Exon3 (Ex3) in Shisa6 (Supplementary Fig. 1). The exon-structure is indicated above the protein structure by alternating light-dark grey lines. Numbers under the protein indicate the beginning and end of the SP, TM and Ex3/4 domain, and the total amino acid length of the protein. b) Biochemical fractionation (homogenate, crude synaptic membranes (P2; with and without microsomes (M)), synaptosomes (SS), synaptic membranes (SM) and postsynaptic density fraction (PSD; Triton X-100 insoluble fraction) of mature mouse hippocampus reveals an enrichment of Shisa6 in the PSD together with GluA2, NR2A, PSD-95, and distinct from Synaptophysin (Syp). c) Immunofluorescence staining of cultured hippocampal neurons (DIV21). Boxed area enlarged (right) shows staining for endogenous Shisa6 (red), colocalized at puncta (arrows) enriched for the synaptic markers GluA2 (green) and Homer1 (blue) with dashed arrows (5 µm) showing the axis of lines (#1–3) used to generate fluorescence line scans (e). Scale bars: 10 µm (left) and 5 µm (right; enlarged area). d) Immunostain for each protein is shown separately (left), in addition to a color-overlaid image (right). Arrows indicate sites of mutual enrichment. (e) Fluorescence line scans show concomitant enrichment of Shisa6 with GluA2 and Homer1 immunostaining at synaptic sites. f,g) Shisa6 colocalizes with AMPARs in native hippocampal protein complexes. Western blot analysis of immunoprecipitated GluA2 complexes reveals the co-precipitation of Shisa6 (f). The reverse immunoprecipitation of Shisa6 complexes confirms the interaction with GluA2, and identifies GluA1 and GluA3 as additional interaction partners. No signal was obtained in the Shisa6 KO (g). h) Flag-Shisa6 (~61 kDa) binds directly to homomeric GluA1, GluA2 and GluA3 receptors, while having minimal affinity for GluK2, as shown by co-precipitation, using a Flag antibody, from HEK293 cells. The 75 kDa band is indicated.
Shisa6 reduces AMPAR-mobility in a PDZ-ligand motif dependent manner

Because Shisa6 is localized synaptically, is interacting with the AMPAR, and can potentially bind PDZ-containing scaffold proteins through its C-terminal EVTV motif, it might affect receptor mobility at the synaptic membrane. To assess this, we tracked in real-time the movement of native GluA2-containing AMPARs at the surface of 12 days in vitro cultured hippocampal neurons using quantum dots (QDs) coupled to specific antibodies directed against the extracellular domain of GluA2 (Fig. 2). We expressed Homer1C-GFP to label synaptic compartment either alone (control), or with Shisa6.

**Figure 2.** Shisa6 decreases AMPAR mobility through its PDZ ligand binding consensus sequence.

a) Representative trajectories of quantum dot (QD)-GluA2 membrane diffusion in control hippocampal neurons (blue/black) or in hippocampal neurons expressing Shisa6 protein (red/light grey). b,c) Median diffusion of GluA2 subunit in control (Crtl) hippocampal neurons (blue) or in neurons expressing Shisa6 (red) or Shisa6ΔEVTV (green). Displayed are results for the synaptic domain (b), labelled by Homer1C-GFP (control n=311 QDs; Shisa6 n=133 QDs; Shisa6ΔEVTV n=171 QDs) and the extrasynaptic domain (c, control, n=2126 QDs; Shisa6, n=865 QDs; Shisa6ΔEVTV n=1109 QDs), as tested by Kruskal-Wallis test. d) Mean proportion of immobile QD-GluA2 in control condition (35.58%±2.84, n=35 neurons) or after expression of either Shisa6 (57.94%±4.35, n=24 neurons) or Shisa6ΔEVTV (42.95%±4.72, n=19 neurons). Shisa6 increases the immobile pool of receptors, whereas expression of Shisa6ΔEVTV restores the corresponding mobile pool, as tested by Bonferroni’s multiple comparisons test. e) Frequency distributions of the diffusion coefficient calculated from the pooled synaptic and extrasynaptic trajectories of QD-GluA2 in control or after expression of Shisa6 or Shisa6ΔEVTV. Expression of Shisa6ΔEVTV increases the diffusion coefficient to values comparable to the control conditions (see b,c). f) Cumulative distribution of the diffusion coefficient of QD-GluA2 in control neurons (blue) or in neurons expressing Shisa6 (red) or Shisa6ΔEVTV (green), with those for Shisa6 being significantly different (P<0.001; Kruskal-Wallis test) from those for control. All values were obtained from 4 independent experiments. All tests: * P<0.050, *** P<0.001.
Characterization of Shisa6

As previously described [111, 132], AMPARs exhibit different surface diffusion movements ranging from immobile to diffusing freely, and trapped within confined domains. Representative trajectories from GluA2 showed that Shisa6 significantly decreases GluA2 mobility (Fig. 2a), in both synaptic (Fig. 2b; diffusion coefficient (µm²/s): control, 0.0128 (±0.0005/0.049 IQR) Shisa6, 0.0006 (±0.0001/0.008 IQR); P<0.0001) and extrasynaptic compartments (Fig. 2c; control, 0.0378 (±0.002/0.114 IQR); Shisa6, 0.0034 (±0.0002/0.115 IQR) P<0.0001). The frequency distributions of GluA2 trajectories diffusion coefficients revealed that expression of Shisa6 decreases the pool of mobile receptors at the advantage of the immobile pool (Fig. 2d–f). After expression of Shisa6, the immobile fraction (57.94%±4.35) was higher than in control conditions (35.58%±2.85, P<0.001) (Fig. 2d). In conclusion, expression of Shisa6 decreased GluA2 surface mobility in both the extrasynaptic and synaptic compartments.

To study the impact of interactions with PDZ-containing proteins, we performed GluA2 diffusion experiments in neurons expressing Shisa6 deleted for the last four amino acids (Shisa6∆EVTV) (Fig. 2b–f). Expression of the C-terminal deletion mutant of Shisa6 increases the mobility of GluA2 compared to wild type Shisa6 both in the synaptic (Fig. 2b; diffusion coefficient (µm²/s): 0.0082 (±0.0002/0.041 IQR) P<0.001) and extrasynaptic compartments (Fig. 2c; 0.0363 (±0.0008/0.11 IQR) P<0.001). This was confirmed by measuring the proportion of immobile receptors in the presence of Shisa6∆EVTV that was not significantly different from non-transfected control cells (Fig. 2d; 42.95%±4.72; P=0.191) and lower than in cells expressing wild type Shisa6 (P= 0.025), (Fig. 2d: F(2,75)=9.69, P=0.0002). This effect was similarly apparent on the frequency plot of diffusion coefficient distribution (Fig. 2e). Finally, the cumulative distribution curve comparing the distributions of the two experimental and control situations (Fig. 2f) showed that expression of Shisa6 immobilizes GluA2-containing AMPARs via an interaction through its PDZ ligand-binding motif.

Native Shisa6 interacts directly with the postsynaptic scaffold protein PSD-95 in a PDZ-ligand motif dependent manner

Given that Shisa6 affects the mobility of synaptically localized AMPARs, we tested whether it can interact with the organizers of the postsynaptic density, i.e., PDZ-containing proteins. First, using an immunoprecipitation on homogenates of the hippocampus with a Shisa6 antibody, we showed that the scaffolding protein PSD-95 (DLG4) is the main PDZ-containing interactor (Fig. 3a, Supplemental Table 1). Second, using a direct yeast two-hybrid assay, we confirmed that Shisa6 is able to directly interact with PSD-95, and that binding is dependent on the C-terminal EVTV domain (Fig. 3b).
Figure 3. Shisa6 interacts with PSD-95 in vitro and in living hippocampal neurons. a) PSD-95 is associated with Shisa6 in native hippocampal protein complexes upon immunoprecipitation of Shisa6, and is the main PDZ-containing scaffolding protein (Supplemental Table 1). b) Direct two-hybrid assay of the C-terminal part of Shisa6 (amino acids 202–557), or that in which the last 4 amino acids (EVTV) were removed, with the control vector or the vector carrying the first two PDZ domains of PSD-95 transformed and grown under stringent nutritional selection (–LTAH). The red coloration results from the cell’s inability to activate the adenine reporter gene. c) FRET scheme: The eGFP inserted in PSD-95 between PDZ domains 2 and 3 is in close proximity with the mCherry inserted on the intracellular C-terminus domain of Shisa6 when the two proteins are bound, and eGFP can transfer its energy to the mCherry (yellow arrow). d) Sample images of neurons expressing PSD-95::eGFP (n=8) and Shisa6::mCherry (n=10) or Shisa6ΔEVTV::mCherry (n=8). Scale bar is 20 µm. e) Sample images showing dendrites with dendritic spines (left) and the same images in which each pixel is color coded with its corresponding eGFP lifetime value (right). Scale bar is 1 µm. f) Lifetime of eGFP (mean±SEM) is decreased (ANOVA: spines, F(2,935)=72.54, P<0.0001; dendritic shafts, F(2,131)=7.97, P=0.0005) in spines (upper panel) and dendritic shafts (lower panel) of neurons expressing Shisa6::mCherry. This effect is not observed in neurons expressing Shisa6ΔEVTV::mCherry. Post-hoc Newman-Keuls test: ** P<0.010, *** P<0.001.
Based on these results, we developed a FRET (Förster resonance energy transfer) approach [212] to access the subcellular localization of the interaction between Shisa6 and PSD-95. In particular, a FRET pair between PSD-95::eGFP (FRET donor) and Shisa6::mCherry (FRET acceptor) (Fig. 3c) was designed. Overexpression of PSD-95::eGFP and Shisa6::mCherry in cultured hippocampal neurons and Fluorescence Lifetime Imaging Microscopy (FLIM) was then used to measure the difference of FRET through the decrease in eGFP lifetime compared with control neurons overexpressing PSD-95::eGFP only. We observed a robust FRET between PSD-95::eGFP and Shisa6 WT::mCherry in dendritic spines (Lifetime eGFP in ns; control: 2.381; Shisa6: 2.254; P<0.001) and dendritic shaft (control: 2.563; Shisa6: 2.461; P<0.001) of living neurons that differed from control (P<0.001). Importantly, we observed no difference in FRET from control upon expression of Shisa6∆EVTV in both these compartments (dendritic spines: Shisa6∆EVTV: 2.366; dendritic shaft: Shisa6∆EVTV: 2.538). Thus, Shisa6 interacts with PSD-95 in dendritic spines and the dendritic shaft via a binding on the PDZ domains of PSD-95.

**Shisa6 modulates AMPAR fast kinetics in HEK293 cells**

Since Shisa6 and AMPARs are partners of the same hippocampal protein complex, they interact in vitro, and Shisa6 traps AMPARs synaptically, we examined whether Shisa6 can also affect biophysical properties of AMPARs. AMPAR-mediated currents were measured in response to glutamate applications in the presence and absence of Shisa6 in HEK293 cells. Expression of Shisa6 in HEK293 cells by itself did not give rise to a glutamate-induced current upon glutamate application (Supplemental Fig. 3). Co-expression of Shisa6 and AMPAR subunits prolonged the decay time of homomeric GluA1 currents, homomeric GluA2 currents, as well as GluA1-GluA2 heteromeric AMPAR currents, induced by a 1-ms glutamate application (Fig. 4; Supplemental Fig. 3). In addition, Shisa6 reduced the current density of GluA1-GluA2 heteromeric AMPAR currents (Fig. 4b). AMPAR current rise times remained unchanged in the presence of Shisa6. Unlike other AMPAR modulatory proteins [194], Shisa6 did not alter the rectification properties of homomeric AMPARs (Supplemental Fig. 3). In addition, Shisa6 did not alter properties of GluK2 kainate receptors (Supplemental Fig. 4).
Figure 4. Shisa6 affects AMPAR decay time and increases deactivation. a) Peak-scaled example traces of whole-cell recording from HEK293 cells expressing heteromeric AMPAR channels in the absence (grey) or presence (red) of Shisa6. Currents were evoked by direct application of 1 mM glutamate during 1 ms. b) Bar graphs (mean±SEM) summarize changes in rise time (1.06±0.06 vs. 1.20±0.06 ms, P=0.101), current density 265.3±25.7 vs. 161.5±36.8 pA/pF, P=0.002) and decay time (4.50±0.28 vs. 5.81±0.35 ms, P=0.005) of AMPAR currents mediated by heteromeric AMPARs in HEK 293 cells in the presence and absence of Shisa6. c) Example traces of miniature EPSC recordings from CA1 pyramidal cells of Shisa6 KO animals and WT littermates. d,e) Superimposed spontaneous synaptic currents (d), and average synaptic currents (e) of Shisa6 KO animals and WT littermates. f) Bar graphs (mean±SEM) of Shisa6 KO and WT animals (n=19 cells per genotype, from 4 WT and 4 KO animals) represent rise time and decay time of synaptic activity, showing that both parameters are affected ex vivo. g) Peak-scaled example trace of whole-cell recordings from HEK293 cells expressing a heteromeric AMPAR channel in the absence (grey) or presence (red) of Shisa6. Currents were evoked by direct application of 1 mM glutamate during 1 s. h) Bar graphs (mean±SEM) summarize changes in rise time (1.60±0.08 vs. 2.04±0.12 ms, P=0.072), desensitization time constant and steady-state AMPAR-mediated currents. * P<0.050, ** P<0.010, *** P<0.001 (t-test).
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**Shisa6 affects AMPAR function at synaptic sites**

To test whether Shisa6 affects AMPAR function in the hippocampus, we recorded AMPAR spontaneous miniature EPSCs in CA1 pyramidal cells in acute hippocampal slices of Shisa6 WT and KO mice (Fig. 4c–f). In WT pyramidal neurons, both the rise- and decay kinetics of mEPSCs were slower than in KO (rise time (ms): 1.10±0.03 vs. 0.98±0.03, P=0.013; decay time (ms): 5.43±0.36 vs. 4.27±0.15, P=0.007). There was no significant difference in mEPSC amplitude and frequency (Supplemental Fig. 5). Immunoblotting in hippocampal synaptic membrane fraction for Shisa6 WT and KO mice revealed no difference in the number of (subunits of) AMPAR, NMDAR, PSD-95 or TARPs present at the synapse (Supplemental Fig. 5). These findings show that the presence of Shisa6 specifically alters the kinetics of synaptic AMPAR currents.

To test whether Shisa6 affects the function of AMPARs at the soma, we pulled nucleated patches from hippocampal CA1 pyramidal neurons recorded in acute slices and applied glutamate (1 mM for 1 ms; Supplemental Fig. 6). We observed no differences in the kinetics of the AMPAR currents in nucleated patches from WT and Shisa6 KO neurons, suggesting that Shisa6 does not functionally interact with AMPARs expressed at the soma membrane.

Finally, we investigated whether Shisa6 interacts with AMPAR in CA1 pyramidal cell dendrites. To that end, we tested the effect of local glutamate uncaging on hippocampal (CA1) dendrites in Shisa6 WT and KO mice. CA1 pyramidal cell dendrites were visualized by adding Alexa-488 to the patch solution. A small, localized puff of Rubi-glutamate was applied to dendrites 1 s before uncaging with light. Light-induced currents were completely abolished by DNQX (10 µM). In Shisa6 KO animals, decay times of light-induced AMPAR currents were shorter than in WT animals (Fig. 5a–c), whereas rise times did not change. These findings confirm that Shisa6 alters the functional properties of AMPAR current at hippocampal dendrites. Combined with the fact that Shisa6 protein is clustered at synaptic sites (Fig. 1, Supplemental Fig. 6), we conclude that in hippocampal pyramidal neurons Shisa6 interacts with synaptic AMPARs to change the properties of synaptic glutamatergic transmission.

**Shisa6 affects AMPAR slow kinetics**

Since AMPAR decay time is prolonged by Shisa6 both in HEK cells and in slices, and deactivation and desensitization are closely related processes, we next investigated whether Shisa6 modulates AMPAR currents in response to prolonged desensitizing glutamate application (1 s, 1 mM) in HEK293 cells (Fig. 4g,h). In the presence of Shisa6, both heteromeric GluA1-GluA2 and homomeric GluA1-containing AMPARs displayed slower desensitization kinetics (Fig. 4h, Supplemental Fig. 7; desensitization α (ms) GluA1-GluA2: 2.94±0.16 vs. 3.58±0.29, P=0.014) and reduced desensitization, observed as an enhanced steady-state conductance in response to 1-s applications of glutamate (Fig. 4h, Supplemental Fig. 7; % of peak conductance; GluA1-GluA2, 4.59±0.04 vs. 12.25±2.28, P<0.001). AMPAR current rise times
remained unchanged for both receptor types (Fig. 4h; Supplemental Fig. 7). Thus, Shisa6 increases sustained AMPAR currents that do not desensitize in the presence of glutamate. We next asked whether this enhanced steady-state current in the presence of Shisa6 is limited to AMPARs in HEK293 cells or also occurs at AMPARs in the hippocampus. In the local glutamate uncaging experiments (Fig 5a), light-induced AMPAR currents were large (100 – 500 pA) and had rise-times that were about 5 times slower than synaptic currents (Fig 5a,b). These currents typically lasted hundreds of milliseconds, with a decay time constant of about 150 ms (Fig 5a), most likely reflecting the time course of glutamate clearing. The fact that in Shisa6 KO animals the decay times of light-induced AMPAR currents were shorter than in WT animals (Fig. 5b,c) indicates that Shisa6 decreased the extent of AMPAR desensitization and increases the slow sustained component of the AMPAR current in hippocampal pyramidal neurons.

The desensitization properties of AMPAR may be engaged physiologically during high frequency synaptic stimulation. To test whether Shisa6 induced modifications of AMPAR properties affects frequency dependent synaptic responses, we applied extracellular stimulation trains of Schaffer collaterals at different frequencies during whole-cell recordings from CA1 pyramidal neurons to repeatedly activate glutamatergic inputs to these neurons, while blocking GABARs with gabazine (Fig. 5d). With only two stimulation pulses, we did not observe significant differences in the paired-pulse ratios at any stimulation frequency (Fig. 5d–g). With stimulation trains of 10 pulses at low frequencies (2 Hz; Fig. 5e), we also did not observe a change in synaptic depression. However, at 20 and 50 Hz stimulation, Shisa6 KO synapses displayed stronger depression than WT synapses (Fig. 5f,g). To exclude the possibility of an underlying presynaptic mechanism, we studied depression of synaptic NMDAR currents with the same stimulation protocol, while inhibiting AMPAR currents with NBQX (Supplemental Fig. 8). We did not find differences in NMDAR-mediated current kinetics, neither in responses between WT and Shisa6 KO synapses at any of the stimulation frequencies. This suggests that in WT glutamatergic synapses, AMPAR currents maintain larger amplitudes during repeated synaptic activation. Enhanced synaptic depression observed in Shisa6 KO synapses most likely resulted from a reduced sustained component of the postsynaptic AMPAR current, i.e., enhanced levels of AMPAR desensitization. These findings identify a role of Shisa6 in maintaining glutamatergic synaptic transmission at high frequencies.
Figure 5. Shisa6 affects decay time and deactivation and desensitization of AMPAR currents.
a) Experimental design used for glutamate uncaging. Caged-glutamate (red) was puffed into the slice onto the dendrites of CA1 pyramidal cells. A 532-nm laser (green) was used to uncage glutamate. b) Superimposed example recordings from glutamate uncaging experiments of CA1 pyramidal cells of Shisa6 KO animals (n=14 from 5 animals) and WT littermates (n=17 from 5 animals). Currents were evoked by 10-ms laser uncaging of Rubi-glutamate (1 mM). Light-induced currents were completely abolished by DNQX (10 µM; n=3, not shown) c) Bar graphs (mean ± SEM) summarize changes in rise time (WT 5.13±0.096 vs. KO 5.34± 0.108 ms, P=0.170) and decay time (142.67± 28.93 ms vs. KO, 74.87± 9.35 ms, P=0.044) of the AMPAR currents. d) Superimposed example traces of whole-cell recordings voltage clamped at –70 mV from CA1 pyramidal neurons of Shisa6 KO (blue) animals and WT littermates (black) in response to 50-Hz stimulation of synaptic inputs from Schaffer collateral fibers. e–g) Pulse ratios of electrically-evoked EPSCs from CA1 pyramidal neurons (at –70 mV) of Shisa6 KO (from 5 animals) animals and WT littermates (from 6 animals) at 2 (e), 20 (f) and 50 (g) Hz. At 20 Hz there was a trend for genotype effect (Gen, F(1,450)=3.52, P=0.067), and a significant effect of time (F(9,450)=17.81, P<0.001), as well as a genotype x time interaction (Gen-time, F(9,450)=3.91, P<0.001). The 50 Hz trains revealed a significant genotype effect (F(1,243)=7.33, P=0.012), time effect (F(9,243)=39.87, P<0.001) and a genotype x time interaction (F(9,243)=6.29, P<0.0001). Cell numbers used are indicated. * P<0.050 (Bonferroni post-hoc test).
Discussion

We identified Shisa6 as an intrinsic auxiliary subunit of AMPAR complexes in the mammalian brain. Physical association of Shisa6 with the pore-forming GluA proteins modulates receptor properties by slowing synaptic AMPAR current activation and deactivation. Shisa6 traps AMPARs at the post synapse in vivo, slow desensitization kinetics and favors a sustained open state upon prolonged activation. Together these processes affect short-term synaptic plasticity.

