Characterization of the Shisa family of potential AMPA receptor modulatory proteins

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Introduction

AMPA receptors are ligand gated ionotropic receptors that mediate the majority of fast excitatory neurotransmission in the brain. AMPA receptors regulate the flow of sodium, potassium, and calcium ions across the cell membrane in response to glutamate. Until recently, the AMPA receptors were thought to largely regulated by posttranslational modifications and via specific interactions with intracellular proteins. This view on AMPA receptors has changed with the discovery of several AMPAR-associated proteins. The first discovered was the auxiliary subunit – TARP γ2 (174), which is critical for the proper function of the AMPA receptors in cerebellar granule neurons (175-177). The discovery of TARP has inspired the search for other auxiliary subunits. Using sensitive proteomics analyses, a number of new transmembrane proteins have been identified that may also serve as AMPAR's auxiliary subunits (195, 197, 199, 229) (fig. 1). The best-studied AMPAR auxiliary subunits are TARPs, Cornichons and the recently added Shisa9 (initially named CKAMP44) (fig. 1). Shisa9 is a member of the Shisa family, which is the focus of the study presented in this thesis.

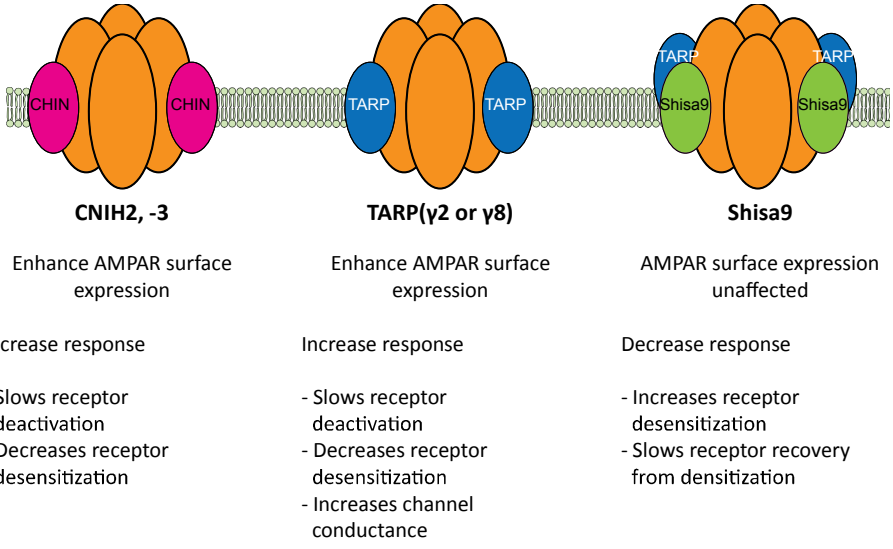

Figure 1. Three families of AMPA receptor auxiliary subunits. A schematic represents Cornichons, TARPs and Shisa9 proteins and summarizes effects of these proteins on AMPA receptor gating properties. Shisa9 and TARP γ2 are possibly constituents of the same AMPA receptor complexes. In the figure, the stoichiometry of interaction is not implied. Figure adapted from (308) and reprinted with permission from AAAS.

The Shisa family of the AMPA receptor auxiliary subunits

The founding member of the Shisa family, *Xenopus* Shisa1, functions in head formation as an inhibitor of Wnt and fibroblast growth factor (FGF) signalling (309). Recently, a brain-specific Shisa protein, Shisa9 was shown to play important role in the modulation of synaptic short-term plasticity by influencing the kinetics and channel properties of the AMPAR in the mouse brain. Shisa9 increases receptor desensitisation, slows down deactivation and recovery from desensitisation, and reduces steady-state current (195).

Shisa6 and -7, two other members of the Shisa family, were also shown specifically expressed in the mouse brain. Shisa6 is an established AMPA receptor auxiliary subunit too (Klaassen *et al.*, 2014, submitted). Opposite to the Shisa9 effect, Shisa6 decreases receptor desensitization and increases steady-state current. Both Shisa9 and Shisa6 prolong deactivation time. Whereas Shisa9 slows down recovery from desensitisation, Shisa6 has no effect on it (Klaassen *et al.*, 2014, submitted). It is plausible that Shisa7 plays a role in modulation of kinetics of AMPA receptors as well, but this needs further study.

