Discussion

Changes in synaptic strength are thought to be the basis for learning and memory. In glutamatergic excitatory synapses the number of AMPAR determines the strength. The protein family of PSD-MAGUKs has long been implicated in targeting glutamate receptors to the synapse. This study provides new insight and clarification into the function of PSD-MAGUK proteins in the synaptic trafficking and targeting of glutamate receptors. It also compares the two most widely used loss of function strategies – RNAi and gene targeting by homologous recombination. Their strength and short comings will be discussed below.

The three PSD-MAGUKs - PSD-95, PSD-93 and SAP102 - we studied play the central role in targeting AMPARs to the excitatory synapse in the hippocampus. In adult brain PSD-95 and PSD-93 work jointly to target AMPARs to the synapse. Other targeting mechanisms may play a minor role. In immature synapses this role is taken by SAP102. Interestingly, knock-out animals show that PSD-95, PSD-93 and SAP102 can partially compensate for loss of the other isoforms. Acute knock-down of protein expression by RNAi eliminates the systems ability to compensate and allows a direct look at each isoforms function. Nevertheless, RNAi has two significant disadvantages. First, it is almost impossible to exclude minimal left over expression of the targeted protein. Second, extensive experiments are necessary to prove targeting efficiency and specificity as dramatically shown for SAP97 in this study.

Previous findings with overexpression of PSD-MAGUKs

Previous studies have used overexpression of the PSD-MAGUKs to shed light on their function. Our group has reported strong enhancement of AMPA mediated synaptic transmission when PSD-95, PSD-93 or SAP102 are overexpressed. The same studies show that overexpression of SAP97 did not have an effect on synaptic AMPAR targeting. In contrast, others showed that overexpression of SAP97 had a modest effect on AMPAR EPSCs in a splice variant specific manner. The effective splice variant was also used in the study conducted in our own group, however. In a recent study, SAP97 isoforms were overexpressed in neurons lacking PSD-95. Under these conditions SAP97 can deliver AMPAR to the synapse (42). As SAP97 overexpression does not have any effect in wild type neurons (72) it is difficult to draw final conclusions. Recently SAP97 has also been implicated in the coordination of post- to presynaptic adhesion (73), the true role of SAP97 remains uncertain.

Overexpression of PSD-95 not only leads to enhanced AMPA EPSCs (72) but also blocks long term potentiation (LTP) and enhances long term depression (LTD) (90). This effect is normally explained by the slot hypothesis. The assumption is made that a given synapse has a set number of slots that can be filled by AMPARs. A synapse undergoing LTP will fill more of these slots. LTD will decrease the number of slots occupied. If PSD-95 is overexpressed, AMPARs are moved to the synapse without stimulation and thus a higher number of receptors is present at the synapse. Since all slots are occupied,

further stimulation normally resulting in LTP has no effect. In contrast, stimulation leading to LTD will remove a larger amount of AMPARs from the synapse. This manifests in enhanced LTD. It is unclear however if PSD-MAGUKs control the slot number under physiological conditions or if the observed results are gain of function artifacts brought about by the massive increase in PSD-MAGUK protein available. The tools developed in this study should shed light on the role of PSD-MAGUKs in synaptic plasticity.

Specificity of RNAi and role of SAP97

The method of RNA interference has been broadly applied for a few years. Adapting this technology for neuroscience has been slow mainly due to technical problems regarding RNAi delivery. Recent development of viral based delivery systems allows for shRNA based studies in neurons. I developed effective and specific shRNA constructs against three of the four PSD-MAGUKs – PSD-95, PSD-93 and SAP102. I carried out a series of experiments to prove target specificity. The first experiments were carried out in heterologous expression system. After the initial screen provided the most promising targeting sequences for each of the four PSD-MAGUKs, I compared these targeting sequences to a non-specific control sequence. The selected targeting sequences all appeared effective in comparison to this control sequence. One other concern is an indirect effect of the targeting sequence on protein expression. I proved the direct mechanism on the targeted mRNA by introducing translationally silent pointmutations into each cDNA. These pointmutations were positioned at the

annealing site of the shRNA. RNAi is highly sequence specific and mismatch of as little as one base pair can prevent knock-down. I was able to rescue each knock-down in this manner. The last test in the heterologous system was to prove target specificity. None of the targeting sequences interfere with the other three PSD-MAGUKs. Isoform specificity was proven with this experiment. A lot of studies will settle for this approach and assume that indeed shRNAs are performing in primary cells with similar efficiency as in the heterologous system.

But is this assumption always correct? To ensure knock-down of the native mRNA, I infected neuronal cultures with lentiviruses carrying shRNAs. I showed that shRNAs targeting PSD-95, PSD-93 and SAP102 perform knock-down specifically and efficiently in neurons. However, the selected shRNA SAP97 construct - effective in heterologous cells - had no effect on SAP97 expression in neurons. I used multiple antibodies to detect SAP97 – with the same negative results. I also generated more targeting constructs against SAP97, among them one published construct (91). There was no significant knock-down of SAP97 in neurons with any of these constructs. Another group recently published a study on SAP97s role in AMPA targeting (42) and was also unable to reproduce previously published results on knock-down of SAP97 (personal communication). When SAP97 expression is diminished, a modest reduction of AMPAR trafficking is observed (91). It is noteworthy however, that the same authors published a study shortly later that excluded SAP97 form the AMPAR protein complex (92, 93). It is possible that SAP97 mRNA is not susceptible to degradation through the RNAi machinery.