Shisa6 protein: a novel auxiliary subunit of AMPARs

Shisa6 qualifies as a bona fide auxiliary subunit of the AMPAR according to criteria as outlined by Yan et al. [213]. First, Shisa6 is a non-pore-forming subunit; expression of Shisa6 alone in HEK293 cells did not lead to a current when activated with glutamate. Second, Shisa6 has a direct and stable interaction with GluA pore-forming subunits; immunoprecipitation experiments using a GluA2 antibody detected Shisa6, and reverse, anti-Shisa6 antibody showed an interaction between AMPA receptors and Shisa6, both in vitro and in the brain. Third, Shisa6 modulates channel properties: both in vitro experiments and gene deletion of Shisa6 in vivo led to affected kinetics of AMPAR currents. In addition, Shisa6 affected AMPAR mobility. Fourth, Shisa6 is necessary in vivo: gene deletion of Shisa6 showed affected rise and decay times of AMPAR currents in the hippocampus, and affected AMPAR-dependent short-term synaptic plasticity.

Shisa6-AMPAR-scaffold interaction stabilizes AMPAR at synapses

Shisa6 limits AMPAR receptor diffusion and induces strong AMPARs stabilization at synaptic sites. Under basal conditions, most AMPAR are not stable in synapses but alternate constantly between immobile and mobile states and mobile AMPARs exchange between synaptic and extrasynaptic sites within seconds [21, 111, 132]. On average, about 50 % of synaptic AMPARs are immobile at a given point in time, being concentrated in small nanoscale clusters, while they are highly mobile in between these clusters [214]. AMPAR surface diffusion and synaptic stabilization are highly regulated by neuronal activity [105, 175] and thought to be one of the main mechanism for activity dependent regulation of AMPAR concentration at synapses, a process at the origin of many forms of synaptic plasticity [98]. The precise molecular mechanisms of the activity dependent, reversible AMPAR stabilization at synapses is still unclear as it does not seem to directly depend on AMPAR subunits [111]. Rather, AMPAR stabilization at PSDs involves interactions of auxiliary subunits with intracellular scaffold proteins. The best-established example of the activity-dependent stabilization of AMPARs is through binding of the C-terminus of the auxiliary subunit TARP gamma2 (also called stargazin) to PSD-95, mediated by CaMKII-dependent stargazin phosphorylation [22, 158, 175]. At rest, reversible binding between Stargazin and PSD-95 allow AMPAR to alternate between
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diffusive and immobile states and synaptic trapping of pre-existing surface receptors through rapid CaMKII-induced phosphorylation of TARPs is proposed to be one of the first events during synaptic potentiation [98, 105, 158, 175]. Here, we show using both single molecule tracking of AMPAR movement and FRET between Shisa6 and PSD-95 that Shisa6 can also bind to PSD-95 and immobilize AMPARs. Whether Shisa6 and TARP act synergistically or competitively to control AMPAR stabilization and whether Shisa6-AMPAR binding to PSD-95 is distinctly regulated by neuronal activity from TARP-AMPAR binding to PSD-95 will be interesting to determine.

**Shisa6-AMPAR interaction affects AMPAR-mediated signaling**

Shisa6 slows entry of AMPAR in the desensitized state and increases steady state currents in the prolonged presence of glutamate. This may be viewed as stabilization of the open state by impairing channel closure probably induced by a conformational process. In that respect, Shisa6 is analogous to TARPs and CNIH in its action on AMPAR. This is in contrast with the effect of its homolog Shisa9 (CKAMP44) that rather prolongs the AMPAR desensitized state [30].

Interestingly, we showed previously that AMPAR surface mobility is key to allow recovery from frequency-dependent synaptic depression at glutamatergic synapses by allowing the exchange of desensitized AMPARs for naive ones [97]. Activity dependent immobilization of AMPARs at synaptic sites leads to increased desensitization of glutamatergic synaptic currents during paired-pulse stimulation, resulting in stronger synaptic depression [29, 33]. Synapses thus have to face the conundrum that by having more stable AMPARs, for example after potentiation, they become sensitive to high frequency induced depression due to AMPAR cumulative desensitization. We found that synaptic AMPARs trapped by Shisa6 are less desensitized by repeated synaptic activation. This sustained activated state in the presence of glutamate might serve as a Shisa6-mediated mechanism to protect synaptic AMPARs from full desensitization upon repeated synaptic activity. Expression of Shisa6 thus allows synapses to sustain higher stimulation frequencies by preventing AMPAR desensitization.
Methods

Animals
Mice were bred in the facility of the VU University Amsterdam. Mice were group-housed in standard type 2 Macrolon cages enriched with nesting material on a 12/12 h rhythm (lights on at 7:00 AM). The housing area had a constant temperature of 23±1 °C and a relative humidity of 50±10%. Food and water was provided ad libitum. All the experiments were performed between 9:00 AM and 5:00 PM. Protein samples and RNA were prepared from 8–14 week old male and female C57/BL6J mice, derived from Charles River. Immunoprecipitations were performed on hippocampi of 8–14 week old male and female WT vs. KO mice. Electrophysiology on CA1 neurons was performed on 8–12 week old males, except for nucleated patches and glutamate uncaging experiments, for which 2–3 week old male mice were used. All experiments were performed in accordance to Dutch law and licensing agreements using a protocol approved by the Animal Ethics Committee of the VU University Amsterdam.

Shisa6 KO generation – The mouse chromosome 11 sequence (nucleotide # 66,299,000–66,379,000) was retrieved from the Ensembl database and used as reference. The mouse RP23-26O19 BAC DNA was used for generating the homology arms and conditional region for the gene targeting vector, The 5’ homology arm (~3.0 kb), 3’ homology arm (~4.0 kb), and conditional region (~1.0 kb) were generated by PCR and fragments were cloned in the LoxFtNwCD vector. The final vector contained loxP sequences flanking the conditional KO region (~1.0 kb), the Neo expression cassette (for positive selection of the ES cells) flanked by FRT sequences (for the subsequent removal of the Neo cassette), and a DTA expression cassette (for negative selection of the potentially targeted ES cells). The final vector was confirmed by both restriction endonuclease digestion and by end sequencing analysis. SwaI was used for linearizing the final vector prior to electroporation, and 30 µg of NotI-linearized final KO vector DNA was electroporated into ~107 C57BL/6 ES cells and selected with 200 µg/mL G418. From 192 ES clones selected for PCR based screening, two targeted clones were confirmed to be correctly targeted and have a single Neo insertion. Blastocyst injection resulted in chimeric offspring, which was finally backcrossed with FLP-mice mice to get rid off the Neo insertion. Subsequently, this line was crossed with Cre-mice to generate full KO mice.

(Real-Time) Polymerase Chain Reaction
Primers – Primers for PCR and real-time PCR were generated using Primer3.0. The final sets of primers are listed in Table S2.
RNA isolation and cDNA synthesis – RNA from several tissues was extracted as previously described [215]. (See supplementary information)
PCR for exon 3 – PCR reactions on two WT samples were generated with Ex1-Ex6 primers with 0.5 U Phusion (New England Biolabs, UK) in a 50-µL reaction using the HF buffer according to the manufacturer’s protocol.
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**Real-time qPCR** – Real-time qPCR reactions (20 μL; ABI PRISM 7700) on pooled tissue (3 mice) were performed using a 96-well format with transcript-specific primers (300 nM) on cDNA corresponding to ~20 ng RNA [215] and SYBR Green reagents (Applied Biosystems) (see supplementary information).

**DNA expression constructs**
The full-length coding DNA for the exon3-containing mouse Shisa6 was obtained from Gen-script as a pENTR4 construct (reference NM_001034874.3 / NP_001030046.1 + exon3 sequence), and subsequently Gateway-cloned into pTRCGw-IRE52-EGFP (see supplementary information), yielding the Shisa6-pTRCGw-IRE52-EGFP construct. This plasmid was modified to FLAG-Shisa6-pTRCGw-IRE52-EGFP by PCR-mediated insertion of a tandem FLAG-tag (sense 5’-GGT GAT TAT AAA GAT CAT GAT ATC GAT TAC AAG GAT GAC GAT GAC AAG CAC-3’, corresponding peptide: GDYKDHDIDYKDDDDKH) between codon 36 (GGG, Glycine) and codon 37 (AAC, Asparagine) of the Shisa6 cDNA.

For the GluK2 plasmid, a 6-myc epitope was inserted after the signal sequence of GluK2 cDNA and cloned in a pcDNA3 vector as described previously [216]. cDNA corresponding to the GluA subunits has been described previously [111]. Homer 1C::EGFP and Homer 1C::DsRed were generated by subcloning Homer 1C cDNA into the eukaryotic expression vector pcDNA3 (Invitrogen).

For FLIM experiments eGFP was inserted at position 253 on PSD-95 wild type (rat, UniProtKB/Swiss-Prot P31016) as previously described [172]. The mCherry tag was inserted 21 amino acids before the stop codon of Shisa6. All DNA constructs were sequence verified before use.

**Antibodies**
Anti-Shisa6 antibody was raised in rabbit against sequence DRYRMTKMHSHPSA (position 494-507 in Shisa6) (Genscript). The antibody was affinity-purified against the antigenic peptide, suspended at 1 mg/mL in PBS containing 0.02% NaN3, and stored at –20 °C. See supplementary information for antibodies used for immunoblotting and immunoprecipitation.

**Immunoblot analysis**
Protein samples were dissolved in SDS sample-buffer (Laemmli), heated to 96 °C for 5 minutes, and loaded onto a 4–15% Criterion TGX Stain-Free gel (Bio-Rad). The gel-separated proteins were imaged with the Gel-Doc EZ system (Bio-Rad), transferred onto PVDF membrane and probed with antibodies. Scans were acquired with the Odyssey Fc system (Li-Cor), and adjusted using Image Studio 2.0 software (Li-Cor).

**Subcellular fractionation**
Subcellular fractions were prepared as described previously [31] with some modifications. See supplementary data.
Immunoprecipitation of Shisa6 and GluA2 protein-complexes from mouse hippocampus
As described in the Supplementary information.

Co-precipitation from HEK293 cells
For protein extraction, HEK293 cells were washed with PBS, resuspended in lysis-buffer (1% Triton X-100, 150 mM NaCl, 25 mM HEPES, and EDTA-free Complete protease inhibitor), and incubated at 4 °C for 1 h while mixing gently. The supernatant was cleared of non-soluble debris by two consecutive centrifugation steps at 20,000x g for 20 minutes. Anti-flag antibody 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 lysis buffer, and bound proteins were eluted by incubation with Laemmli sample buffer.

Yeast Two-Hybrid
A direct two-hybrid assay was performed in PJ69-2 yeast cells between PSD-95 (amino acids 39-262 of NP_031890.1, encoding PDZ domains 1 and 2) and either the wild-type cytoplasmic domain of Shisa6A (Shisa6-cd WT, amino acids 202-557) or the truncated mutant thereof (Shisa6-cd ΔEVTV), as described [31]. Cell-growth was recorded after 4 days of stringent nutritional selection (-Leu, -Trp, -His, -Ade). Methods as described in [31].

Dissociated Hippocampal Neuronal Cultures
Hippocampal neurons derived from 18-day old rat embryos of either sex were cultured following the Banker protocol [217]. Briefly, dissociated neurons were plated on poly-L-lysine coated glass coverslips at a density of ~18,000 cells/cm2 and co-cultured over an astroglial feeder layer in Neurobasal medium supplemented with B27.

Immunofluorescence staining of cultured hippocampal neurons
Neurons (DIV21) were fixed for 15 minutes in 4% paraformaldehyde and 4% sucrose in PBS. Cells were rinsed 3 times in PBS and then incubated in 50 mM ammonium chloride in PBS for 10 minutes. Then, cells were rinsed in PBS and permeabilized with 0.2% Triton-X100 in PBS for 5 minutes. After 3 rinses with PBS, cells were incubated for 60 minutes in blocking solution containing 1% BSA (Sigma, A3059) in PBS. Next, neurons were incubated for 60 minutes in primary antibodies against Shisa6 (1/250), Homer1 and GluA2 that were diluted in blocking solution and centrifuged for 5 minutes at 15,000 rpm. Then, cells were incubated in blocking solution again before 30-minute incubation in with Alexa-conjugated goat anti rabbit (Alexa647), goat anti guinea pig (Alexa568) or goat anti mouse (Alexa488) secondary antibodies (Invitrogen) that were diluted (1:1,000) in blocking solution and centrifuged. Images of triple-stained neurons were obtained by epifluorescence microscopy (Leica, DM5000) using a 63x Plan Apochromat NA 1.4 oil objective.
Single nanoparticle tracking (QD) for surface diffusion of AMPAR

The experimenter was blind to the construct used, which was revealed after analysis. For endogenous GluA2 QD tracking, hippocampal neurons were incubated with monoclonal antibody directed against N-terminal extracellular domain of GluA2 subunit (MAB397; Millipore, Paris, France) for 10 minutes followed by 5 minutes 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 50 ms and up to 1000 consecutive frames. Signals were detected using a CCD camera (Quantem; Roper Scientific). QD recording sessions were processed with the Metamorph software (Universal Imaging Corp., Sunnyvale, CA, USA). The instantaneous diffusion coefficient, D, was calculated for each trajectory, from linear fits of the first 4 points of the mean square displacement versus time function using MSD(t) = <r^2>(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. QD-based trajectories were considered synaptic if colocalized with Homer 1C dendritic clusters for at least five frames.

Fluorescence lifetime imaging microscopy experiments

The experimenter was blind to the construct used, which was revealed after analysis. FLIM experiments were performed at 37 °C using an incubator box with an air heater system (Life Imaging Services, Basel, Switzerland) installed on an inverted Leica DMI6000B (Leica Microsystems, Wetzlar Germany) spinning disk microscope and using the LIFA frequency domain lifetime attachment (Lambert Instruments) and LI-FLIM software. Cells were imaged with a HCX PL Apo 100x oil NA 1.4 objective using an appropriate GFP filter-set. Cells were excited using a sinusoidally modulated 1-W 477 nm LED (light-emitting diode) at 40 MHz under wild-field illumination. Emission was collected using an intensified CCD LI2CAM camera (FAICM, Lambert Instruments). The phase and modulation were determined from a set of 12 phase settings using the manufacturer’s software LI-FLIM software. Lifetimes were referenced to a 1 mM solution of fluorescein in saline (pH 10) that was set at 4.00 ns lifetime.

Following FLIM measurements, cells were excited using a 100 mW 491nm DPSS laser (Calypso, Cobolt) for GFP imaging and a 100 mW 561 nm DPSS lasers (Jive, Cobolt) for imaging, and imaged with a HCX PL Apo CS 63x oil NA 1.4 objective. Signals were recorded with a back-illuminated Evolve EMCCD camera (Photometrics, Tucson, USA). Acquisitions were done on the software MetaMorph (Molecular Devices, Sunnyvale, USA).

HEK cell Transfection and cover slipping

HEK293 cells were transfected with GluA-pcDNA (0.8 µg) using Polyethylenimine (PEI 2500), 24–48 hours after transfection, cells were transferred to coverslips that were coated with 100 µg/mL Poly-L-Lysine (Sigma Aldrich, St. Louis, MO, USA) three hours prior to recording. To ensure consistency in the culture, we used HEK293 cells that were passaged no more than
24 times. Cells were passaged at least 3 hours prior transfection in DMEM media (Gibco), 10% FBS (Invitrogen), 1% Penicillin-Streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA) in 2.5 cm dishes. Cells were 60–70% confluent when transfected.

Electrophysiology
For electrophysiological recordings we made use of Clampex 9.0 along with a 700B amplifier and a 1322 digitizer (all from Molecular Devices, Sunnyvale, California, USA). Cells were identified and patched using an Olympus BX50WI DIC microscope with a 40x, 0.8 nA W objective (all Olympus coorporation, Tokyo, Japan). Data was acquired using an internal 4 pole Bessel filter (3 kHz) and a sample frequency of 20 kHz. All stimuli were timed using an Master 8 (A.M.P.I. Jerusalem, Israel). For electrical stimulation we used an isoflex (A.M.P.I. Jerusalem, Israel).

HEK293 cell Recordings
HEK293 cells were perfused with 32 °C standard artificial CSF (aCSF) during the recordings containing (in mM): 126 NaCl, 3 KCl, 10 D-glucose, 26 NaHCO3, 1.2 NaH2PO4, 2 CaCl2 and 1 MgSO4, carboxygenated with 95% O2 and 5% CO2 to obtain pH7.4 and an osmolarity of 300. HEK293 cells were recorded using borosilicate electrodes (OD 1.5 mm, ID 0.86 mm; Harvard Apparatus, Holliston, MA, USA) of 2.5–4 MΩ resistance and filled with intracellular solution containing (in mM): 70 Cs-Gluconate, 70 CsCl2, 10 HEPES, 0.1 Spermine, 0.5 NaGTP, 5 Mg2ATP, 10 EGTA (pH 7.3, 290 osm). HEK 293 cells were gently lifted from the cover slip and placed in front of a Piëzo-driven theta-barrel electrode (TGC 200; Harvard Apparatus, Holliston, MA, USA), filled with standard aCSF on one side and standard aCSF supplemented with 1 mM L-glutamate Hydrochloride (Sigma Aldrich, St. Louis, MO, USA) on the other side [151]. The experimenter was blind for the constructs used, which was revealed after analysis. Access resistance in HEK cells was typically below 8 MΩ. Cells with access resistance above 10 MΩ or with current amplitude below 20 pA were excluded from analysis.

Acute brain slices
Mice were decapitated and the brain was removed from the skull in ice-cold slice solution containing (in mM): 126 NaCl, 3 KCl, 10 D-glucose, 26 NaHCO3, 1.2 NaH2PO4, 0.5 CaCl2 and 7 MgSO4. Acute horizontal hippocampal slices were cut with a thickness of 300 μm using a vibratome (Microm HM 650 V) in ice-cold slice solution and transferred to standard aCSF for a recovery period of at least 1 h prior to recordings.

Electrophysiological Recordings
Glass electrodes of 3–5 MΩ resistance were used for all whole-cell recordings from acute brain slices and pulled using borosilicate glass (OD 1.5mm, ID 0.86mm; Harvard Apparatus, Holliston, MA, USA). Slice recordings were performed using standard aCSF (see above) at 32 °C. During all experiments, the experimenter was blind to the genotype of the animal. Input/access resistances were monitored throughout the recordings. Unless indicated otherwise,
used salts were purchased at Sigma Aldrich (St. Louis, MO, USA) and drugs were purchased at Abcam (Cambridge, UK). The experimenter was blind to the genotype used, which was revealed after analysis. Input resistance changes were monitored throughout the recordings. Our average input resistance for CA1 pyramidal cells was around 165 MΩ. Cells with input resistance changes above 20% within a recording were discarded before analysis. Our typical access resistance was around 10 MΩ. Cells with access resistances above 20 MΩ were not used for analysis.

**Miniature EPSCs:** CA1 pyramidal cells were patched using electrodes of 3–5 MΩ resistance filled with intracellular solution containing (in mM): 125 Cs-Gluconate, 5 CsCl2, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 K-Phosphocreatine, 2 Mg-ATP, 0.3 GTP and 4% Biocytin (Molekula GmbH, Munich, GER) (pH 7.3, 290 osm). Slices were superfused with standard aCSF that was supplemented with 1 µM Tetrodotoxin (TTX) and 10 µM SR-95531 (Gabazine).