Shisa6, -7 and -9 interact with scaffold proteins of the PSD and may play a role in AMPA receptor anchoring

Members of the Shisa family are single transmembrane proteins that contain cysteine-rich domain (cystine-knot) in the extracellular N-terminus and cytoplasmic C-terminus that ends in a PDZ type II ligand motif (196). The interaction between Shisa9 and AMPARs occurs extracellularly (unpublished data), leaving the cytoplasmic domain of 252 amino acids available for interaction with cytoplasmic proteins. It is conceivable that the C-terminal interacting proteins of the Shisa proteins affect function of the AMPAR, for instance by governing the localization of the receptor in the postsynaptic membrane. As a first step towards experiments in that direction a profound insight into the interacting proteins of the Shisas is required.

To decipher the cytoplasmic interactome of brain-specific Shisa proteins we chose the yeast two-hybrid (YTH) system. This method is designed for large-scale screening of direct, often weak and transient interactions (267). Using the YTH approach, we identified 43 binding partners of Shisa9 and more than 200 candidate interactors of Shisa6 and -7. The differences for proteins identified in the screening might be due to two reasons. Shisa6 and -7 have larger cytoplasmic domains, than Shisa9, potentially allowing a larger set of proteins to interact with. The second reason is that a smaller amount of colonies were picked during Shisa9

YTH screen, what possibly led to a smaller number of potential interactors identified.

Among the identified Shisa9 interactors, there are PDZ domain-containing proteins, localized at the postsynaptic density (PSD). PSD95 and PSD93 are scaffold proteins that belong to the MAGUK family (157). These proteins function in the synaptic retention of the AMPA receptor (156). For example, PSD95 directly interacts with TARP γ2 protein, this interaction has a dominant role in the synaptic recruitment of AMPARs (148). Based on our data that Shisa9 and PSD95 interact (chapter 2), we suggest that Shisa9 might also be involved in the immobilization of the AMPA receptors to the PSD. In our study we showed that the Shisa9-PDZ interactions are exclusively dependent on the ligand motif (EVTV) present at the C-terminus of Shisa9. We introduced in our electrophysiological experiments the TAT-Shisa9+EVTV peptide that through competition may impair the anchoring of the AMPA receptor at the PSD and therefore might affect diffusion of the AMPA receptors in and around the PSD. Upon addition of the mimetic peptide we observed changes in the basic functional properties of the AMPA receptors: receptor deactivation kinetics sped-up and recovery from desensitization slowed-down. Thus, trapping the AMPAR at the PSD, or not, affects AMPAR function. Thus, Shisa9 has a dualistic role in modulating the function of the AMPAR, first in directly affecting the receptors biophysical properties, and second, via determining the AMPAR' spatial localization. Our mimetic peptide approach only allowed us to suggest that the Shisa9 protein interaction with the scaffold is of importance to AMPAR function and synaptic plasticity (decreased facilitation). These experiments were validated by actual measurements of AMPAR mobility. The interaction disrupted by the mimetic peptide will include that of Shisa9 with PSD95, but might include other PDZ-containing scaffold proteins as well.

Shisa6 was also shown to be a direct partner of PSD95 and that binding is also dependent on the C-terminal EVTV domain. Moreover, single nanoparticle (QD) tracking experiment showed that Shisa6 decreases AMPAR mobility through its PDZ ligand binding consensus sequence (Klaassen *et al.,* submitted). Altogether, our data suggest that the Shisa proteins are likely involved in the immobilization of the AMPA receptors at the cell surface of the PSD.

Shisa6, -7 and -9 interact with proteins involved in the AMPA receptor trafficking and endocytosis

GRIP1 and PICK1 proteins, identified in the YTH screening of Shisa6, -7 and -9, are key players in the process AMPA receptor trafficking (chapters 3 and 4). The function of GRIP1 is to anchor AMPA receptors at synaptic and intracellular

locations via the interaction with the GluA2 subunit (243-245). In contrast to GRIP1, PICK1 is involved in long-term depression (251, 252) by stimulating AMPAR internalization (253, 254). Both GRIP1 and PICK1 interact with Shisa7 and -9 via the PDZ domain, indicating that only one of the two can bind to a molecule of Shisa protein at the same time. The Shisa6ΔEVTV mutant did not lose the interaction with GRIP1 and PICK1, suggesting an alternative mode of binding to Shisa6. The exact molecular mechanism by which Shisa6, -7, and -9 trap AMPA receptors at cell surface is unknown. GRIP1 and PICK1 might compete for the interaction with Shisa6, -7, -9. This will depend on their cellular expression profile. In particular, Shisa9 is specifically expressed in the dentate gyrus of the hippocampus. One model is that when the interaction between Shisa6, -7, or -9 and GRIP1 is favoured, they might play a role in anchoring AMPA receptors at the cell surface. Alternatively, the Shisa's bind PSD95 and may anchor the AMPAR via this PDZ protein interaction. Also, local regulatory mechanisms, such as specific posttranslational modifications, might be determining factors in this.