**Short-Term Plasticity:** Whole-cell recordings of CA1 pyramidal cells were achieved using intracellular solution containing: 125 Cs-Gluconate, 5 CsCl2, 4 NaCl, 10 HEPES, 0.2 EGTA, 2 K-Phosphocreatine, 2 Mg2-ATP, 0.3 GTP and 4% Biocytin (pH 7.3, 290 osm). CA1 pyramidal cells were stimulated in the stratum radiatum of the hippocampus around 80–150 µm distance to the soma at dendrite proximal locations. Dendrites were visualized using Alexa488 (Life Technologies, Carlsbad, CA, USA) in the intracellular recording solution. Electrical stimulation was performed inputs using an extracellular stimulation electrode of 2–3 MΩ resistance filled with standard aCSF. Moderate stimulation intensity was assessed during an I/O protocol at the beginning of each recording. Synaptic changes were recorded in response to 10 pulse presynaptic stimulation at frequencies between 2 Hz and 50 Hz at a holding current of –70 mV. Slices were superfused with standard 32 °C aCSF which was supplemented with 10 µM SR-95531 (Gabazine). NMDA currents were measured as indicated above in the additional presence of 2 µM NBQX voltage clamped at –30 mV. NMDA receptors were blocked by the addition of 20 µM APV to visualize the nature of the remaining current (Supplemental Fig. 9). Recovery from desensitization: Equipment and experimental setup used is the same as in short-term plasticity experiments. A 40-pulse 100 Hz pulse train was used to desensitize the synapses completely followed by recovery pulses following 200, 300 and 500 ms after the train. During the recordings acute brain slices were superfused with standard aCSF supplemented with 10 mM SR-95531.

**Nucleated patches/ glutamate uncaging:** CA1 pyramidal cells were patched using electrodes of 2.5–4 MΩ resistance. The intracellular solution contained (in mM): 70 Cs-Gluconate, 70 CsCl2, 10 HEPES, 0.5 NaGTP, 5 Mg2-ATP, 10 EGTA and 10 K-Phosphocreatine (pH 7.3, 290 osm). In glutamate uncaging experiments the same intracellular solution was supplemented with 2 µM Alexa 488 (Life Technologies, Carlsbad, CA, USA) and 4% Biocytin (Molekula GmbH, Munich, GER) for cellular identification. Glutamate was applied to nucleated patches and HEK293 cells with a Piezo-driven double-barrelled pipette as described.
above. In the glutamate uncaging experiments 1 mM RuBi-Glutamate (Abcam, Cambridge, UK) was continuously puffed by a light protected glass electrode of 2.5-3.5 ΩM resistance into the close proximity of the apical dendrite for the duration of 4 seconds. At 2.5 s into the glutamate puff, glutamate was uncaged using a 50 µm multimode fiber (Thorlabs, Newton, New Jersey, USA) close to the application electrode. A 10-ms laser pulse (532 nm wavelength; ML III - 532, ~300W; CNI, Changchun, 130103, P.R. China) was used to uncage glutamate. Glutamate uncaging experiments were performed in the presence of 1 µM TTX and 10 µM SR-95531.

Statistics
Data are presented as mean±SEM or medians±interquartile range (IQR) defined as the interval between 25%–75% percentile. All tests were two-sided. Replicates were biological in nature.

Cell-imaging – Normally distributed data sets were compared using the paired Student’s test and unpaired Student’s t-test. Statistical significance (Prism 4.0 (GraphPad, La Jolla, CA)) between more than two normally distributed datasets was tested by one way ANOVA variance test followed by a Newman-Keuls test to compare individual pairs of data. Non-Gaussian data sets were tested by non-parametric Mann-Whitney test.

Electrophysiology – Data was analysed using custom made Matlab (Mathworks, Natick, MA, USA) software and MiniAnalysis (Synaptosoft, Decatur, GA, USA) for the analysis of miniature EPSCs. Rise times were measured as the time that it took to get from 20% to 80% of the maximal amplitude. Current decays are reported as Decay time (90–10 %) when stated, otherwise the desensitization time constant that was determined by fitting double exponential curves, is reported. Statistical significance was assessed using Graphpad Prism 5 software (GraphPad Software, La Jolla, CA). A Student’s t-test was applied when data passed the Kolmogorov-Smirnov test for normality. If not, significance was determined using a Mann-Whitney U test. Significance of short-term-plasticity data was assessed using a 2-way ANOVA with Bonferroni post-hoc testing.
Characterization of Shisa6

Acknowledgements

The authors thank Joost Hoetjes and Robert Zalm for genotyping, Frank Koopmans for proteomics support, the Bordeaux Imaging Center, part of the FranceBioImaging national infrastructure, for support in microscopy, the IINS cell biology core facility for providing neuronal cultures. ABS, HDM, SS, received support from HEALTH-2009-2.1.2-1 EU-FP7 ‘SynSys’ (#242167). RVK was funded by NWO-ALW grant (#ALW2PJ/12048), JS was funded by EU Marie Curie ITN CerebNet (MEST-ITN-2008-238686). ABS, HDM, and LJMS were funded by the Dutch Neuro-Bsik Mouse Pharma Phenomics consortium (grant BSIK 03053 from SenterNovem, PRR was funded by EU Marie Curie ITN ‘Neuromics’ (MEST-CT-2005-020919). DC, ASH, FC, JP and CR were supported by funding from the Ministère de l’Enseignement Supérieur et de la Recherche, Centre National de la Recherche Scientifique, the Conseil Régional d’Aquitaine and the ERC grant nano-dyn-syn to DC.
Supplementary materials

(Real-Time) Polymerase Chain Reaction

**Primers** – Primers for PCR and real-time PCR were generated using Primer3.0. The final sets of primers are listed in Table S2.

**RNA isolation and cDNA synthesis** – RNA from several tissues was extracted as previously described (Spijker et al. 2004). (See supplementary data) Samples were DNase-I treated according to the manufacturer’s instructions (20 U/μg RNA; Boehringer, Germany) to remove traces of genomic DNA, which was verified by using intron-specific PCR primers (data not shown). RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), and the integrity of RNA was checked by gel electrophoresis (1%-TBE-agarose gel). Random-primed (25 pmol; Eurofins MWG Operon, Germany) cDNA synthesis was performed on individual RNA samples (~1 μg total RNA).

**PCR for exon 3** – PCR reactions on two WT samples were generated with Ex3-Ex5 primers with 1 U Phusion (New England Biolabs, UK) in a 50-µL reaction using the HF buffer according to the manufacturer's protocol.

**Real-time qPCR** – Real-time qPCR reactions (20 μL; ABI PRISM 7700) on pooled tissue (3 mice) were performed using a 96-well format with transcript-specific primers (300 nM) on cDNA corresponding to ~20 ng RNA [215] and SYBR Green reagents (Applied Biosystems). (See supplementary data) Amplification efficiencies of primer sets (Eurofins MWG Operon, Germany) were tested by qPCR and analyzed for amplification efficiency. Only primers with proper efficiency [215, 218] were used. Cycle threshold (Ct) values were used to calculate the relative level of gene expression, where Ct value is the fractional cycle number at which the fluorescent signal of a reaction passes the threshold (reaching intensity above background). Expression level of three housekeeping genes (GAPDH, α-actin, HPRT) was measured as reference for input. Expression is denoted using normalized Ct values on a log2-scale. Let normalized Ct-values be denoted by Ctnormx (where x represents Shisa6 expression, y represents the geometric mean of Ct-values of the housekeeping genes, and i represents a given sample), Ctnormxi then is given by Ctnormxi = Ctxi – Ctyi. As a bigger Ct-value correlates with a lower expression level, for practical purposes, Ctnormxi values were converted into conCtnormxi values, calculated as conCtnormxi= –Ctnormxi +15. Due to this conversion, the final positive value of Ct is positively correlated with relative gene expression level, which makes the visualization simpler. Relative gene expression levels were expressed as conCt-norm-values ±SEM.
Characterization of Shisa6

DNA expression constructs
pTRCGw-IRES2-EGFP is an adapted version of the pTRCGw vector, created by insertion of a Gateway-cloning pDEST recombination site (Invitrogen) and pIRES-EGFP element, upstream of the existing WPRE and pA modules. Glutamate receptor 1 isoform 1 (NP_001106796.1) and Glutamate receptor 2 isoform 1 (NP_001077275.1) were Gateway-cloned into the (pDEST) pTRCGw-IRES2-EGFP vector, yielding respectively GluA1-pTRCGw-IRES2-EGFP and GluA2-pTRCGw-IRES2-EGFP.

Antibodies
Antibodies used for immunoblotting were anti-GluA1 (Abcam, ab109450, 1:20,000), anti-GluA2 (Neuromab, 1:1,000), anti-GluA3 (Abcam, ab40845, 1:500), anti-GluK2 (Santa Cruz, C-18, 1:1,000), anti-GluN2A (Abcam, ab14596, 1:2,000), anti-PSD95 (Neuromab, 1:50,000), and anti-Synaptophysin (Genscript, 1:2,000). Antibodies used in immunoprecipitation were anti-GluA2 (Neuromab), anti-FLAG M2 (Sigma) and IgG control (Genscript, whole protein). Antibodies used in immunocytochemistry were guinea pig anti-Homer1 (Synaptic Systems, 160-004, 1:500) and mouse anti GluA2 (NeuroMab, 75-002, 1:500).

Subcellular fractionation
Subcellular fractions were prepared as described previously [31] with some modifications. All steps were performed at 4 °C and in the presence of EDTA-free Complete protease inhibitor (Roche). Hippocampal tissue was homogenized in buffer containing 0.32 M Sucrose and 5 mM HEPES, pH 7.4 (Homogenate fraction), and centrifuged at 1,000x g for 10 min. The supernatant was either (1) centrifuged at 18000x g for 30 minutes, yielding a crude synaptic membrane pellet (P2 fraction), (2) centrifuged at 120,000x g for 2 h, yielding the crude synaptic membrane + microsome pellet (P2+M fraction), or (3) layered onto a discontinues 0.85/1.2 M sucrose density gradient and centrifuged at 120,000x g for 2 h. The 0.85/1.2 M sucrose interface was collected as the synaptosome fraction. Synaptosomes were hypotonically shocked by dilution in 5 mM HEPES pH 7.4 and subjected to a second round of centrifugation on a 0.85/1.2 M sucrose density gradient. The synaptic membrane fraction was collected at the 0.85/1.2 M sucrose interface. Alternatively, synaptosomes were mixed with Triton X-100 (0.32 M sucrose and 5 mM HEPES, pH 7.4) to a final concentration of 1%, incubated for 1 hour, and layered on top of a 1.0/1.5/2.0 M sucrose density gradient. After 2 h centrifugation 120000x g, the preliminary PSD fraction was collected at the 1.5/2.0 M interface. A second 1% Triton X-100 extraction and subsequent 1.5/2.0 M sucrose density gradient purification yielded the final PSD fraction.
Immunoprecipitation of Shisa6 and GluA2 protein-complexes from mouse hippocampus

All subsequent steps, excluding protein-elution, were performed at 4 °C. Hippocampal tissue from WT and Shisa6 KO mice was homogenized in buffer A (0.32 M sucrose, 10 mM HEPES pH 7.4, and EDTA-free Complete protease inhibitor) and centrifuged at 1,000x g for 10 minutes. Centrifuging the supernatant at 18,000x g for 30 minutes yielded the crude synaptic membrane pellet. This pellet was resuspended to 5 mg protein/mL in buffer B (1% DDM, 150 mM NaCl, 25 mM HEPES, and EDTA-free complete protease inhibitor), incubated for 1 h while mixing gently, and centrifuged at 20,000x g for 20 minutes. This extraction-procedure was repeated once more on the remaining pellet. The supernatant from both extractions was pooled and subjected to a second round of centrifugation at 20,000x g for 20 minutes. Antibody (Anti-Shisa6 antibody, anti-GluA2 antibody, or IgG control) 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 buffer C (0.1% DDM, 150 mM NaCl, 25 mM HEPES pH7.4) and bound proteins were eluted by incubation with Laemmli sample buffer.

In-gel Tryptic digestion

The free cysteine residues of Laemmli buffer-eluted proteins were blocked by addition of acrylamide (3.75% final concentration) for 30 minutes at room temperature. Proteins were resolved on a 10% SDS polyacrylamide gel, fixed, and stained with colloidal Coomassie Blue G-250. Sample lanes were cut into 3 segments, destained, and the proteins in-gel digested with Trypsin/Lys-C mix (Promega) during overnight incubation at 37 °C. The peptides were extracted twice with 50% acetonitrile + 0.1% trifluoroacetic acid for 40 minutes, once with 80% acetonitril + 0.1% trifluoroacetic acid for 20 minutes, dried by speedvac, and stored at -80 °C.

HPLC-MS-MS

Peptide samples were redissolved in 0.1% acetic acid and loaded onto a PepMap100 C18 precolumn (300µm i.d., 5 µm particle size; Dionex) connected to an UltiMate 3000 HPLC system (Dionex). Separation was achieved on a 200 mm Alltima C18 in-house packed column (100 µm i.d., 3 µm particle size) by using an aqueous-organic gradient of 5% to 40% acetonitrile + 0.1% formic acid for 27 minutes at a flow rate of 400 nL/min. Eluates were electro-sprayed directly into a TripleTOF 5600+ system (Absciex) operated in Information Dependent Acquisition mode. One full-scan cycle consisted of a precursor ion scan (m/z range of 350 to 1250, charge state +2 to +5, 90 cps intensity treshold, and 0.25 seconds accumulation time) followed by MS-MS analysis of the top 20 most abundant ions (m/z range 200 to 1800, 0.09 seconds accumulation time). Former target ions were excluded for 10 seconds.
MS data analysis
TripleTOF 5600+ output files were converted from wiff to mzML (centroid) format using the AB SCIEX MS Data Converter utility and searched against the mouse UniProtKB/Swiss-Prot canonical sequence database (version 2014-03-21) by means of the Morpheus search engine (version 1.0.0.0) [219]. Methionine oxidation and protein N-terminal acetylation were selected as variable modifications, and propionamide was set as fixed cysteine modification. The maximum mass tolerance for precursor and product ions was 25 ppm and 0.025 Da respectively. Trypsin (no proline rule) was selected as protease, allowing up to one missed cleavage. A target-decoy database was created on the fly and the maximum Peptide Spectrum Match (PSM) false discovery rate (FDR) was set to 1%. In-house developed software was used to import the PSM results from the Morpheus pepXML files into ProteinProphet [220], where the final protein inference and protein validation was performed (protein FDR of 1%). The summed spectral count value of all unique peptides was used for relative protein quantification. The statistical significance of the results was analyzed using a non-normalized beta-binomial test [221], a method developed specifically for the analysis of spectral count-based datasets, where results do not follow a normal distribution.
Supplementary Figure 1. Shisa6 structure and tissue expression. a) Schematic representation of the Shisa family, featuring a signal peptide (SP; blue), an extracellular domain with conserved cysteine-rich motif, single transmembrane region (TM; green), and intracellular domain with PDZ-ligand motif (EVTV/AVTV; red). An alternative spliced exon (Ex; brown) is present in Shisa9, Shisa6 and Shisa7, albeit of different size and exon number. Shisa6 and Shisa7 form a subfamily with Shisa9 and Shisa8. Shisa4 and Shisa5, as well as Shisa2 and Shisa3 form separate families (Pei et al., 2012). Numbers under the protein indicate the beginning and end of the SP, TM and alternatively spliced exon domain, and the total amino acid length of the protein. b) Sequence alignment of the typical Cysteine-rich domain found in the N-terminal extracellular part of Shisa proteins, containing 8 highly conserved cysteine residues. c) Quantitative PCR shows that the Shisa6 gene is specifically expressed within the brain (note the log2-scale), and virtually absent in pancreas and testes. d) RT-PCR on cDNA generated from hippocampal RNA using primers flanking exon 4 of Shisa6. Using this RT-PCR on WT mice in duplicate, we showed a single Shisa6 transcript that contains exon 3. The exon 3-less form of Shisa6 (GenBank NM_001034874.3/ NP_001030046.1) was not detected in the hippocampus. Sequence analysis confirmed the presence of exon 4 (black letters) between exons 2 and 4 (gray letters). The amino acid sequence is indicated above the nucleotide sequence.
Characterization of Shisa6

Supplementary Figure 2. Shisa6 is enriched within the hippocampus and cerebellum. a) Representative picture of Shisa6 in situ hybridization signal (probe RP_060321_02_E02) and expression energy from Allen Brain Atlas (Lein et al. 2007) in the hippocampus showing Shisa6 expression in the entire hippocampus and cerebellum. b) The different molecular weights (~73, ~66, and ~59 kDa; arrow heads) of the Shisa6 protein that has a mature calculated mass of ~59 kDa is highly enriched within the hippocampus and cerebellum. The ~48 kDa signal is not specific to Shisa6 (see d). Lower panel depicts the loading control (total protein). c) Representation of Shisa6-null mice generation: The Shisa6 locus around exon 1, encoding the N-terminal part of the Shisa6 protein including the start-site (ATG), with essential restriction sites (pink) and the 5’- and 3’- probes used for Southern blotting, is shown (top). Mice with a null-allele were generated by deletion of most of exon 1, in which the remaining Loxp and FRT sites are present after homologous recombination. d) Specificity of our Shisa6 antibody for immunoblotting was confirmed by absence of signal in hippocampal and cerebellar crude synaptic membrane fractions from Shisa6 KO animals. Arrowheads indicate the apparent molecular weights of endogenous Shisa6 (see b). e) Electrophoretic mobility of hippocampal Shisa6 under reduced and denatured (SDS-PAGE) conditions was higher than expected based on theoretical protein size alone (expected mature weight 58.7 kDa, putative signal peptide 30 amino acids), potentially due to protein glycosylation. Treatment with PNGase-F reduced the observed molecular weight substantially to a single band of ~59 kDa, confirming the presence of N-linked glycans on native Shisa6.
Supplementary Figure 3. Shisa6 increases AMPAR decay time of homomeric AMPAR currents.

a,c) Peak-scaled example traces of whole-cell recording from HEK293 cells expressing homomeric GluA1 (a) or GluA2 (c) AMPAR channels consisting of this AMPAR in absence (black/gray) or presence (dark/light red) of Shisa6. Currents were evoked by direct application of 1 mM glutamate during 1 ms. b,d) Bar graphs (mean±SEM) summarize changes of AMPAR currents mediated by homomeric GluA1 (b) or GluA2 (d) AMPARs in HEK293 cells in the presence and absence of Shisa6 in terms of rise time (0.77±0.02 vs. 0.78±0.02 ms; P=0.708; 1.49±0.07 vs. 1.74±0.08, P=0.051), current density (405.9 ± 81.8 vs. 328.8 ± 68.2 pA/pF; P=0.171; 13.98±1.58 vs. 10.71±1.77, P=0.143) and decay time (3.41±0.15 vs. 5.83±0.29 ms; P<0.001; 7.94±0.63 vs. 14.30±1.35, P=0.001). e) IV-curve of homomeric GluA1-containing AMPAR expressed in HEK293 cells in the presence (n=4) and absence (n=5) of Shisa6 shows that Shisa6 does not alter rectification of GluA2-lacking AMPARs. f) Expression of Shisa6 in the absence of an AMPAR (orange; n=3) does not lead to currents when stimulated by 1 s glutamate, indicating that Shisa6 is not a pore-forming subunit. *** P<0.001 (Student’s t-test).
Supplementary Figure 4. Shisa6 does not alter kainate receptor kinetics. a) Peak-scaled example traces of whole-cell recordings from HEK293 cells expressing GluK2 (GluR6) homomeric receptors in the absence (black; n=15) or presence (dark green; n=15) of Shisa6. Currents were evoked by direct application of 1 mM glutamate during 1 s. b) Zoom-in of panel a to visualize changes in kinetics of GluK2 (GluR6). c–f) Bar graphs (mean±SEM) summarize changes in rise time (c), current density (d), decay time (e) and steady-to-peak ratio (f) of currents mediated by homomeric kainate receptors in HEK293 cells in the presence and absence of Shisa6.
**Supplementary Figure 5.** Shisa6 deletion does not lead to compensatory effects on synaptic AMPAR expression. a) Bar graphs (mean±SEM) summarize absence of significant changes in EPSC amplitude (16.21±0.52 vs. 14.73±0.65 pA, P=0.085) and frequency (0.75±0.15 vs. 0.54±0.13 Hz, P=0.290) recorded in CA1 pyramidal neurons in acute hippocampal slices of Shisa6 KO and WT littermates (n=19 cells per genotype, from 4 WT and 4 KO animals). b) Immunoblots from hippocampal synaptic membrane fractions from WT and Shisa6 KO mice (n=6 each) do not reveal differences in abundance of AMPAR (GluA1, P=0.216; GluA2, P=0.924), NMDAR (GluN1, P=0.101; GluN2a, P=0.565; GluN2b, P=0.481), PSD-95 (P=0.862), or TARPs (γ2, P=0.545; γ8, P=0.611), when expressed as fold change over WT samples. Signal was normalized to total protein level as measured by TCE intensity.

**Supplemental Figure 6.** Shisa6 is only expressed at synaptic sites. a) Picture of a nucleated patch from a CA1 pyramidal cell. b) Superimposed example recordings from nucleated patches of CA1 pyramidal cells of Shisa6 KO animals (n=12 from 3 animals) and WT littermates (n=12 from 5 animals). Currents were evoked by a 1-ms application of 1 mM glutamate. c) Bar graphs (mean±SEM) summarize changes in rise time and decay time of nucleated patch recordings (rise time (ms): WT, 0.79±0.05 vs. KO 0.84±0.08, P=0.500; decay time (ms): WT, 8.32±0.66 vs. KO 8.56±0.72, P=0.800). The absence of differences suggests that Shisa6 does not interact with functional AMPARs at the cell soma.
Supplemental Figure 7. Shisa6 affects the desensitization of AMPAR currents. a) Peak-scaled example trace of whole-cell recordings from HEK293 cells expressing homomeric AMPAR channels in absence (black) or presence (red) of Shisa6. Currents were evoked by direct application of 1 mM glutamate during 1 s. b) Bar graphs (mean±SEM) summarize changes in rise time (1.48±0.10 vs. 1.25±0.09 ms, P=0.230), desensitization time constant (4.78±0.16 vs. 6.02±0.43, P=0.016), steady-state AMPAR-mediated currents of homomeric AMPARs (5.18±0.91 vs. 11.30±9.25 % of peak current, P=0.004) in absence (black) or presence (red) of Shisa6. * P<0.050, ** P<0.010, (Student’s t-test).
Supplemental Figure 8. Shisa6 has no presynaptic effect as measured by NMDAR currents. a) Diagram showing the recording site and stimulation electrode used for stimulation of Schaffer collateral synapses in the presence of NBQX and Gabazine. b) Example trace recorded from CA1 pyramidal neurons of WT animals in absence (black) or presence (brown) of 20 µM APV in response to 50-Hz stimulation of synaptic inputs from Schaffer collateral voltage clamped at −30 mV. c) Example trace recorded from CA1 pyramidal neurons of WT animals (black) or KO (blue) in response to 50-Hz stimulation of synaptic inputs from Schaffer collateral voltage clamped at −30 mV, showing that rise and decay times of NMDAR currents, measured in the presence of 2 µM NBQX, are not affected in Shisa6 KO (P>0.500). d) Superimposed example traces recorded from CA1 pyramidal neurons of Shisa6 KO (blue) animals and WT littermates (black) in response to 50 Hz stimulation of synaptic inputs from Schaffer collateral voltage clamped at −30 mV. e–g) Pulse ratios of electrically-evoked EPSCs (at −30 mV) from CA1 pyramidal neurons of Shisa6 KO (blue, n=15 from 4 animals) animals and WT littermates (n=12 from 5 animals) at 2 (e), 20 (f) and 50 (g) Hz. Cell numbers used are indicated.
Supplemental Table 1. PSD-95 is the main PDZ-domain containing interactor of hippocampal Shisa6. Mass spectrometry analysis of native Shisa6 complexes immunoprecipitated from the hippocampi of Shisa6 WT and KO animals (crude synaptic membranes; n=3 IPs per genotype), revealed PSD-95 (Dlg4) as most abundant PDZ domain-containing interactor. Shisa6 is given in blue, interacting AMPAR subunits are given in light yellow, PDZ-containing interactors detected are given in orange. Selection criteria were: 1) For protein identification, all WT samples should have ≥ 1 unique peptide found. 2) The average WT/KO spectral-count should be ≥ 1, indicating enrichment in the WT samples. 3) Performing a beta-binomial test, enrichment in the WT samples should reach P≤0.01 (green). Failing a particular selection criterion resulted in an exclusion marker (*). Percent coverage based on peptides found vs. total measurable peptides generated, as a proxy for protein abundance, is given.
### Table S2

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**Supplemental Table 2.** Sequence of DNA primers used. For PCR experiments to detect the presence or absence of exon 3 in Shisa6, as well as for real-time PCR, different primer sets were used. The forward and reverse primer, as well as the amplicon size generated, are indicated.
Shisa6 limits plasticity of hippocampal glutamate synapses

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Abstract

Shisa6 has been described as a novel AMPAR auxiliary protein. Among other things, the presence of Shisa6 at AMPARs leads to altered deactivation and desensitization properties and reduces the probability of AMPARs to desensitize upon glutamate application (Chapter 2). This effect might be accounted for by aberrant synaptic AMPAR movements, postsynaptically trapped when associated with Shisa6. Here we present evidence, that Shisa6 plays a crucial role in the formation process of synaptic plasticity. In response to theta burst stimulation of Schaffer collateral (SC) fibres, CA1 pyramidal neurons show enhanced LTP formation in adult Shisa6 KO mice compared to wildtype (wt) littermates. These findings underline the importance for Shisa6 in regulating AMPARs at synapses in response to glutamate, and might indicate the restriction of AMPAR movement at synapses during plasticity-inducing stimuli. With recent studies indicating the co-decoration of AMPARs with both Shisa and TARP subunits, it will be of great interest to see, whether Shisa6 also coexists with TARP subunits of overlapping hippocampal expression pattern such as γ-8.
Shisa6 and LTP

Introduction

Synaptic targeting and trapping of AMPARs at the PSD are essential for both long- and short-term plasticity [24, 97, 111]. Today it is well established, that AMPARs are anchored to their synaptic PSD location via a set of auxiliary proteins [22, 111]. A textbook example of such interaction is the interaction between AMPARs and Stargazin, which in turn interacts with PSD-95, the predominant protein at the PSD [22, 111]. Interestingly, the binding between these two proteins and thus the synaptic anchoring of AMPARs, is affected by synaptic activity and was shown influenced by kinases such as CaMKII or specific MAGUKs through posttranslational modifications [75, 141, 175].

Recently, Shisa6 has been described as a newly discovered bona fide AMPAR auxiliary protein [213]: a non-pore-forming, transmembrane protein which associates with native AMPAR complexes and changes its subcellular behaviour and biophysical properties (see chapter 2). The presence of this protein at AMPA receptor complexes slows the deactivation and desensitization kinetics of AMPARs in heterologous cells and alters their synaptic depression properties in hippocampal tissue. Furthermore, data convincingly indicates that Shisa6, like Stargazin, serves as adapter protein, which links decorated AMPARs to postsynaptic scaffolding proteins via its C-terminal PDZ- ligand motif ([22, 111], chapter 2). Removal of this interaction site from Shisa6 leads to an increase in synaptic AMPAR- mobility at synapses of neuronal hippocampal cultures, probably as a result of impaired synaptic anchoring of AMPARs to PSD proteins, such as PSD-95 or SAP102 (chapter 2). As a consequence, the biophysical properties of AMPARs are altered. However, whether the process of long-term potentiation of glutamate synapses is affected by the presence of Shisa6 subunits is not known. Since the incorporation of AMPARs into the PSD is essential for the formation of LTP, we subjected CA1 pyramidal neurons of Shisa6 KO and wild type background to LTP stimulation to test whether AMPARs decorated with Shisa6 play a role in this process.

Results

Apart from changing the conductance properties of decorated AMPARs, Shisa6 traps AMPARs at synaptic sites most probably through interactions with the PSD (Chapter 2). Since such interactions are known to be essential for the formation of long-term plasticity, we hypothesized that Shisa6 might play a role in this process. To test this hypothesis, we made whole-cell recordings from CA1 pyramidal cells of Shisa6 KO and wild type littermates. Glutamate inputs were activated by extracellular electrical stimulation of the Schaffer-collaterals (SC) fibers.
The evoked EPSPs showed similar rise times (in ms: wt (n=15) 3.93 ± 0.2437 vs KO (n=15) 3.32 ± 0.1669, p= 0.0501), decay times (in ms: wt (n=15) 63.56 ± 3.90 vs KO (n=15) 58.15 ± 3.600, p= 0.3158) and amplitudes (in pA: wt (n=15) 3.51 ± 0.21 vs KO (n=15) 4.19 ± 0.33, p= 0.0961) in both genotypes (figure 1C). The slope measured during baseline between the two genotypes was comparable (in pA: wt (n=15) 0.65 ± 0.06 vs KO (n=15) 0.86 ± 0.09, p= 0.0683; figure 1E). These results are similar to our observations from evoked EPSCs where we failed to observe changes in kinetics between wildtype and KO animals when stimulating SC inputs to CA1 pyramidal neurons (chapter 2).

After establishing a stable baseline of EPSP amplitude and slope, a theta-burst stimulation paradigm was used to induce LTP [218]. In short, this stimulation paradigm consisted of 3 repetitions of 10 action potential bursts at 5 Hz frequency, with each burst consisting of 5 action potentials evoked at 100Hz frequency, as described by Buchannan et al [218]. In both WT and KO neurons, an increase in slope was induced after the theta-burst stimulation. 5 minutes after LTP induction, the observed changes in EPSP slope showed no significant differences between genotypes (slope norm. to baseline, (wt, n= 15) 1.25 ± 0.10 vs (KO n = 15) 1.50 ± 0.20, p = 0.2854), arguing for the early phase LTP induction being independent of Shisa6 (figure 1F). However, 15 minutes following LTP induction the increase in EPSP slope of CA1 pyramidal neurons of Shisa6 KO animals was significantly larger compared to wt cells (slope norm. to baseline, (wt, n= 15) 1.40 ± 0.10 vs (KO n = 15) 1.78 ± 0.11, p = 0.0163). This change persisted over the period of 25 minutes post induction (slope norm. to baseline, (wt, n= 15) 1.47 ± 0.09 vs (KO n = 15) 1.87 ± 0.13, p = 0.0168; figure 1F). These data indicate that Shisa6 plays a role in the process of LTP formation and/or maintenance.

Similar results were obtained with EPSP amplitude after LTP induction. In baseline recordings we found no differences in amplitude between wt and KO animals (wt, n= 15) 3.51 ± 0.21 vs (KO n = 15) 4.19 ± 0.33, p = 0.0961; figure 1G). Five minutes after the induction of LTP, both genotypes show a similar increase in EPSP amplitude (slope norm. to baseline, (wt, n= 15) 1.29 ± 0.07 vs (KO n = 15) 1.56 ± 0.15, p = 0.1485; figure 1 H). As for the EPSP slope, the change in EPSP amplitude became significant 15 minutes following LTP induction (wt, n= 15) 1.53 ± 0.13 vs (KO n = 15) 1.94 ± 0.15, p = 0.0460). However, 25 minutes post induction the difference was no longer significant (wt, n= 15) 1.66 ± 0.13 vs (KO n = 15) 2.106 ± 0.19, p = 0.0635; figure 1.H).

We observed no changes in 20-80% rise and decay kinetics in EPSPs between the genotypes for the entire course of the experiment (suppl. Fig1B).
Figure 1. Shisa6 influences synaptic potentiation

A. Schematic drawing of the recording setup. CA1 pyramidal cells were patched in whole-cell configuration. Monosynaptic EPSPs were evoked through electrical stimulation of Schaffer collateral fibers about 120 – 150 microns away from the soma. B. 2 min bin averages of EPSP slope values in neurons of Shisa6 wildtype (black) and KO (blue) animals. Slopes were calculated as the first 2.5 ms of the EPSP rise time. Error bars represent the SEM. After 8 minutes of baseline, a theta burst protocol induced LTP (grey bar). C. Bar graphs represent the EPSP kinetics of rise and decay (mean ± SEM) before the induction of LTP. D. Example traces of EPSPs from CA1 pyramidal cells evoked through stimulation of the Schaffer collateral fibers before (grey) and after (black) the induction of LTP. E. Bar graph represents the difference (mean ± SEM) in EPSP slope between Shisa6 wildtype (black) and knockout (blue) animals under baseline conditions. F. Bar graphs represent the changes in EPSP slope at 5, 15 and 25 minutes after the induction of LTP observed between wildtype and Shisa6 KO animals. p<0.05
Discussion

In this study we addressed the question whether the auxiliary AMPAR subunit Shisa6 affects the induction of long-term potentiation at hippocampal synapses. Interestingly, animals that lack Shisa6 showed an increase in synaptic potentiation compared to wild type animals. AMPAR containing synapses potentiate more readily without Shisa6. In the light of the prior findings that Shisa6 restricts the synaptic movements of AMPARs at synapses in neuronal cultures (Chapter 2), this result seems counterintuitive.

One possible explanation for this phenotype is that the number of AMPARs at synapses is generally reduced in Shisa6 knockout animals (compared to wildtype animals) providing the opportunity for bigger synaptic changes upon LTP-stimulation. However, recordings of spontaneous EPSCs in both genotypes showed similar amplitudes, arguing for intact synaptic incorporation of AMPARs (see chapter 2).

A second possible explanation is that AMPARs decorated with Shisa6 are generally immobilized at synapses through PSD interaction and therefore not able to participate in activity-dependent motions. In this scenario, only those AMPARs can move into synapses upon LTP stimulation that are not associated with Shisa6. The absence of Shisa6 in knockout animals would therefore lead to a general increase of AMPAR mobility at the cell-membrane and might thus underlie greater changes upon synaptic stimulation.

This hypothesis gains importance with our findings that the presence of Shisa6 prevents AMPARs from entering a desensitized state, preventing synaptic depression upon high-frequency stimulation (see chapter 2). The synaptic role of Shisa6 might therefore be rather in ensuring basic AMPARs transmission regardless of the general state of the synapse than to mediate synaptic changes. In this regard, investigation of long-term depression in the presence and absence of Shisa6 becomes a necessity. In both mentioned scenarios, absence of Shisa6 decorated AMPARs will most likely result in increased numbers of surface AMPARs which are decorated with competing auxiliary subunit, including TARPs. Therefore, the observed phenotype of enhanced LTP in the absence of Shisa6 might be due to the LTP effects of the auxiliary subunits that replace Shisa6 in its absence.

In general, the findings that AMPAR auxiliary proteins are involved in the process of long term potentiation is not new [75, 112]. In particular their ability to interact with PSD proteins has been regarded as a key process in anchoring AMPARs to the postsynaptic membrane during synaptic plasticity [24].
Characterization of Shisa6

In the hippocampus, the predominant TARP subunit γ-8 has been shown to directly influence the formation of LTP. Studies using γ-8 knockout mice showed reduced LTP in CA1 pyramidal synaptic currents [75, 112]. Interestingly, even though the involvement of PSD-95 in the process of LTP formation has been extensively studied, γ-8 seems to influence LTP independently of its PDZ ligand motif, as mice that carried a deletion of this motif showed normal synaptic potentiation [24, 138, 139, 219]. The exact mechanisms of these findings need to be investigated. Whether and how PDZ ligands of Shisa6 play a role in the enhanced synaptic potentiation of Shisa6 is unknown. We hypothesize that the PDZ binding sites, which are occupied with Shisa6 under wildtype conditions, might be occupied by other auxiliary proteins and the overall mobility of AMPARs enhanced. It is to be noted that since the KO of Shisa6 enhances synaptic potentiation, Shisa6 has an opposing effect to potentiation to TARP γ-8 [75, 112, 202]. Interestingly, Shisa9, the only described member of the Shisa- family, showed no effect on LTP in DG granule cells upon stimulation of the medial perforan path and opposing effects on Short-term-plasticity compared to TARP γ-8 [202]. Since a recent study indicated the coexistence of Shisa9 and TARP γ-8 at the same AMPAR complexes [202], it will be interesting to see, whether the same holds true for Shisa6 and how both proteins differentially regulate hippocampal LTP.

Material & Methods

Electrophysiology setup:
For electrophysiological recordings we made use of Clampex 9.0 along with a 700B amplifier and a 1322 digitizer (all from Molecular Devices, Sunnyvale, California, USA). Cells were identified by eye and patched using an Olympus BX50WI DIC microscope with a 40x, 0.8nA W objective (all Olympus cooporation, Tokyo, Japan). Data were acquired using an internal 4 pole Bessel filter (3 kHz) and a sample frequency of 20 kHz. All stimuli were timed using a Master 8 (A.M.P.I. Jerusalem, Israel). For electrical stimulation we used an isoflex (A.M.P.I. Jerusalem, Israel).

Recordings
Electrophysiological recordings were made using borosilicate electrodes (OD 1.5mm, ID 0.86mm; Harvard Apparatus, Holliston, MA, USA) of 3 – 5 MΩ resistance that were filled with intracellular solution containing (in mM): 110 K-Gluconate, 10 KCl, 10 HEPES, 0.4 NaGTP, 4mM Mg2ATP, 10 K- Phosphocreatine and 0.5% biocytin (pH 7.3, 290 osm). Cells were superfused with aCSF containing (in mM): 126 NaCl, 3 KCl, 10 D-glucose, 26 NaHCO3, 1.2 NaH2PO4, 2 CaCl2, 1 MgSO4 , and 0.1 Glycine, carboxygenated with 95% O2 and 5% CO2 to obtain pH7.4 for the duration of the experiments. Stimulation electrodes were pulled using the same glass but with a resistance of 2-3 MΩ and filled with aCSF.
Chapter II

Slices
8-13 week old male mice were decapitated and the brain removed in ice-cold slice solution containing the following salts (in mM): 126 NaCl, 3 KCl, 10 D-glucose, 26 NaHCO3, 1.2 NaH2PO4, 0.5 CaCl2 and 7 MgSO4. A microtome was used to cut horizontal hippocampal brain slices which were cut with a thickness of 300nm in ice-cold slice solution. Immediately after slicing, the acute slices were transferred to a slice chamber with aCSF, which was pre-heated to 37 degrees Celsius and left at room temperature for at least one hour prior to recordings.

LTP induction
Hippocampal CA1 pyramidal cells were identified by eye using light microscopy. The stimulation electrode was placed along the Schaffer-collateral fibers in the CA3/2 region around 150-250 microns away from the recording site. Initially, we recorded a step protocol to assess the spiking properties of the recorded neuron. Next, an input-output protocol was used to ensure medium synaptic stimulation. After this, we recorded at least 8 minutes of baseline EPSPs. LTP-induction took place within 15 minutes after achieving whole-cell configuration. A theta burst protocol was used to induce LTP as described by Buchanan et al [218]. Following LTP-induction cells were recorded for 40 minutes to assess LTP.

Analysis
Data were analysed using custom-written Matlab scripts (Mathworks, Natick, MA, USA). Recordings with input resistance changes above 20 percent throughout the recordings were excluded from analysis. Stability of the baseline recordings was assessed using linear regression analysis. Recordings with baseline stability < 5 mins were removed from analysis. Slope values of the first 2.5 ms of the rising phase of the EPSPs were normalized to the average slope value during the baseline period. Readout frame for plasticity changes was set at 5, 15 and 25 minutes after theta burst stimulation to ensure (semi) long-term synaptic changes. Overall EPSP rise times were calculated as the time required for a 20 – 80% to the peak amplitude, and decay times represent the time required for a 90 -10 % amplitude change. The data are displayed as mean ± SEM. Statistical significance was assessed using a student’s t-test when the data passed a Kolmogorov Smirnoff test for normality. If not, a Mann-Whitney U test was used. Significance is indicated using the following coding: * = p < 0.05.
Supplementary Data

**Suppl. Figure 1. EPSP kinetics remain stable during the formation of LTP**

A-D. Bar graphs represent 20 – 80% rise- and 90 – 10% decay times (mean ± SEM) of EPSPs over time at baseline and 5, 15 and 25 minutes after LTP induction (see methods). Values can be found in table one. E. Bar graph (mean ± SEM) represents the mean input resistance measured from Shisa6 KO and wt animals F. example of changes in input resistance of CA1 pyramidal neuron over the time of an LTP recording. Grey lines indicate 20 % change in input resistance.
Table 1. EPSP kinetics before and after the induction of LTP. Rise times reflect 20 – 80% rise times of EPSPs, decay times the time required for a 90 - 10% amplitude change (see methods).