GIPC1, another protein identified in the YTH screening, is a cargo adapter, which interacts with myosin VI (255-257), involved in the regulation of the trafficking of the postsynaptic AMPA receptors (258-260). The direct interaction between Shisa6 and GIPC1 points towards involvement in the process of AMPA receptor trafficking, which is ultimately bound to regulate synaptic activity, since the number of AMPARs available in the synapse sets the strength of synaptic transmission.

Shisa6, -7 might serve as mediators in the process of AMPA receptor ubiquitination

The ubiquitination of membrane proteins serves as a signal for their internalization (281). The faith of the ubiquitinated protein is dependent on what kind of ubiquitin chain is added. It is thought that monoubiquitination serves as a signal for internalization whereas polyubiquitination targets uniquitinated proteins for the degradation by the proteasome or lysosome (283, 310).

The ubiquitination of the AMPA receptors is currently under intense study. It was shown that AMPA receptors, and in particular the GluA1 subunit, undergoes activity-dependent ubiquitination, which is followed by receptor internalization and degradation by either the proteasome (279) or the lysosome (280). Yet, the detailed mechanism remains unknown. Alternatively, ubiquitinated AMPA receptors may be deubiquitinated and recycled back to the plasma membrane. In HEK293T cells expressed GluA1 subunits are ubiquitinated by endogenous E3 ubiquitin ligase NEDD4. In neuronal cultures the ubiquitination of GluA1 subunit

occurs only upon stimulation of the AMPA receptor by its agonist, otherwise the ubiquitination is absent (280). Therefore it is suggested that in neurons there must be a mechanism of specific recruitment of NEDD4 to the GluA1 subunit after stimulation of the AMPAR. It is proposed that an unknown intermediate protein with capability to bind both GluA1 and NEDD4 is involved in the mediation of activity-dependent ubiquitination of the AMPA receptor. We identified NEDD4 In the YTH screen as interactor of the cytoplasmic domain of Shisa6 and -7 proteins and confirmed the interaction between the two in HEK293T cells, and in the cerebellum of the mouse brain. We showed that the PPsY motif within the C-terminus of Shisa7 is a ligand motif for binding of NEDD4. Thus, we propose the Shisa6 and -7 proteins might form an intermediate scaffold for GluA1 ubiquitination (chapter 5). However, specific experiments that are directed towards the necessity of Shisas for GluA1 ubiquitination to occur needs to be confirmed.

To determine the mechanism of activity-dependent AMPA receptor ubiquitination, several additional experiments will be required. It is important to establish the involvement of Shisa6, -7 in this process in a neuronal context. Overexpression or Shisa6, -7 knockout in neurons should have an effect on the intensity of the ubuquitination of the AMPA receptor. Moreover, we showed that Shisa6, -7 might be the substrates of ubiquitination themselves. Upon addition of inhibitors of proteasome and deubiquitinases, we observed the increase in molecular weight of Shisa6, -7 in HEK293T cells. These observations need further validation. Based on the preliminary data of chapter 5, the working model is as follows. Under basal conditions Shisa6 and -7 are residing at the cell surface in the PSD and anchor AMPARs, thereby decreasing their mobility. Upon AMPAR activation, the receptor undergoes a conformational change. Shisa6, -7 then recruit NEDD4 and serve as scaffolding platform for NEDD4 interaction, with GluA1, likely to facilitate ubiquitination. Then, Shisa6-7 become ubiquitinated by an unknown E3 ligase, possibly NEDD4, and dissociates from the AMPA receptor complex. The ubiquitinated AMPA receptor becomes less stable at the surface and internalizes.

Future perspective

With the discovery of AMPA receptor auxiliary subunits, it became evident that regulation of the AMPA receptor is a complex process. It is important to determine the stoichiometry of association and the spatial and temporal molecular composition of AMPA receptor complexes in order to better define the function of diverse auxiliary subunits and the potential synergy of their function. Moreover, the definition of molecular complexes of auxiliary subunits themselves is as important. Given the example of the Shisa proteins it is clear that different Shisa-

AMPAR-cytosolic-interactor complexes must exist. Shisa proteins have only one PDZ domain ligand motif and potentially numerous PDZ domain-containing interactors to bind to, indicating that association between the Shisa proteins and their partners has to be strictly regulated and that this may possibly vary per synapse, synaptic compartment and/or moment in time, depending on the neuronal activity. New technological advancements, e.g., in microscopy and mass spectrometry, will aid in the further dissection of the intricate role of the AMPAR auxiliary proteins.