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<th>p-value</th>
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C-terminal interactors of the AMPA receptor auxiliary subunit Shisa9


* These authors contributed equally

Abstract

Shisa9/CKAMP44 has been identified as auxiliary subunit of the AMPA-type glutamate receptors and was shown to modulate its physiological properties. Shisa9 is a type-I transmembrane protein and contains a C-terminal PDZ domain-binding site that potentially interacts with cytosolic proteins. In this study, we performed a yeast two-hybrid screening that yielded eight PDZ domain-containing interactors of Shisa9, which were independently validated. The identified interactors are known scaffold proteins residing in the neuronal postsynaptic density. To test whether C-terminal scaffolding interactions of Shisa9 affect synaptic AMPA receptor function in hippocampus, we disrupted these interactions using a Shisa9 C-terminal mimetic peptide. In the absence of scaffolding interactions of Shisa9, glutamatergic AMPA receptor-mediated synaptic currents in the lateral perforant path of the mouse hippocampus had a faster decay time, and paired-pulse facilitation was reduced. Furthermore, disruption of the PDZ interactions between Shisa9 and its binding partners affected hippocampal network activity. Taken together, our data identifies novel interaction partners of Shisa9, and shows that the C-terminal interactions of Shisa9 through its PDZ domain interaction motif are important for AMPA receptor synaptic and network functions.
Introduction

The AMPA-type glutamate receptor (AMPAR) is widely expressed in the brain and mediates the majority of fast excitatory neurotransmission. The AMPAR is a transmembrane glutamate-gated ion channel comprised of four pore-forming subunits GluA1–4 [220]. The subunit stoichiometry determines aspects of AMPAR function, including channel conductance, receptor trafficking and subcellular localization [55]. In addition, a group of auxiliary transmembrane proteins regulates membrane expression and function of AMPAR. These include TARPs [28], the Cornichons CNIH-2 and CNIH-3 [190]. Recently, the AMPAR-modulating protein Shisa9, initially named CKAMP44, was added to this list [30]. Shisa9 modulates synaptic short-term plasticity by influencing kinetics and channel properties of AMPAR via direct interaction. Shisa9 is a type-I transmembrane protein, which is localized postsynaptically and predominantly expressed in neurons of the hippocampal dentate gyrus and in the cerebral cortex. Shisa9 belongs to the Shisa protein family [199], which is characterized by the presence of a cystine-knot motif in the extracellular N-terminus and a PDZ type II binding motif Glu-Val-Thr-Val (EVTV) at the distal intracellular C-terminus.

AMPARs are known to anchor at their postsynaptic site in order to align with the presynaptic transmitter release machinery. Anchoring of AMPARs at the postsynaptic density (PSD) occurs mostly through proteins that associate with the intracellular domains of AMPAR subunits. For instance, PDZ domain-containing scaffold proteins interact directly with the AMPAR GluA2 subunit, through binding of GRIP1 [221] and PICK1 [222], and to GluA1 via SAP97 [223]. Alternatively, AMPARs bind indirectly to the postsynaptic scaffold proteins, e.g. PSD95, via direct interaction with TARP that simultaneously binds to AMPARs and PSD95 [27, 111]. Albeit that AMPARs are anchored postsynaptically, they are highly mobile receptors. They undergo constitutive and activity-dependent translocation to, and removal from, synapses, which is determined by guided lateral diffusion [224], and receptor endo-/exocytosis events [45]. These processes also involve AMPAR associated proteins. For instance, TARP is involved in the lateral insertion of new AMPARs at the postsynaptic membrane [177]. Changing the number of AMPARs residing at the postsynaptic membrane underlies synaptic plasticity and the expression of memory [225]; increases in the amount and function of synaptic AMPAR lead to LTP [69, 72] and, conversely, removal of AMPAR from postsynaptic density mediates LTD [95] [226]. It is conceivable that auxiliary subunits transiently interacting with AMPARs are of importance for anchoring the receptor at the postsynaptic site.

In this study, we aimed at identifying cytosolic C-terminal interacting partners of Shisa9, which might be important for anchoring the protein at the postsynaptic membrane, and elucidating the involvement of these interactors in AMPAR synaptic and network functions.
Results
Shisa9 interacts with PDZ domain-containing proteins in a PDZ-ligand motif-dependent manner

To identify cytosolic proteins potentially involved in the interaction with Shisa9, $6.2 \times 10^6$ clones of a mouse brain cDNA library were screened in a yeast two-hybrid system using the Shisa9 cytoplasmic domain (cd) as bait (fig. 1A). Out of the 426 yeast cell transformants that induced cell growth under nutritional selective conditions, 384 were processed for prey-protein identification, by prey plasmid isolation and sequencing (see Methods). Blasting prey library-plasmids against the IPI protein-database (ipi.MOUSE.v3.37) resulted in the identification of 146 cDNA clones (E-value < 0.001), 84 of which featured both a correct reading frame and a lack of internal stop codons. Combined, the collapsed sequences represented 43 different putative Shisa9-cd interactors (table S1), including several proteins that contained the anticipated PDZ domains. For follow-up studies, the postsynaptic scaffold-components PSD93, PSD95, MPP5 and GRIP1 were selected, in addition to synaptic-trafficking elements PICK1, LIN7b and GIPC1 (table 1). DYNLT3, a well-represented, but PDZ domain-lacking protein, was also taken along. The specificity of putative Shisa9 interactors was confirmed with a direct two-hybrid assay using representative clones of each protein and the empty bait vector as control (fig. 1B, table 1). None of the interactors was able to induce cell-growth in the absence of the Shisa9-cd while cultured under high-stringent selective conditions (–Leu, –Trp, –His, –Ade).

Table 1. Putative Shisa9 interactors

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Table 1. Putative Shisa9 interactors (Gene symbol, recommended Uniprot name) selected for validation, as identified by yeast two-hybrid. The “clone count” represents the number of hits in the screen, the “start position” refers to the first amino acid of the protein’s reference sequence (Protein Refseq) conserved within the direct two-hybrid clone, and the “PDZ domains” column lists the number of complete PDZ domains anticipated within that clone.
C-terminal interactions of Shisa9

To establish the involvement of the Shisa9 C-terminal PDZ-ligand motif in protein-protein interaction, we re-tested interaction after deletion of the PDZ interaction motif. Indeed removal of the C-terminal EVTV sequence completely disrupted cell growth for PDZ domain-containing proteins, while in the case of DYLNLT3, cell growth did occur (fig. 1A, B). This confirms that the distal EVTV motif indeed is involved in and essential for the specific interaction of Shisa9 with all identified PDZ domain-containing binding partners.

![Diagram of Shisa9 domains](image)

**Figure 1.** The cytoplasmic side of Shisa9 interacts with multiple PDZ domain-containing proteins in a PDZ-ligand motif dependent manner. A. Schematic representation of Shisa9 and the two Shisa9 cytoplasmic domains (cd) used within the yeast two-hybrid screen and direct two-hybrid assay (SP, signal peptide; TM, transmembrane domain; EVTV, C-terminal PDZ-ligand motif. B. Direct two-hybrid assay performed under stringent nutritional selection (–LTAH). The absence of growth results from the cell's inability to activate the adenine reporter gene. pBD WT and pACT2 WT labels refer to the empty plasmids.

Independent validation of putative Shisa9 interactors by means of co-immunoprecipitation from HEK293T cells

To validate the interactions identified in the yeast two-hybrid system, we overexpressed HA-tagged Shisa9WT or HA-Shisa9ΔEVTV proteins and V5-tagged interactors in HEK293T cells and performed co-immunoprecipitations using anti-HA antibody. We confirmed that Shisa9 interacts with PSD95, PSD93, PICK1, GRIP1 and LIN7b via the PDZ domain present in these interacting partners, since Shisa9ΔEVTV lost possibility to establish interaction with these proteins. MPP5, DYNLT3 and GIPC1 failed to show interaction with Shisa9 in the co-immunoprecipitations (fig. 2). Given that the tested interactors of Shisa9 are highly expressed in PSD, we conclude that PSD95, PSD93, PICK1, GRIP1 and LIN7b might represent true interacting partners of Shisa9.
PSD95 is present in brain-derived Shisa9 complexes

The PSD is a synaptic protein structure that is very densely packed, which makes it difficult to bring into solution. For this reason, proteins that were identified in this study as Shisa9 interactors have not been identified in our previous experiments based on mass spectrometry analysis [30]. Here we used immunoprecipitation of native Shisa9 complexes from brain tissue followed by immunoblotting, which is a more sensitive technique than mass spectrometry for the identification of a pool of endogenously interacting proteins. We performed this experiment on two different brain regions, the hippocampus and the cortex. We were able to demonstrate that Shisa9 binds to PSD95 in hippocampus and in cortex (fig. 3).
A TAT-tagged peptide of the Shisa9 C-terminus disrupts interaction between Shisa9 and PSD95

To establish whether the interaction of Shisa9 and PSD95 has a role in AMPAR function in hippocampus, and to resolve what these interactions of Shisa9 might mean to synaptic function, we aimed at disrupting the interaction in acute hippocampal brain slices. For this, we generated a synthetic peptide that fuses the TAT-sequence (GRKKRRQRRRPQ) to the 19 C-terminal amino acid residues of Shisa9. This mimetic peptide is designed to compete for the C-terminal interaction with the interactor of Shisa9. The TAT sequence carries fused sequences into neuronal cells in vivo [228, 229].

We first tested whether this C-terminal TAT-mimetic peptide of Shisa9 is able to compete for interaction with PSD95. For this, biotinylated Shisa9+EVTV peptide coupled to NeutrAvidin beads, was allowed to interact with recombinant mouse PSD95 protein via interaction of the EVTV motif in the peptide and the PDZ domain in PSD95. Subsequently, a molar excess of TAT-tagged Shisa9 mimetic peptide was added in order to disrupt the interaction between PSD95 and biotinylated Shisa9 peptide. As control, we used a TAT-tagged Shisa9 peptide lacking the EVTV stretch (analogous to the Shisa9ΔEVTV protein used in figs. 1–2) and TAT-scrambled peptide. Samples were subjected to analysis by SDS-PAGE and stained with trichloroethylene (fig. 4). The intensities of PSD95 were quantified by Image Lab software (BioRad) and nor-
ormalized to the amount of PSD95 in the TAT-scrambled lane. The TAT-Shisa9+EVTV peptide disrupted approximately 50% of the interaction between the existing PSD95 and the biotinylated Shisa9 peptide in comparison to the TAT-Shisa9∆EVTV peptide. This indicates that TAT-Shisa9+EVTV peptide is capable of competing for interaction between PSD95 and Shisa9 (fig. 4). From this experiment, we extrapolated that a peptide concentration of 10 µM should be used in hippocampal slice experiments to interfere with Shisa9-PSD95 interactions.

**Figure 4. TAT-Shisa9 C-terminus mimetic peptide disrupts interaction between Shisa9 and recombinant PSD95.** A. The TAT-tagged Shisa9-EVTV C-terminus mimetic peptide, but neither TAT-scrambled nor TAT-Shisa9∆EVTV peptide, competes off the interaction between recombinant PSD95 and the biotinylated Shisa9+EVTV peptide. The 100 kDa molecular weight marker is indicated. B. Quantification of the PSD95 band in the presence of TAT-scrambled, TAT-Shisa9+EVTV or TAT-Shisa9∆EVTV peptide. PSD95 band intensities were normalized to the intensity of PSD95 band in TAT-scrambled peptide lane. All experiments were performed 3 times.
C-terminal interactions of Shisa9

Shisa9-PDZ interactions affect synaptic AMPAR function in hippocampus

In mouse brain, Shisa9 is expressed in the dentate gyrus of the hippocampus [30]. In hippocampal neurons, Shisa9 overexpression prolongs the decay kinetics of AMPAR mediated currents [30]. Given that Shisa9 has PSD interaction partners interacting via PDZ domains, we hypothesized that Shisa9 will exert this function at hippocampal AMPARs when having these protein-protein interactions intact. To test this, we recorded from dentate gyrus granule cells in acute hippocampal slices of the mouse and stimulated glutamatergic projections of the lateral perforant path (LPP; fig. 5A). We interfered with Shisa9-PDZ interactions by applying the TAT-Shisa9 mimetic peptide (TAT-Shisa9+EVTV), and using the modified peptide (TAT-Shisa9ΔEVTV) as control (see methods; fig. 3). Neither the presence of a scrambled peptide, nor untreated wild type slices showed differences to the TAT-Shisa9ΔEVTV control (suppl. fig. 1).

In the presence of the Shisa9-PDZ interfering TAT-Shisa9+EVTV, AMPAR mediated synaptic currents showed faster deactivation kinetics than in the presence of the control peptide (TAT-Shisa9ΔEVTV (n = 23) 6,1 5 ± 0,3 ms; TAT-Shisa9+EVTV (n = 21) 5,01 ± 0,2 ms; p = 0,007, student's t-test). AMPAR current rise times were not different between the two conditions (TAT-Shisa9ΔEVTV (n=23) 1.944±0.94 ms; TAT-Shisa9+EVTV (n = 21) 1,829 ± 0,829 ms; p = 0,377, student's t-test) (fig. 5B-D). These data show that disrupting C-terminal PDZ domain interactions of Shisa9, through which it interacts with PSD proteins, affects synaptic AMPAR current properties in the hippocampus. AMPAR-mediated glutamatergic synaptic currents in Shisa9 knockout animals show slower recovery from desensitization observed by reduced paired-pulse facilitation in dentate gyrus granule cells [30].

To test whether Shisa9-PDZ interactions are involved in recovery from desensitization of synaptic AMPARs, we tested the effect of the TAT-Shisa9 mimetic peptide on paired-pulse facilitation in whole cell recordings from dentate gyrus granule cells stimulated in the lateral PP. Interference with Shisa9-PDZ interactions reduced paired-pulse facilitation (fig. 5E, F; Two-way ANOVA: peptide treatment, F(1, 326) = 36.00, p < 0.0001; stimulation interval, F (9, 326) = 54.41, p < 0.0001; interaction, F(9, 326) = 4,27, p < 0.0001; TAT-Shisa9ΔEVTV n = 19, TAT-Shisa9+EVTV n=23). At the 20, 50 and 100 ms inter-pulse interval, paired-pulse facilitation was significantly (all p < 0.001) reduced by the TAT-Shisa9+EVTV peptide (50 ms: 1.62 ± 0.04 to 1.27 ± 0.02 in the presence of the control peptide). Thus, these data show that interference with Shisa9-PDZ interactions slowed recovery from desensitization. Together, our findings demonstrate that protein interactions at the C-terminus of Shisa9 affect AMPAR kinetics and synaptic facilitation.
Figure 5. Partial disruption of C-terminals Shisa9 interaction affect AMPAR mediated currents in dentate granule cells. A. Diagram showing the recording site and the electrically stimulated fibers of the lateral perforant path (dark blue; adapted from [230]). B. Example traces of AMPAR-mediated EPSCs after incubation with either the TAT-Shisa9+EVTV (active) or the TAT-Shisa9∆EVTV (control) peptide. Traces were aligned to the onset of the current. C, D. Bar graphs (mean ± SEM) summarize the changes in rise and decay kinetics vs. the kinetics of the control pulse. **p < 0.01 (Student’s t-test). E. Representative recordings of a paired-pulse protocol at different stimulation intervals after incubation with either the TAT-Shisa9+EVTV (purple) or the TAT-Shisa9∆EVTV peptide. F. Averages (±SEM) summarizing the differences in paired-pulse ratio facilitation at different inter-event-intervals. ***p < 0.001, *p < 0.05 (Post-hoc testing).
Shisa9-PDZ interactions shape hippocampal network oscillations

Synchronization of hippocampal neuronal activity relies on fast synaptic transmission via AMPARs [231, 232]. Given that Shisa9 interactions affect synaptic AMPAR function in hippocampus, we hypothesized that tuning of AMPAR function by Shisa9-PDZ interactions would affect synchronization of neuronal activity. To test this, we recorded network oscillations induced by the metabotropic glutamate receptor agonist DHPG (10 µM) in acute hippocampal slices (fig. 6A). Interference with Shisa9-PDZ protein interactions by application of the TAT-Shisa9+EVTV peptide altered several parameters of hippocampal network oscillations. The mimetic peptide significantly increased the power spectral amplitude of DHPG-induced hippocampal oscillations both compared to no peptide application (control 0.49 ± 0.07 μV²/Hz, n = 20; TAT-Shisa9+EVTV 1.24 ± 0.2 μV²/Hz, n = 9, p = 0.006, fig. 6C), as well as compared with inactive peptide (TAT-Shisa9ΔEVTV 0.38 ± 0.09 μV²/Hz, n = 11, p = 0.0007). Interference with Shisa9-PDZ interactions by the TAT-Shisa9+EVTV peptide showed no significant effect on the average frequency of oscillations (control 21.3±0.4 Hz vs. TAT-Shisa9+EVTV 19.7 ± 0.8 Hz, p = 0.07, control vs. TAT-Shisa9ΔEVTV 21.5 ± 0.6 Hz, p = 0.87, fig. 6D). Application of the mimetic peptide significantly narrowed the spectral half-width with respect to control conditions (control 6. 0 ± 0.4 Hz, TAT-Shisa9+EVTV 4.3 ± 0.3 Hz, p = 0.01, fig. 6E). There was no effect on the spectral half-width of the inactive peptide when compared to control conditions (5.5 ± 0.5 Hz for TAT-Shisa9ΔEVTV, p = 0.87, fig. 6E). These data show that PDZ protein interactions of Shisa9 that tune synaptic AMPAR function are involved in setting the properties of hippocampal neuronal network activity and synchronization.
Figure 6. Shisa9 increases the synchrony of DHPG-induced hippocampal oscillations via PDZ domain interactions. A. Wavelet display of recorded field potentials of DHPG-induced oscillations under the 3 experimental conditions: Control (no peptide application, top trace), PDZ interacting peptide TAT-Shisa9+EVTV (middle) and inactive form of the peptide TAT-Shisa9ΔEVTV (bottom). Warmer colors indicate higher oscillation amplitude (dimension-less units). B. Comparison of the power spectral density of the DHPG-induced oscillations in the 3 experimental conditions: control (light blue), TAT-Shisa9+EVTV (purple), TAT-Shisa9ΔEVTV (dark blue). C. TAT-Shisa9+EVTV peptide significantly increases the spectral amplitude of DHPG-induced hippocampal oscillations with respect to no peptide application, as well as with respect to the inactive peptide. D. Application of TAT-Shisa9+EVTV peptide has no significant effect on the frequency. E. TAT-Shisa9+EVTV peptide significantly narrows the spectral half-width with respect to control conditions. *p < 0.05 (Student’s t-test).
Discussion

The aim of this study was to identify novel cytosolic Shisa9-interacting proteins, and to establish the relevance of these protein-protein interactions for Shisa9-mediated modulation of glutamatergic synaptic transmission. We have previously reported that Shisa9 is enriched within the postsynaptic density, a protein-packed structure that is notoriously difficult to solubilize while maintaining protein complex integrity, a requirement for immunoprecipitation-based proteomics. The yeast two-hybrid approach to interactor identification circumvents these limitations, while offering improved chance at the discovery of more transient associations.

In this study, we successfully applied the yeast two-hybrid method in the identification of novel putative Shisa9 interacting proteins. Our findings confirm that Shisa9 can associate with several PDZ domain-containing proteins, such as PSD95 and LIN7b, and that this binding is dependent upon Shisa9’s distal PDZ-ligand motif (EVTV). The well-established importance of PDZ domain-containing proteins in glutamatergic synaptic plasticity, ranging from receptor trafficking to receptor immobilization/scaffolding [98], and the clearly defined site of Shisa9 association, led us to focus in the follow-up characterization on these interactors.

In the two-hybrid screening using the Shisa9 intracellular domain, we identified 43 putative binding partners. We selected proteins based on the presence of a PDZ domain (PSD95, PSD93, MPP5, PICK1, GRIP1, LIN7b and GIPC1). These proteins are all well known for their presence in the postsynaptic density [233-236]. This indicated that Shisa9 could potentially interact with PSD scaffold proteins. In addition, we selected DYNLT3, which does not have a PDZ domain. All selected proteins were tested for autoactivation in a direct mating assay, and were confirmed to be Shisa9 interactors (fig. 1). Furthermore, we created the Shisa9ΔEVTV mutant, which lacks the PDZ domain ligand; we confirmed by co-immunoprecipitation from HEK293T cells that Shisa9 interacts with PSD95, PSD93, PICK1, GRIP1 and LIN7b specifically via its PDZ domain, since Shisa9ΔEVTV completely loses interaction with these proteins (fig. 2).

We identified PSD95 in Shisa9 complexes derived from hippocampus and cortex (fig. 3). We visualized PSD95 by means of immunoblotting of immunoprecipitation samples. Immunoblotting is a more sensitive method of protein identification than mass spectrometry and probably explains why PSD95 was not previously found [30]. The fact that the other Shisa9 protein interactors were not found using this method does not exclude them from being binding partners in the PSD in vivo, but rather suggests that these could be regulated in a plasticity-dependent manner.

We addressed the issue of whether protein interactions through the C-terminus of Shisa9 affect synaptic AMPA receptor function. To resolve this issue, we made use of TAT-fusion
peptides, which have been shown to successfully disrupt protein interactions at AMPAR [225, 229, 237]. We interfered with the interaction between Shisa9 and its partners by applying a C-terminal TAT-tagged mimetic Shisa9+EVTV or a control TAT-Shisa9ΔEVTV peptide. We found that C-terminal protein interactions of Shisa9 tune the functional properties of AMPARs. Interfering with the PDZ-interaction between Shisa9 and its binding partners affected basic functional properties of the AMPA receptors: it sped-up de-activation and slowed-down recovery from desensitization (fig. 5). Our data are in agreement with previous findings, in which paired-pulse ratios and current decay times of the AMPA receptor in hippocampal CA1 neurons were affected by the overexpression of Shisa9 in this area [30]. Knocking out Shisa9 in dentate gyrus granule cells resulted in increased paired-pulse facilitation of the lateral perforant path inputs [30]. We found that only interfering with C-terminal protein interactions of Shisa9 in granule cells, leaving Shisa9 itself unaltered, reduced paired-pulse facilitation. Stripping Shisa9 of its C-terminal interactions apparently alters short-term facilitation in opposite direction from removing Shisa9 entirely.

Based on our data that Shisa9 and PSD95 interact, these findings suggest that Shisa9 might be involved in anchoring of the AMPA receptors to the PSD. In our experiments, the introduction of the TAT-Shisa9+EVTV peptide may impair the anchoring of the AMPA receptor at the PSD and therefore might affect diffusion of the AMPA receptors in and around the active zone. Our mimetic peptide approach only allows us to suggest that the Shisa9 protein interaction with the scaffold is of importance to AMPAR function and synaptic plasticity (decreased facilitation). The disrupted interaction will include that of Shisa9 with PSD95, but may include other identified PDZ-containing scaffold proteins, the latter of which cannot be identified easily by immunoprecipitation from brain samples due to the resistance to solubilization of the PSD.

We found that the tuning of functional properties of synaptic AMPAR by Shisa9 and its protein interactions shaped hippocampal neuronal network oscillations. Hippocampal network oscillations are the result of balanced excitatory and inhibitory synaptic transmission [232]. Interference with Shisa9-PDZ interactions increased the power of network oscillations and narrowed the frequency range of oscillations. Possibly, the longer synaptic AMPAR currents with slower decay kinetics that occur when Shisa9-PDZ interactions are intact, allows the hippocampal network to synchronize at a broader range of frequencies, resulting in a wider power spectral density distribution covering more frequencies. Disrupting Shisa9-PDZ interactions would speed up synaptic AMPAR currents and limit the frequency range at which the network synchronizes, and as a result, increases the power at this limited frequency range. Excitatory glutamatergic synaptic inputs received by interneurons, in particular to those that are parvalbumin-positive and cholecystokinin-positive, are important for hippocampal network oscillations [238, 239]. Whether Shisa9 is also expressed by hippocampal interneurons and whether AMPAR kinetics in interneurons is affected by Shisa9 remains to be determined. Regardless, Shisa9 is expressed in dentate gyrus granule cells [30] and we show that synaptic
C-terminal interactions of Shisa9

AMPAR current properties in dentate gyrus granule cells are tuned by Shisa9-PDZ protein interactions. Disruption of these Shisa9-PDZ interactions in dentate gyrus neurons may underlie the effects we observed on hippocampal network activity.

The first auxiliary subunit of the AMPA receptor – stargazin (γ2) – was discovered in the late 90-s [155]. Since then it was shown that stargazin belongs to the family of the transmembrane AMPA receptor regulatory proteins – TARPs [158]. Identification of the TARPs stimulated the discovery of the cohort of AMPAR’s auxiliary subunits – CNIH2 and 3 [190], Shisa9 (CKAMP44; [30], SynDIG1 [240], GSG1L [241]. The list of potential AMPA receptor auxiliary subunits keeps growing [153, 241]. The expanding set of auxiliary subunits raises the question how a large number of structurally unrelated and functionally different proteins regulate the AMPA receptors. In this study, we found that the AMPAR interacting protein Shisa9 binds to well-known PSD proteins, and we established the Shisa9-PSD95 interactions to be present in the brain. In addition we found that affecting the anchoring of Shisa9 via its C-terminal tail in brain slices affects AMPAR function, synaptic plasticity and neuronal network synchronization in the hippocampus. This indicates that Shisa9 not only modulates the biophysical properties of the receptor by direct association but also affects function through controlling its synaptic localization.
Materials and methods

**Yeast two-hybrid screen** – The yeast two-hybrid screen was performed according to the methods reported by Walhout and Vidal [227]. For bait-construction, the PCR-amplified Shisa9 C-terminal domain (amino acids 172 (KLGL) – 424 (EVTV) of NCBI Refseq NP_082553.2) was inserted into the EcoRI–SalI-digested pBD-GAL4 vector (Stratagene). The screen was performed by high-efficiency transformation of a pACT2-contained mouse brain Matchmaker cDNA library (Clontech) into bait construct-positive PJ69-2a yeast cells (displaying no intrinsic reporter activity). Transformed cells were selected for 4 to 6 days on plates supplemented with 3 mM 3-amino-1,2,3-triazole and lacking Leu, Trp and His (–LTH), followed by a secondary selection under Leu, Trp and Ade-depleted conditions (–LTA). Growth-positive transformants were picked on days 4, 7 and 10, and subjected to another 15 days of –LTA selection. For the prey protein identification, yeast colonies were resuspended in 15 µL of Zymolyase solution (4 mg/mL Zymolyase T-100 (Seikagaku corporation), 1.2 M Sorbitol and 0.1 M sodium phosphate buffer pH 7.5), incubated for 1 hour at 37 °C, and heated to 98 °C for 10 min. pACT2 inserts were PCR-amplified from the crude-lysate (forward: 5’-GATGATGAAGATAC CCCACCAAACCC-3’, reverse: 5’-GCACGATGCACAGTTGAAGTGAACTTG-3’), used as template in BigDye™ terminator 3.1 sequence reactions (Applied Biosystems) (primer: 5’-TCTGTATGGCTTACCCATACGATGTTCC-3’), and analyzed on an Applied Biosystems 3730 DNA Analyzer. Sequence files were blasted against the IPI protein database (ipi.MOUSE.v3.37), frame-checked and validated to contain no stop-codons upstream of the prey-protein coding region.

**Direct two-hybrid assay** – The Shisa9-cd (cytoplasmic domain) and ΔEVTV bait-construct was PCR amplified, inserted into the EcoRI–SalI-digested pBD-GAL4 vector, and transformed into the PJ69-2a yeast strain. Selected prey-clones were rescued from yeast using the RPM kit (MP Biochemicals, according to the manufacturer’s instructions), amplified in Escherichia coli (DH5αF), and transformed into the PJ69-2a yeast strain. The identity of each isolated clone was confirmed by sequence analysis, and blasting against the NCBI reference proteins database. Bait and prey transformants were grown under respectively -Trp (-T) and –Leu (-L) selective conditions, diluted to an OD600 of 0.5, mixed according to the direct two-hybrid matrix, and spotted on rich medium YPD plates. The cells were allowed to grow for 48 hours, followed by replica-stamping onto –LT selective medium. After 3 days the plates were analyzed for cell-growth, replica-stamped onto -LTAH plates (high stringency selection) and incubated for 10 days. Cell-growth was recorded at days 4, 7 and 10. The identity of the bait and prey proteins was re-confirmed at the end of the direct two-hybrid assay by insert amplification and sequence analysis (as described in the yeast two-hybrid screen section).
C-terminal interactions of Shisa9

DNA constructs – cDNA fragments encoding mouse full length and ΔEVTV HA-tagged Shisa9 were amplified by PCR from a previously designed plasmid containing HA-tagged Shisa9. The HA-tag was introduced between the signal peptide and the N-terminus. PCR products were subcloned into the pTRCGW-CMV-IRES2-EGFP-Dest vector. The mouse cDNA encoding full length proteins of putative Shisa9 interactors (PSD93, PSD95, MPP5, PICK1, GRIP1, GIPC1, LIN7b, Dynlct3) were amplified by PCR and subcloned into pcDNA3.2V5/Dest vector (Invitrogen) to obtain V5-tagged proteins. All constructs were sequence verified and used for transfection of HEK293T cells.

Transfection of HEK293T cells – HEK293T cells were transfected using PEI 2500. Cells were passed the day before transfection in DMEM media (Gibco), 10% FBS (Invitrogen), 1% Penicillin-Streptomycin (Gibco) in 10 cm dishes. On the day of transfection cells were 60–70% confluent. The medium was refreshed 2 h before transfection; 5 μg DNA was mixed with 250 μL PBS, after which 35 μL PEI 2500 was added to the DNA-PBS mixture. The transfection mixture was gently vortexed, incubated for 10 min at RT and dropwise added to HEK293T cells. After transfection (48 h), cells were harvested in 1 mL of lysis buffer (25 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, EDTA-free protease inhibitor cocktail (Roche)) with 1% DDM (Thermo Scientific) or 1% Triton X-100 (Roche, for PICK1), incubated (45 min, rotating) at 4 °C, spun down at 20800x g for 10 min at 4 °C. The obtained supernatant was used for co-immunoprecipitation.

Co-immunoprecipitation from HEK293T cells – Anti-HA-tag antibody (2 µg; ab9110, Abcam) was added to the obtained HEK293T cells lysates and incubated (overnight, rotating) at 4 °C. Subsequently, protein A/G beads (30 μL; Santa Cruz) were added and samples were incubated (1 h, rotating) at 4 °C and washed 3 times with lysis buffer containing 0.1% TritonX-100. SDS sample buffer (50 μL) containing 10% 2-mercaptoethanol was added to the obtained pellets and boiled for 5 min prior to analysis using SDS-polyacrylamide gel electrophoresis and immunoblotting.

Animals – C57Bl6J mice (Charles River), 7 a.m. lights on/7 p.m. lights off, with water and food ad libitum (immune-precipitations: male and female of > 10 weeks; electrophysiology: males of 2–4 weeks) were handled in accordance to the Dutch law using a protocol approved by the Animal Ethics Committee of the VU University Amsterdam.

Co-immunoprecipitation from mouse hippocampus and cortex – Mouse cortex or hippocampus was homogenized with a potter and piston at 900 rpm on ice, for twelve times up and down motion in 30 mL homogenization buffer (25 mM HEPES/NaOH, pH 7.4, 0.32 M sucrose, 1x Roche protease inhibitor). The extract was centrifuged at 1000x g, 10 min at 4 °C. The supernatant was removed, centrifuged at 10000x g, 2 h to obtain a pellet P2-fraction, which was resuspended in HEPES buffer to 10 μg/μL protein, and mixed with equal volume of lysis buffer with 2% DDM. After incubation (45 min, rotating) at 4 °C, the sample
was centrifuged (20000 \times g 15 \text{ min}) at 4 °C. The pellet was resuspended in lysis buffer with 1% DDM (300 \mu L), incubated for another 45 \text{ min} rotating at 4 °C, and again centrifuged. The obtained supernatants (1425 \mu L, 6 \text{ mg} protein) were pooled, and anti-Shisa9 antibody (12 \mu g, PA5-21058, Thermo Scientific) was added and incubated overnight (rotating at 4 °C). Agarose-protein A/G beads were added and incubated for 1 \text{ h} at 4 °C. After washing 4 times in lysis buffer with 0.1% DDM, proteins were eluted off the beads with 60 \mu L SDS sample buffer, and were loaded (10 \mu L) on Criterion Precast gel (BioRad).

**Western blotting** – Immunoblotting was done overnight at 40 V onto PVDF membrane (BioRad). For immunostaining of co-immunoprecipitation samples from HEK293T cells the following antibodies were used: anti-V5 (Abcam, 1:1000), anti-HA (3F10, Roche, 1:1000) in 5% milk TBST, incubation was done overnight at 4 °C on a shaking platform. For co-immunoprecipitation samples from brain anti-PSD95 antibody (75-028, Neuromab, 1:10000) was used. The secondary antibodies used were goat-anti-mouse-HRP (DAKO, for anti-V5 and anti-PSD95) and goat-anti-rat-AP (Southern Biotech, for anti-HA). The membranes were developed by means of ECF (Amersham) or ECL femto (Thermo Scientific) according to the manufacturer’s instructions.

**Purification of recombinant PSD95** – Mouse His-tagged PSD95 was produced in E. coli BL21AI strain (Invitrogen) transformed with pDEST17-PSD95 plasmid. The expression of PSD95 was induced at OD600 = 0.6–0.8 with 0.2% arabinose (Sigma). Cells were harvested 3 \text{ h} after induction by spinning down at 20000 \text{ g} for 30 \text{ min} at 4 °C. Pellets were resuspended in lysis buffer 25 mM HEPES pH 7.4, 150 mM NaCl with 25 mM Imidazole, frozen in liquid nitrogen and stored at – 80 °C. Upon use, resuspended pellets were thawed (30 °C) and EDTA-free protease inhibitor cocktail (Roche) was added. Cells were cracked by means of One Shot system (Constant Systems Limited) at 1.7 kbar, 3 times, after which lysates were spun down at 20000 \text{ g}, 15 \text{ min} at 4 °C. Supernatant was filtered (0.45 \mu m filter; Millipore) before loading on 1 mL HisTrap column (GE Healthcare) equilibrated with lysis buffer containing 25 mM Imidazole. Purification was performed on AKTA system (GE Healthcare). PSD95 was eluted from the column with a linear gradient of Imidazole up to 500 mM. Fractions were collected, pooled together, frozen in liquid nitrogen, and stored at – 80 °C. PSD95 was concentrated (Amicon ultracentrifuge filter unit 10000 MW cut-off; Millipore) to 1.4 mg/mL, aliquoted, frozen in liquid nitrogen and stored at – 80 °C until needed.

**Peptide competition assay** – Biotin- and TAT-Shisa9+EVTV peptides are identical to last 19 amino acids of the C-terminal part of Shisa9. 0.5 \mu M biotinylated Shisa9+EVTV peptide (biotin-HFPPTQPYFITNSKTEVTV) or Shisa9-∆EVTV peptide (biotin-HFPPTQPYFITNSKT; GenScript Corporation) was incubated with NeutrAvidin beads (100 \mu L; Thermo Scientific) for 10 \text{ min} at RT rotating. Unbound peptide was washed away (3 times) with lysis buffer containing 0.05% Tween-20. Recombinant PSD95 (0.1 \mu M) was added in total volume of 1 mL and incubated for another 10 \text{ min} at RT rotating. The TAT-tagged Shisa9 peptide (10 \mu M; TAT-Shisa9+EVTV
C-terminal interactions of Shisa9

– TAT-HFPPTQPYFITNSKTEVTV; TAT-Shisa9ΔEVTV – TAT-HFPPTQPYFITNSK or TAT-scrambled –
TAT-YPNETKQTIFVSVTPHPFT, GenScript Corporation) was added to the beads-PSD95 mixture
and incubation continued for another 2 h at RT. Unbound PSD95 was washed away with
cold lysis buffer containing 0.1% Triton X-100 (Roche), washing was performed 4 times, and
at the last step beads were transferred to a new tube. To the obtained bead pellet 75 µL
SDS-sample buffer was added and boiled for 5 min prior to SDS-PAGE. Samples were loaded
on Criterion Precast gel (BioRad) and PSD95 band was visualized by means of 2,2,2-Trichlo-
roethanol present in the precast gels.

Electrophysiology – Synaptic plasticity and network oscillations. Acute horizontal hippo-
campal slices, 300 µm or 400 µm thick, were prepared from either 21 to 30 or 12 to 17
days-old C57BL/6 mice to perform either synaptic plasticity or network oscillations experi-
ments respectively. After decapitation, the brain was quickly removed and sliced in ice cold
aCSF containing (in mM): 110 choline chloride, 25 NaHCO3, 11.6 Na-ascorbate, 10 D-glu-
cose, 7 MgCl2, 3.1 Na-pyruvate, 2.5 KCl, 1.25 NaH2PO4, 0,5 CaCl2 for synaptic plasticity
recordings and 126 NaCl, 3 KCl, 10 D-glucose, 26 NaHCO3, 1.2 NaH2PO4, 1 CaCl2 and 3
MgSO4, for oscillations recordings. In both cases, aCSFs were carboxygenated with 95% O2
and 5% CO2 (pH 7.4). Slices were transferred to a bath of carboxygenated modified aCSF
containing (in mM): 2 CaCl2, 1 MgCl2 and 25 Glucose for the synaptic recordings or 2 CaCl2
and 2 MgSO4 for the oscillations recordings. Slices were incubated for at least 1 h prior to
recording with 10 µM of TAT-Shisa9+EVTV, TAT-Shisa9ΔEVTV or TAT-scrambled peptide. Ex-
periments were performed at 31 ± 1 °C. Whole cell recordings of dentate gyrus granule cells
were performed using borosilicate electrodes with a resistance of 3–5 MΩ filled with internal
solution containing (in mM): 120 Cs-gluconate, 10 CsCl, 8 NaCl, 10 HEPES, 10 phospho-
creatine-Na, 0,3 Na3GTP, 2 Mg-ATP, 0,2 EGTA, and 4% Biocytin, pH 7.3. Input resistances
were monitored throughout recordings. Lateral perforant path inputs were stimulated using
electrical stimulation. Local field potentials were measured at the CA3 hippocampal area by
means of multi-electrode arrays consisting of 60 electrodes spaced at 100 µm. Oscillations
were chemically induced by the addition of DHPG (10 µM). Data analysis was performed by
custom-made software developed in Matlab®.

Statistics – Data is presented as average ± SEM. Statistical significance was tested with the
student's t-test (α = 0.05). Correction for multiple comparisons was applied for the oscilla-
tions’ analysis. A two-way ANOVA was performed for the paired-pulse-ratio analysis using
Bonferroni post-hoc testing. Significance is marked with asterisks as ***p < 0.0001, **p <
0.01 and *p < 0.05. All data was normally distributed.

Acknowledgements

Grip1 cDNA is a kind gift of prof. dr. Casper Hoogenraad.
Supplementary material

**Figure S1.** A. Data show the paired pulse facilitation (mean ± SEM) at different inter-event intervals upon lateral perforant path stimulation (TAT-Shisa9ΔEVTV (n = 9), TAT-scrambled peptide (n = 12), TAT-Shisa9WT (n = 10). No significant changes could be observed between the tested groups. B,C. Bar graphs (mean ± SEM) summarize the data on rise- and decay kinetics between all tested groups (TAT-Shisa9ΔEVTV (n = 9), TAT-scrambled (n = 12), TAT-Shisa9WT (n = 10). The tested groups showed no significant differences.
C-terminal interactions of Shisa9

Table S1. Putative Shisa9-cd interactors identified by the yeast two-hybrid screening

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Chapter 5

General Discussion
5. General Discussion

5.1 Introduction

In recent years AMPAR research has sharpened our understanding of the glutamate synapse and AMPAR function in particular. We have gained insights into AMPAR trafficking and membrane insertion, regulatory modifications and how synaptic changes of AMPARs underlie synaptic plasticity (for review see [20, 29, 65]). The probably most important breakthrough, however, came with the discovery of AMPAR auxiliary proteins [26, 165]. These proteins assist AMPARs virtually on every step in their lifespan, ranging from receptor processing in the ER, surface expression, synaptic mobility, enhancing the agonist affinity of AMPARs to tuning their conductance properties (for review see [28]). The influence of these auxiliary proteins on AMPAR function can roughly be divided into two general working mechanisms. First, auxiliary subunits directly influence the biophysical properties of AMPARs, as seen in studies from heterologous cells, in which the co-expression of auxiliary proteins with AMPARs leads to changes in kinetics and pharmacology [30, 190, 192, 194, 246]. These changes are most likely due to e.g., conformational changes of crucial AMPAR domains/structures, such as the ligand binding domain or channel pore [167]. Secondly, auxiliary proteins execute indirect influence on the behaviour of AMPARs by acting as essential adapter proteins, which link AMPARs to numerous secondary interactors without the necessity for these proteins to directly interact with the receptor. Also several of these proteins can do both, i.e., affect receptor function directly and indirectly. The most studied adapter function is the interaction of the prototypical TARP Stargazin with PSD-95, the predominant synaptic scaffolding complex [22]. Until the discovery of Stargazin, several studies had linked PSD-95 closely to AMPAR function, however without establishing a direct interaction between the two proteins [23, 24, 139]. Not only did the discovery of Stargazin provide a missing link between these two proteins, it also provided insights into the regulatory mechanism underlying the bidirectional influence of AMPAR and PSD-95 [75, 111, 175].

Needless to say, the discovery of auxiliary proteins has boosted our understanding of AMPARs at synapses and therefore glutamate synaptic transmission. The purely qualitative descriptions of AMPAR movements and behaviour at synapses could be linked to structural components and existing models of synaptic plasticity were refined. For instance, the number of AMPARs present at synapses has been shown to correlate with the strength of a particular synapse; the discovery of Stargazin and its activity-dependent interaction with PSD structures, provided a mechanism underlying these phenomena [14, 16, 22, 75].

Despite the great advancements in AMPAR research over the past decade, we are only at the start of understanding AMPARs transmission in all its detail. Following the description of the first AMPAR auxiliary proteins numerous studies set out to discover novel members of
native AMPAR complexes and ultimately more AMPAR interacting proteins [153]. As a result of these efforts, the list of proteins known to interact with AMPARs is growing [153]. Initially, several proteins have been described as AMPAR auxiliary proteins, even though their precise role in affecting AMPAR function is elusive. To put a stop to this, Yan and Tomita, published a paper that listed exclusive criteria that every potential auxiliary protein of ligand-gated ion-channels needs to fulfill in order to be labeled as one [213]. Following these, a candidate protein needs to be non-pore forming, in direct and stable interaction with a pore-forming subunit, modulate the properties of decorated channels and necessary for proper ion-channel function in-vivo. Today, in the field of AMPAR research three protein families are widely accepted to match these criteria in mice: Transmembrane-AMPAR-regulatory proteins (TARPs), Cornichons 2 and 3 (CNIH) and Shisa9 (currently the only described member of the Shisa-family [28]).

In this thesis, I present the first experimental evidence that Shisa6 is a bona fide AMPAR auxiliary protein. Being closely related to Shisa9 this protein associates with AMPARs to regulate kinetics and trafficking behaviour. Furthermore, we were able to show that the absence of Shisa6 in knockout animals results in aberrant expression of synaptic plasticity. Even though we were able to cover numerous aspects of Shisa6 ranging from molecular to physiological level, many important questions in regard to function, behavior and regulation remain unanswered.

5.2 Shisa6 vs. Shisa9

With Shisa6 being the second Shisa-family member being characterized as AMPAR auxiliary protein, a functional comparison between Shisa6 and Shisa9, the other described member, is inevitable. On first sight, Shisa9 and Shisa6 are structurally related proteins which both associate with AMPARs at synaptic sites. However, on closer look the notion of two functionally distinct proteins becomes clearer.

Shisa6 and Shisa9 display only partially overlapping expression patterns ([30], chapter 2). Shisa9 is predominantly present in the dentate gyrus whereas Shisa6 is most abundant throughout the hippocampal formation and in the purkinje cell layer of the cerebellum. Moreover, experimental evidence strengthens the view that Shisa6 and Shisa9 serve different functions: in response to prolonged glutamate applications, Shisa6 slows the desensitization time whereas Shisa9 quickens it [30]. In addition, Shisa6 seems to prevent AMPARs from entering a desensitized state, where for Shisa9 there is no such indication. Also the presence of Shisa9 does not affect the formation of LTP, whereas we were able to show a reduction in LTP in the presence of Shisa6 ([202], chapter3). Similarly, Shisa6 changes only the kinetics of mEPSCs, Shisa9 affects solely the frequency and amplitude of synaptic transmission. Furthermore, Shisa9 slows the recovery time from desensitization, whereas Shisa6 does not affect it ([30],
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Although these data may imply two functionally rather unrelated proteins, the data of both studies are hard (if not impossible) to compare. For instance, when assessing AMPAR-currents in response to prolonged glutamate applications, we have been working with whole-cell recordings from HEK293 cells that co-expressed the flip splice-variant of AMPAR subunits with Shisa6. In contrast, von Engelhardt et al. first used CA1 pyramidal cells and dentate gyrus granule cells of Shisa9 overexpression and knockout animals in outside-out configuration for their study [30]. Though they later used overexpression in the dentate gyrus to confirm their hippocampal findings, the difference in the outline of both studies still stands [202]. Despite the difference in cell type and patch configuration, even the AMPAR subunits found in both experimental setups were different. Classically, the flip splice-variant of AMPARs is known to be exchanged for the flop variant around birth [247]. Interestingly, only the flip variant displays a steady-state current in response to prolonged glutamate application [5, 247, 248]. Though a more recent study presents evidence for both splice variants to be present in adult mice, somatic AMPARs in adult animals seem to predominantly contain flop variants. [247, 249].

These qualitative differences seem to pervade throughout both studies making a real comparison of the two proteins hard to do. More comparable studies using the same paradigms and recording conditions are needed to reach a final conclusion on the functional differences and similarities of Shisa6 and Shisa9.
5.3 More about Shisas?

With now two members of the Shisa- family being characterized as AMPAR auxiliary protein, the next step will be to identify more family members that could serve a similar function. An overview picture of the known Shisa- family members and their phylogenetic relationship is presented in figure 1. In- situ hybridizations of the Allan brain institute already indicate the expression of multiple Shisa- family members in the brain, among which Shisa5 and Shisa7 [279]. Although, Shisa5 seems to be expressed throughout the brain at moderately high levels, it is only distantly related to Shisa9 and Shisa6 and lacks potentially vital domains such as the PDZ- ligand motif or the alternatively spliced exon 4 and might therefore not be the most straightforward target of investigation. However, even the TARP- family is structurally divided into Type I and Type II TARPs based on the their C-terminal sequence and potency to rescue surface expression through loss of Stargazin, with both subgroups interacting with - and modifying AMPARs ([158, 182, 183, 188]). Thus albeit their diversity in sequence, structurally diverse TARPs show a functional relationship. Therefore, it cannot be ruled out that the Shisa5, despite its structural diversity from Shisa9 and Shisa6, still serves as an AMPAR auxiliary subunit.

**Figure 1. Shisa family proteins and homology.** A. Proteins of the Shisa- family share a number of common structural motifs and domains including a signal peptide (SP) and a cysteine-rich motif on the N-terminus (yellow), a transmembrane domain (TM, green), as well as a potentially spliced exon (light blue) and a PDZ ligand motif (red) on their C-terminus (blue). B. Phylogenetic tree of Shisa proteins by MOLPHY. Protein names are shown as Shisa subfamily names followed by species name abbreviations (Bf, Branchiostoma floridae; Dr, Danio rerio; Gg, Gallus gallus; Hs, Homo sapiens; Om, Oncorhynchus mykiss; Oa, Ornithorhynchus anatinus; Xt, Xenopus tropicalis; and Xl, Xenopus laevis.) With permission of the publisher [199].
Shisa7 on the other hand is highly homologous to Shisa9 and Shisa6, shares a similar domain structure and is expressed throughout the hippocampus and the cerebral cortex making it an attractive target for investigation. Preliminary studies confirm that Shisa7 is indeed able to bind to multiple AMPAR subunits and suggest its involvement in synaptic transmission and long-term plasticity (Klaassen and Ruiperez-Alonso, unpublished).

Given the partial overlapping expression patterns of Shisa6 and other Shisa- proteins (e.g. Shisa7), the question of functional redundancies among Shisa- family members is inevitable. It has been demonstrated that certain TARP- family members are capable of rescuing stargazer mice, indicating a functional overlap among TARP- family members [158]. It will be interesting to see whether similar results can be obtained in the Shisa- family. This question could be particular interesting for knockout studies and the explanation of observed phenotypes. The generation of double knockout animals will help clarify these matters.

5.4 AMPAR complexes – it is getting complex

With the growing list of AMPAR auxiliary proteins and associated interacting proteins, two major questions need to be addressed. First, how can the AMPARs host such a variety of proteins and how does the decoration of the AMPAR with multiple proteins translate into the functional properties of decorated receptors. In a model addressing the variety of proteins associated with an AMPAR, Schwenk divided the receptor complex into an inner and an outer core (see Figure 2) [153]. The inner core is constituted by the four AMPAR subunits, associated with major auxiliary subunits (such as TARPs and Cornichons), which are proposed to bind the AMPAR outside of the membrane plane at four binding sites. Here, Cornichons and TARP proteins seem to compete for two of these bindings sites, with two bindings sites being constitutively occupied by type I TARPs [153, 250]. The outer- core interactors bind to the receptor outside of the inner-core binding sites and outside the membrane plane. Whereas TARPs are regarded as constitutive interactors, the presence of all other auxiliary subunits is arbitrary.

This model takes into account the main physiological findings from the field of AMPAR research. For instance, in this model TARP and CNIH proteins compete for the same AMPAR binding site (figure 2, red circle). This is in line with findings in which CNIH competitively reduce TARP stoichiometry but still preferentially bind to AMPAR in complex with TARPs, specifically TARP γ-8 [153]. Secondly, the constitutive decoration of AMPARs with TARPs seems likely, as surface trafficking of AMPARs is abolished in the absence of TARPs [27, 112, 157, 251].
Figure 2. Model for the assembly of native AMPARs as proposed by Schwenk et al. [153]. According to this model, AMPAR complexes can be divided into an inner- and outer core. The inner core interaction sites are depicted as solid circles in red and grey and are predominantly occupied by auxiliary subunits of the TARP and CN1H families. TARP family proteins are constitutively part of the receptor complex. The outer core interaction sites are depicted in red open circles and may host Shisa9 (CKAMP44). Secondary proteins interactors may become part of the receptor complex through binding to inner or outer core proteins. Figure taken from Schwenk et al., Neuron 2012, with permission of the publisher.

However, even though this model gives way for the decoration of AMPARs with multiple auxiliary subunits, it does not reveal the stoichiometry of these proteins, and does not render any explanation of how the co-decoration influences AMPAR properties. In general, the idea how the co-decoration of AMPARs with auxiliary subunits affects AMPAR properties is not well developed. Best understood is the co-decoration of CA1 pyramidal cell-AMPARs with TARP γ-8 and CN1H2 [192, 193]. Unlike prior believes, the surface trafficking of these AMPAR seems to be solely dependent on the presence of the TARP subunit. However, the slowing of deactivation kinetics, which is also feature of TARP associated AMPARs, is enhanced when Cornichon is also present at these complexes [192]. Another example of co-decorated AMPARs and involving Shisa9 and TARP γ-8 has been published recently [202]. The functional changes induced by the presence of either subunit seem less clear. Where TARP γ-8 is necessary for long-term potentiation, Shisa9 is not. On the other hand, Shisa9 and TARPs seem to have opposite effects on desensitization and short-term plasticity, with Shisa9 increasing and γ-8 decreasing it. However, co-expression in HEK cells showed that GluA1 fused to TARP constructs decreased desensitization properties when Shisa9 was co-expressed indicating the coexistence of both proteins at the same receptor complex, a finding that was later confirmed in hippocampal tissue [202]. Additional data is needed on how the specific effects of each protein orchestrate on / tune a single receptor.
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In general, the question which auxiliary subunits coexist at the same AMPA receptor complex and how they mutually influence and tune AMPAR responses, deserves more attention. Co-expression studies in heterologous cells have proven to render valuable insights into this matter [193, 252]. However, since auxiliary proteins are likely to be influenced by members of their respective interactomes, expression studies in neuronal cells are needed. Also the search for binding sites and crystallization studies could possibly provide valuable insights into the assembly structure of AMPAR tetramers and auxiliary subunit binding, which will help form a more realistic model of AMPAR complexes.

5.5 Why another addition?

With this thesis, the list of AMPAR auxiliary proteins has a new member, Shisa6. With many of these auxiliary proteins serving similar functions, e.g. with respect to enhanced surface trafficking or the increase in deactivation time, and in regard to Darwin’s evolution theory, it seems appropriate to ask why multiple proteins in the brain would serve such similar functions and even coexist at the same receptor complex. The answer is: multi-level tuning. With glutamate being the principal excitatory neurotransmitter and AMPARs the most abundant glutamate receptor in the central nervous system, the necessity for close regulation of these receptors comes into play.

AMPA receptor auxiliary proteins have provided valuable insights into AMPAR physiology and amplified our understanding of AMPAR responses. Over the past decade they have been strongly associated with the regulation of key AMPAR features/behaviours including ER- forward trafficking (TARP, CNIH), surface trafficking (TARPs), subunit composition (CNIH, TARPs), kinetics (TARP, CNIH, Shisa), agonist affinity (TARP, CNIH) and surface diffusion (Shisa) (for review see [28], chapter 2). With every auxiliary protein providing a unique feature of tuning to the receptor complex and the likelihood of multiple of such proteins co-decorating the same AMPAR complex, these proteins most likely account for the huge variety of AMPARs, depending of the tissue, cell type and potentially even activation patterns.

In this regard the close investigation of native AMPAR complexes needs more attention. Novel imaging techniques and blue native gel electrophoresis of AMPAR complexes have proven to be useful tools in order to solve important aspects of native AMPAR complexes like co-decorations and stoichiometry [153, 253]. On this basis, physiology experiments on native AMPAR complexes along with heterologous expression studies of AMPARs that are decorated with multiple auxiliary subunits in native stoichiometry, will render more insights into the physiological mechanisms that tune AMPAR responses in various brain areas. Ultimately, the generation of a computer-based model that integrates all available AMPAR data might eventually allow the prediction of AMPAR composition based on physiological phenotypes.
5.6 What are the remaining mysteries?

Although we were able to show that the presence of Shisa6 at an AMPAR complex alters its conductance properties, e.g. deactivation and desensitization kinetics, the mechanism underlying this phenomenon remains elusive. Upon initial description of Shisa9 it was proposed that a cysteine-rich motif might form into an elaborate tertiary structure through formation of numerous cysteine-bridges, conserved in all Group I Shisa proteins. These were suggested to resemble structural properties of conotoxins [254]. However, the conotoxins have very different cysteine spacing from the Shisa proteins. In line with this, these toxins have been found to directly interfere with different proteins, i.e., the pores of Sodium and Calcium channels. In order to gain clarifying insights into the association between Shisa and AMPAR proteins and provide answers to the question how Shisa6 influences the biophysical properties of AMPARs, structural data, such as crystallizations of AMPARs’ channels in complex with Shisa6 are needed.

In addition, our understanding of how Shisa6 is present at AMPAR complexes is still very basic. We do not know much about the ratio of Shisa6-decorated receptors to total AMPARs nor about the stoichiometry of Shisa6 at AMPAR complexes. The latter has been shown to be of great functional impact for the effect size of such auxiliary subunit on the decorated receptors, as seen in TARPs which seem to be present at AMPARs in numbers of two or four at decorated AMPAR receptors. Interestingly, these numbers seem to vary between cell type and tissue [180, 252, 253].

Additionally, we lack any knowledge on the possible coexistence of Shisa6 with other AMPAR auxiliary proteins. Since Shisa6 overlaps in expression pattern with known proteins such as Cornichons or TARP γ-8, the co-decoration of the same AMPAR seems possible [112, 158, 190]. Interestingly, for TARP γ-8 and CNIH2 such co-decoration has been proven. Here the surface trafficking seems to be relying on the TARP subunits, whereas both proteins mutually modify the conductance properties [191, 192, 198]. Recently, a similar co-decoration has been described for Shisa9 and TARP γ-8 in the dentate gyrus [202]. Following this aspect, immunoprecipitations revealed that Shisa6 might indeed be present at the same complexes as TARP γ-8 or SynDIG-4, but not with other Shisa-family members nor CNIH (Pandya, Klaassen unpublished). The functional meaning of such co-decoration will need to be addressed in order to gain a better understanding of native AMPAR complexes and their regulation/tuning. Co-expression experiments in heterologous cells with Shisa6 cross-linked to certain AMPAR subunits, might provide crucial insights into the stoichiometry of Shisa6 at AMPARs and can prove to be helpful in assessing effects by additional auxiliary subunits. In this regard, it will also be interesting to see, whether Shisa6 shows a subunit preference for any of the AMPAR subunits. Similar results were obtained for CNIH and certain TARPs and might influence our view on the functional role of Shisa6 in activity-dependent processes [198, 255, 256].
Another important aspect of AMPAR auxiliary proteins is their role in regulating AMPARs through posttranslational modifications. In this process, the auxiliary proteins might either interact with proteins that modify themselves, the AMPAR or both. A well described mechanism in this regard is the phosphorylation of AMPARs and Stargazin during synaptic plasticity [75, 175]. Interestingly, immunoprecipitations of Shisa6 yielded a list of numerous protein interactors, which have been directly linked to the regulation of synaptic plasticity, among which kinases and several MAGUKs and Ubiquitin ligases (Klaassen, unpublished; [138, 175, 257, 258]). An involvement of either of these proteins in the regulation of AMPARs function or localization mediated by Shisa6 could be thinkable. A first step in establishing such regulatory interaction would be the identification of potential posttranslational modification sites in the sequence of Shisa6 along with the identification of such modifications in vitro and vivo. Since the aspects mentioned above have not been part of our initial description, their answers remain elusive. However, addressing them will ultimately lead to a better in depth understanding of the working mechanism of Shisa6 and refine our view on AMPAR-mediated glutamate synaptic transmission.

5.7 How does Shisa6 work?

We identified Shisa6 as a bona fide AMPAR auxiliary protein. It is a non-pore forming protein that associates with and modifies native AMPARs. We were able to show that the presence of Shisa6 at AMPARs in heterologous cells results in slowed deactivation and desensitization properties in response to brief and prolonged glutamate application respectively. Furthermore, the level of desensitization in response to prolonged application of glutamate is lower in the presence of Shisa6. Interestingly, these reduced levels of desensitization are reflected in the depression properties of synapses in response to high-frequency stimulation where synapses show enhanced levels of depression in the absence of Shisa6. Moreover, our study indicates that Shisa6 decorated AMPARs are immobilized at synapses through interactions between Shisa6 and the PSD. Given the fact that AMPARs and PSD proteins are two key components in the formation and maintenance of synaptic plasticity and Shisa6 is acting as a mediator between these two players, we hypothesized that the absence of Shisa6 would lead to alterations in synaptic plasticity. In accordance with this hypothesis, we were able to show that the absence of Shisa6 leads to enhanced levels of LTP upon synaptic stimulation. These findings might be explained by an increase in the mobility of synaptic AMPARs in the absence of Shisa6 and the simultaneous increase in AMPARs decorated with competing auxiliary subunits, such as TARPs, Cornichons and possibly other Shisa- family members.
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Taking these major aspects of Shisa6 physiology into account, we propose a simple working model for Shisa6 at glutamate synapses (figure 3). In this model, Shisa6’ key role is to enhance the resolution of glutamate synapses by ensuring that AMPARs in the active zone do not desensitize upon high frequency stimulation of the presynapse. Synapses that incorporate Shisa6 are thus able to translate trains of presynaptic activity into postsynaptic signals and will therefore greatly facilitate signal transduction at synapses.

Figure 3. Synaptic working model of Shisa6. A. In response to trains of presynaptic activity, AMPARs which are decorated with Shisa6 do not desensitize or desensitize to a lower extend then Shisa6 lacking receptors (chapter 2). Note that the postsynaptic response is composed of all AMPARs present at the synapse. B. Potential movement paths of AMPARs at synapses in the presence and absence of Shisa6. The synaptic movement of AMPARs is restricted in the presence of Shisa6 through interactions with the PSD (as shown in chapter 2) C. Shisa6 traps AMPARs at synaptic sites which show reduced level of desensitization and therefore follow the activity patterns of the presynapse, regardless of the general state of the synapse.

Taking these major aspects of Shisa6 physiology into account, we propose a simple working model for Shisa6 at glutamate synapses (figure 3). In this model, Shisa6’ key role is to enhance the resolution of glutamate synapses by ensuring that AMPARs in the active zone do not desensitize upon high frequency stimulation of the presynapse. Synapses that incorporate Shisa6 are thus able to translate trains of presynaptic activity into postsynaptic signals and will therefore greatly facilitate signal transduction at synapses.
General Discussion

Why would Shisa6 be important?

Going by the old Hebbian rule of synaptic plasticity ‘fire together – wire together’, synaptic strength is determined by the activity of a particular synapse [259]. A synaptic connection that shows activity that leads to postsynaptic activity will be strengthened, a connection whose activity does not result in postsynaptic activity might be weakened and can even get lost.

To understand and evaluate the importance of our findings of Shisa- and AMPA receptor auxiliary proteins in general, it is necessary to gain a more complete picture of synaptic function in response to presynaptic neurotransmitter release. In this thesis, we chose to focus on the feed-forward signalling that takes place at glutamatergic synapses and includes the activity of AMPARs [14, 65]. We have seen that these receptors are regulated in response to presynaptic activity through protein interactions, which cannot be disentangled from their respective association with auxiliary proteins [28, 250]. As described on the interplay with NMDARs, the activity and regulation of AMPARs influences the probability of NMDAR activity, which in turn leads to further regulatory processes [14, 65, 260, 261]. These include the synthesis of novel AMPAR as well as the resizing of synaptic boutons among others. However, this feed-forward response is only one part of the synaptic tuning process. Not only that each of the subsequent postsynaptic components may be individually regulated and tuned, but also the synapse is no “one way street”. As glutamate is released upon presynaptic activity, postsynaptic activity will propagate back to the presynaptic terminal through retrograde signalling mechanisms, such as endocannabinoids or NO, which for example may alter its release probabilities on occasions of subsequent neuronal activity [262-266]. Since the amount of released glutamate is directly linked to the postsynaptic response, this feed-back inhibition must be considered when thinking about synaptic plasticity and tuning. Likewise, an action potential that is trigger at the soma will back-propagate into the dendrites and thus provide information about the neuronal activity to the synapses [267].

Apart from the “one-way” view of synaptic activity, we have implied that the activity of one synapse alone can determine whether a signal is propagated from one cell to another in form of an action potential. However, information transfer between two cells does not solely happen on one synapse alone, but rather a plurality of individual synapses that show activity within a specific time window [268]. How much an individual synapse contributes to the propagation of a spike is therefore dependent on many different factors, for instance the general state of the synapse (based on its recent history), the general state of the neuronal network, the position of the synapse on the dendritic tree and the local environment of the synapse itself [269, 270]. Not to mention the occurrence of inhibitory connections, which decrease the propagation of a postsynaptic response. Information transfer between two cells is therefore a multi-level process. Rather than a single telephone connection, a neuron functions like an integrator of many different signals and can, based on the activity patterns on its
synapses, convey a plurality of information to its neighboring cells [268, 271, 272]. However, this information is based on the recent activity of both connected cells and heavily influenced by the first postsynaptic entities that response to presynaptic glutamate signal: AMPARs.

The work in this thesis has shown that Shisa proteins tune AMPAR responses on multiple levels. They affect AMPAR properties through direct interaction, resulting in altered synaptic function, including changing the desensitization properties of synapses (Shisa6, Shisa9) and the deactivation time constant of synaptic transmission (Shisa6). Furthermore, Shisa proteins play crucial roles in positioning AMPARs at synaptic sites, as seen through direct observation (Shisa6, Chapter 2) and by competing for PDZ- domain mediated interactions (Shisa9, Chapter 4).

Considering the central role of AMPARs for excitatory transmission at synapses, the vast impact of its auxiliary proteins on synaptic transmission is no big surprise. Changes in the tuning of AMPARs affect the actions of subsequent receptors and molecules in the signalling cascade, which shape the postsynaptic response to presynaptic activity. With regard to the neuron functioning as a signal integrator, each change in synaptic signal will ultimately alter the signal integration process and therefore tune the neuronal output. In this regard, paired-recordings or behavioural studies will provide more insights into the importance of Shisa proteins in signal transfer between cells. Since the same signal from a synapse in close vicinity to the soma will show heavier impact on a neuronal signal than that of a more distant synapse, it will be interesting to investigate the distribution of Shisa-synapses on a dendritic tree.

5.8 More to come?!

In the past decade, since the concept of AMPAR auxiliary proteins arose, our knowledge and understanding of this receptor has leaped. With the discovery of Stargazin and the subsequent findings of its family members to play similar roles in modulating AMPAR responses, novel AMPAR interactors have emerged. To date, only three families have been accepted as auxiliary proteins, but more candidates are likely to be added. Proteins GSG1 and SynDIG1 have already been described as promising candidates. GSG1 is a relative of tight-junction proteins that was shown to increase the surface expression and influences AMPAR kinetics in heterologous systems [153, 273]. The findings about SynDIG1s effect on AMPAR trafficking and surface expression, however, are more diverse and might be most prominent in developing synapses [244, 274, 275]. Further studies of both proteins are needed to clarify the exact roles of both proteins in native tissue to reach a final verdict about their classification as AMPAR auxiliary subunits.
5.9 Outlook

AMPARs are among the most important and most abundant receptors in the central nervous system [4, 5]. Dysfunctions in glutamate-mediated synaptic transmission and particularly in the AMPAR system have been associated with several neurological and psychiatric disorders such as mental retardation, Alzheimer’s disease or amyotrophic lateral sclerosis (among others) [276]. Resulting from this association, AMPARs have been used as drug targets for neurological treatment [276-278].

With the recent discovery of AMPAR auxiliary proteins and their vast impact on our understanding of AMPAR transmission, it becomes apparent, that our knowledge of glutamate transmission is lacking behind our ambition to interfere with this system. Since AMPARs readily form functional receptors when expressed in heterologous cells, people were tempted to believe that AMPARs are more or less solitary proteins at the cell membrane that might be regulated through different subunit composition and transient protein interactions. Today we know that this is not the case. With the discovery of Stargazin and its severe phenotype, people began to understand that AMPARs, like other ion-channels, are dependent on the help of other proteins in order to fulfil their elaborate function. Today we know that virtually no AMPAR exists on the neuronal surface without the assistance of its auxiliary proteins regardless of neuronal cell type. However, our list of primary AMPAR auxiliary subunits is far from being complete. Three main families are being described here in this thesis, but it is likely that multiple additions will be made in the near future.

With the work presented in this thesis, another addition to the growing list of AMPAR auxiliary proteins has been made. Shisa6 shows overlapping functions with existing auxiliary subunits and might even decorate partially overlapping receptor populations. However, every step in understanding this dominant transmission system in the brain will bring us one step further towards explaining synaptic function and ultimately the brain itself. More in depth research will be needed to clarify and disentangle the distinct function of auxiliary proteins on the one hand and all proteins that are part of AMPAR complexes on the other. Only if we reach a deep understanding of AMPAR complexes and all proteins involved, well-founded concepts of synaptic plasticity and memory formation or even the development of specific drugs for several types of diseases will come into reach.
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Summary

Nerve cell communicate with each other via anatomical connections called synapses. At the presynapse, the “sender” neuron translates an electrical signal into a chemical signal by releasing neurotransmitter into the synaptic cleft. On the postsynaptic site, the “receiver” neuron recognizes the presence of these neurotransmitters via receptor proteins. These proteins transform the chemical back into an electrical signal through the opening of ion-channels. The principle neurotransmitter in the vertebrate brain is called glutamate.

Upon glutamate binding, AMPARs rapidly open their channel structures and mediate the initial postsynaptic depolarization. This activity is then followed by the opening of slower ion-channels, the influx of second messengers and ultimately protein trafficking and synthesis, thus all processes that adapt the synapse in response to activity. Playing such a crucial part in triggering the postsynaptic response, AMPARs have been subject of intense research for the past two decades. It was found that the number of synaptic AMPARs is directly linked to its synaptic strength and that AMPARs of different subunit composition can be found at synapses depending on their particular state. Furthermore, many studies successfully linked AMPARs to interacting proteins that affected the tune-ability of synapses. Moreover, it has been shown that AMPARs immobilize at synapses through interactions with the postsynaptic density (PSD) upon induction of synaptic potentiation. In contrast, AMPARs are removed from the PSD upon the induction of synaptic depression. However, how AMPARs are exactly linked to the PSD remained unknown. This “missing link” was first found with the discovery of AMPAR auxiliary proteins. Rather than being a solitary protein, AMPARs seem to form close interactions with this small group of trans-membrane proteins, which show to affect the behaviour of AMPARs on many different levels, ranging from affecting their synaptic anchoring to their affinity for glutamate.

One such auxiliary protein is Shisa9, the first characterized member of the Shisa-family. Shisa9 is known to be a transmembrane protein, which associates directly with AMPARs and modifies its function. However, though its role as auxiliary subunit was widely accepted, the working mechanisms of its action remained unclear. With Shisa9 incorporating a PDZ-ligand motif, we hypothesized that, like other auxiliary proteins, it may function as a “linker” protein between AMPARs, the structure of the postsynaptic density (PSD) and its associated proteins. This work is subject of Chapter 4 of this thesis. First, we confirm an interaction between Shisa9 and proteins of the PSD, most importantly PSD-95. Next, utilizing a novel peptide-competition-assay to disrupt the interaction between Shisa9 and PSD-95, we surprisingly found that changes in kinetics as well synaptic depression properties are modified through this interaction. Furthermore, we even observed changes in hippocampal brain oscillations when disrupting the interaction between Shisa9 and PSD – 95 underlining the importance of this interaction. These findings corroborate a dual role for Shisa9 in the modification
of decorated of AMPARs: the initially described direct modification of AMPAR properties due to an interaction between the two proteins and a more indirect effect through the linkage of AMPARs to the PSD and its associated proteins. What proteins exactly account for the changes in AMPAR properties remains elusive and needs to be investigated.

After the role of Shisa9 as an AMPAR auxiliary protein has been established, other members of the Shisa- family shifted into focus. Using a proteomic approach, our lab identified Shisa6 as a potential candidate protein to serve a similar function as Shisa9. It shows a high level of structural homology with Shisa9 and interacts with native AMPAR complexes in the hippocampus. These initial findings lead to the hypothesis, that Shisa6 might act as auxiliary protein for AMPARs itself.

In Chapter 2, we generate insights into the working mechanisms of Shisa6 by using a multi-disciplinary approach. First, we characterize the protein on a molecular level. Here we found that Shisa6 interacts with multiple AMPAR subunits in vitro and vivo through direct interactions. Furthermore, we were able to show that this interaction takes place at synaptic locations. Confirming its role as an auxiliary subunit, we next showed that the presence of Shisa6 at AMPARs alone is sufficient to change the conductance properties of the decorated channels using co-expression experiments in heterologous cells. Next, we tried to translate our findings into a more native scheme. Introducing novel Shisa6 KO animals, we showed that basic synaptic transmission and synaptic desensitization properties change in the absence of Shisa6. Strikingly, quantum-dot AMPAR tracking experiments revealed restricted AMPAR movement at synaptic sites in the presence of Shisa6. In short: Chapter 2 adds Shisa6 to the list of AMPAR auxiliary proteins.

With regard to our findings in Chapter 2, we next hypothesized that Shisa6 might affect the formation of long-term potentiation, which is subject of Chapter 3. Here, we utilize a theta-burst stimulation protocol to induce long-term potentiation at hippocampal CA1 neurons. Unlike our hypothesis, we found that the absence of Shisa6 enhances synaptic long-term potentiation. This effect might be triggered through the absence of Shisa6 “treating” of AMPARs to the active zones of synapses. With changes in the numbers of AMPARs underlying changes in synaptic state, the immobilization of receptors is likely to influence the overall status of the synapses. The absence of Shisa6 may therefore lead to a higher mobility of AMPARs and the compensation with competing auxiliary proteins. However, further studies will be needed to understand the mechanism behind these findings.

In Chapter 5 of my thesis, I discuss the current state of knowledge on Shisa proteins and put it into a wider perspective. With the Shisa- family members being a relatively young field of research, many questions are yet to be addressed. With Shisa6 and Shisa9 being two relatively novel AMPAR auxiliary subunits, the working mechanisms of these proteins are not yet fully understood. Furthermore, more research is needed to evaluate the impact and im-
portance of Shisa family proteins. With the initial studies focusing on one auxiliary subunit at the time, it is essential to progress towards AMPAR complexes, that have been shown to incorporate multiple auxiliary subunits to tune AMPAR function. Only if we achieve a good understanding of AMPAR complexes and their behaviour at synapses, we will eventually be able to understand the processes that take place to mediate and tune synaptic activity. With information transfer between neuron relying not on singular synapses, but rather the temporal occurrence of synaptic activity over the whole of the dendritic tree, this work marks a small, but essential step in the process of “decoding” the brain.
Nederlandse samenvatting

Zenuwcellen communiceren met elkaar via anatomische verbindingen die synapsen worden genoemd. Hier wordt een elektrisch signaal in het zendende neuron vertaald in een chemisch signaal door het vrijgeven van neurotransmitters in de synaptische spleet. In de hersenen van gewervelden is glutamaat een van de meest voorkomende neurotransmitters. Het ontvangende neuron herkent de aanwezigheid van deze neurotransmitters via receptoreiwitten, die tevens als ion kanaal fungeren. In het geval van glutamaat is dit de AMPA receptor (AMPARs). Bij binding van glutamaat openen de AMPARs snel hun kanaal structuren en staan zo een inwaardse stroom van positief geladen ionië toe, die zorgen voor een depolarisatie in het ontvangende, “post-synaptische” neuron. Op deze manier transformeren deze receptoren dus het chemische signaal weer in een elektrisch signaal.

Deze initiële activatie van AMPARs wordt gevolgd door de opening van tragere ion kanaalen, de instroom van tweede boodschappers en uiteindelijk eiwittransport en synthese. De cruciale rol van AMPARs in de synaptische overdracht van informatie heeft er toe geleid dat AMPARs over de afgelopen decennia onderwerp van intensief onderzoek zijn geweest. Zo is ontdekt dat het aantal synaptische AMPARs direct gekoppeld is aan de synaptische sterkte en dat AMPARs met verschillende subunit samenstellingen zijn te vinden op synapsen, afhankelijk van hun specifieke toestand. Bovendien hebben meerdere studies aangetoond dat AMPARs zijn gekoppeld aan interacterende eiwitten die het aanpassingsvermogen vermogen van synapsen beïnvloeden. Verder is aangetoond dat AMPARs worden geïmmobiliseerd in synapsen door interacties met de postsynaptische dichtheid (PSD) na inductie van synaptische potentiatie. Daarentegen worden AMPARs juist uit de PSD verwijderd bij de inductie van synaptische depressie. Hoe AMPARs aan het PSD zijn gekoppeld is vooralsnog echter grotendeels onduidelijk. Deze “missing link” werd voor het eerst gevonden met de ontdekking van de zg. AMPAR ‘hulpeiwitten’. In plaats van een eenzame eiwitten te zijn blijken AMPARs namelijk nauwe interacties te hebben met een kleine groep trans-membraaneiwitten, die het gedrag van AMPARs op vele verschillende niveaus kunnen beïnvloeden, waaronder hun verankering in de synaps en hun affiniteit voor glutamaat.

Een dergelijk hulpeiwit is Shisa9, het eerste gekarakteriseerde lid van de Shisa-familie. Van Shisa9 is bekend dat het een transmembraan eiwit is dat direct koppelt aan AMPARs en hun functie en/of gedrag verandert. Hoewel haar rol als AMPAR hulpeiwit algemeen werd aanvaard, waren de werkingsmechanismen hiervan nog onduidelijk. Shisa9 beschikt over een PDZ-ligand motief en dit leidde ons tot de hypothese dat, net als andere ondersteunende eiwitten, dit eiwit kan functioneren als een soort brug-eiwit tussen AMPARs, de PSD en bijbehorende eiwitten. Ons werk over dit onderwerp is gepresenteerd in hoofdstuk 4 van dit proefschrift. In de eerste plaats bevestigen wij een interactie tussen Shisa9 en eiwitten van de PSD, met name aan PSD-95. Gebruikmakend van de zg. ‘peptide- concurrentie assay’ om de interactie tussen Shisa9 en PSD-95 verstoren, kwamen wij er verrassenderwijs achter
dat de kinetiek en synaptische depressie eigenschappen worden gecomprimeerd door deze interactie. Het belang van deze interactie wordt onderstreept door de observatie dat ook het verstoren van de interactie tussen Shisa9 en PSD – 95 leidt tot veranderingen in de hersenen oscillaties van de hippocampus. Deze bevindingen bevestigen een tweeledige rol van Shisa9 bij de modificatie van AMPARs: een directe modificatie van AMPAR eigenschappen en een indirect effect via de koppeling van AMPARs de PSD en de bijbehorende eiwitten. Precies welke eiwitten verantwoordelijk zijn voor de veranderingen in AMPARs eigenschappen is vooralsnog niet duidelijk.

Na het vaststellen van de rol van Shisa9 als AMPAR hulpeiwit, richtten wij ons op andere leden van de Shisa-familie. Met behulp van een proteomics benadering heeft ons lab Shiisa6 geïdentificeerd als kandidaat-eiwit voor een soortgelijke functie als Shisa9. Shiisa6 heeft namelijk een hoge structurele homologie met Shisa9 en het toont interactie met natuurlijke AMPAR complexen in de hippocampus. Deze eerste bevindingen leidden tot de hypothese dat Shiisa6 kunnen fungeren als hulpeiwit voor AMPARs zelf.


In navolging van onze bevindingen in hoofdstuk 2 kwamen wij tot de hypothese dat Shiisa6 van invloed zou kunnen zijn op lange termijn potentiatie van synapsen. Dit onderzoek vormt het onderwerp van hoofdstuk 3. Hier maken wij gebruik van een theta-burst stimulatie-protocol om een lange termijn-versterking van synapsen te induceren in CA1 neuronen van de hippocampus. In tegenstelling tot onze verwachtingen vonden wij dat de afwezigheid van Shiisa6 synaptische lange termijn potentiatie verbetert. Dit effect kan wellicht komen doordat er in de afwezigheid van Shiisa6 geen AMPARs in de actieve zones van synapsen kunnen worden ‘gevangen’. De afwezigheid van Shiisa6 leidt wellicht tot een grotere mobiliteit van AMPARs en de compensatie met concurrerende hulpeiwitten. Verdere studies zijn echter nodig om het mechanisme achter deze bevindingen beter te begrijpen.
In hoofdstuk 5 van mijn proefschrift bespreek ik de huidige kennis over Shisa eiwitten binnen een breder perspectief. Het onderzoek naar de Shisa-familie staat nog in de kinderschoenen en veel vragen moeten nog worden aangepakt. Shisa6 en Shisa9 zijn twee recent ontdekte AMPAR hulpeiwitten waarvan de werkingsmechanismen nog niet volledig zijn begrepen, en meer onderzoek is daarom nodig om hun impact en belang volledig op waarde te kunnen schatten. Tot nog toe hebben de eerste studies zich voornamelijk steeds op een enkel AMPAR hulpeiwit per keer gericht, maar om vooruitgang te boeken is het essentieel om ons in de toekomst ook te richten op de totale AMPAR complexen, waarin meerdere ondersteunende hulpeiwitten samenwerken om AMPAR functie af te stemmen. Alleen met een goed begrip van AMPAR complexen en hun rol in de synaps zullen we in staat zijn om de processen te begrijpen die plaatsvinden bij het afstemmen van synaptische activiteit.
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Here I am writing the final lines of what will become my thesis. Tonight everything has to go to the printer, so I better find the right words to express myself. However, writing this part turns out to be a challenge. It is a bit like writing a love letter: you put your entire soul into it and in the end you are bound to read the words like a perfect stranger. I know that now. I have needed many attempts for this version, and I think I will take this one, not because it expresses exactly what I feel, but rather because I run out of time to give it another try.

I am grateful for many things that happened to me in the past five years, I am struggling to write a list of names that I want to thank for what happened to me. I am blessed with a number of great friends, geographically split to multiple locations and I am really happy that I can say that some of them derive from my Amsterdam-days. However, it was my complete-Amsterdam experience that makes me not wanting to miss it and cannot be reduced to a bunch of people that I call my friends. However, I feel that I officially want to thank those, who made this all possible.

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About the Author

Jasper Stroeder was born and raised in Hamburg. He received his Diploma in Biology from the University of Hohenheim in 2009, with plant physiology as major and genetics and biochemistry as minor subjects. He completed his diploma thesis “The role of subtilisin-like proteases in the defense reaction of solanum lycopersicum” in the lab of Prof. Dr. A. Schaller in 2009. Jasper made his first steps in the field of neuroscience in the lab of Prof. Dr. J.M. Ramirez in Seattle (Seattle Childrens Research Institute, 2009) before starting his PhD in the lab of Prof. Dr. H. D. Mansvelder in the beginning of 2010 at the CNCR in Amsterdam where he got in touch with his second promoter Prof. Dr. A.B. Smit. Jasper became a Marie-Curie fellow (FP-7) in November 2010, associated with the European CEREBNET project. Unfortunately, he did not have time to think about the cerebellum. Yet.