Identification of novel binding partners

The data presented in this study gives indication of common and also distinct functions of PSD-95 and PSD-93. I attempted to identify common as well as specific binding partners of PSD-95 and PSD-93 using preparative immunoprecipitation from hippocampal homogenates from wild type, PSD-95, PSD-93 knock-out or PSD-95/PSD-93 double knock-out animals. This might provide a mechanistic explanation for the subpopulations of synapses observed upon acute loss of PSD-95 or PSD-93. However, no specific binding partners for either protein were identified. The interesting question for the basis for loss of AMPAR from mostly non overlapping synapse populations remains unanswered. One could envision binding partners that connect to other complexes on the presynaptic side that determine the PSD-MAGUK used at the postsynapse. One other unanswered question is the potential preferential binding to TARPs. TARPs were recently identified as auxiliary subunits of AMPARs (94). They bridge between AMPARs and PSD-MAGUKs (74). *In vitro* studies were unable to identify preferences for the interaction of the PSD-MAGUK isoforms with TARP isoforms (87). This might not reflect the in vivo situation.

shRNA mediated knock-down of PSD-MAGUKs

Transfection of shRNA PSD-95 and PSD-93 but not shRNA SAP102 into mature cultures of hippocampal neurons reduces surface GluR2 receptors.

Infection of mature hippocampal slice cultures with shRNA for PSD-95 or PSD-93 resulted in a similar ~50% reduction in AMPAR EPSCs. One interesting difference is the loss of AMPARs from mostly non-overlapping subsets of synapses. This is not reflected in dissociated hippocampal cultures. PSD-95 and PSD-93 staining in these cultures overlap almost completely. An explanation for this difference between dissociated and more native slice cultures has not been established. It might reflect a higher order of organization intact in the tissue. An electron microscopy immunogold labeling study revealed on about 30% overlap of PSD-95 and PSD-93 at hippocampal synapses (95). Thus knock-down of PSD-95 or PSD-93 increased the number of synapses carrying a normal amount of NMDAR but lacking AMPAR, called silent synapses for the absence of AMPAR mediated transmission.

Overexpression of SAP102 in slice culture leads to enhancement of AMPA EPSCs. Knock-down of SAP102 in mature synapses had no effect on surface GluR2 in dissociated neurons or AMPA EPSCs in slice cultures. The role of SAP102 in AMPAR trafficking will be expanded upon below.

Studies in gene targeted deletions of PSD-MAGUKs

Mice with targeted deletions of PSD-95, PSD-93 or SAP97 have been generated. None of these mice show deficits in basic synaptic transmission. The first PSD-95 mutant mouse generated has some deficits in synaptic plasticity. Conclusions from this mouse are complicated by the fact that the mutation yields truncated PSD-95 containing the first two PDZ domains (59).

This truncated form targets properly to synapses (72). We analyzed a newly generated mutant, which completely lacks PSD-95 (89). No effect on AMPAR expression levels or synaptic transmission was detected. Similarly, analysis of the PSD-93 knock-out generated (4) shows normal basic excitatory transmission and expression of AMPARs.

The presented findings with acute shRNA targeting PSD-95 and PSD-93 strongly indicate compensation for loss of one of the PSD-MAGUKs by others. I generated the PSD-95/PSD-93 double knock-out mice to test this hypothesis. Unlike the single knock-outs, which are indistinguishable from their littermates, PSD-95/PSD-93 double knock-out fall behind in size and weight at around two to three weeks after birth. When weaned at three weeks, they require special attention to survive and are hypokinetic. By four to five weeks they are about half the body size compared to their littermates.

The results in dissociated and slice cultures from hippocampi prove that PSD-95 and PSD-93 are most important for proper targeting of AMPARs at mature excitatory synapses. Loss of both of these isoforms results in loss of about half of AMPARs from PSD enriched, synaptic fractions and redistribution to extra synaptic sites as concluded from solubility assays and Western blot analysis. Accordingly, AMPAR mediated synaptic transmission is reduced by 55% with no effect on NMDARs. Normalcy of NMDAR mediated current with loss of mEPSC frequency but not amplitude indicates an increase in silent synapses in double knock-out animals. However, individual knock-down of either isoform resulted in roughly the same reduction in AMPAR EPSCs. I found that

total as well as synaptic SAP102 is significantly upregulated in mice lacking PSD-95 and PSD-93 suggesting compensation. Indeed, knock-down of SAP102 in PSD-95/PSD-93 double deficient mice results in further loss of AMPA mediated responses by 53%. This translates to about 15% when compared to wild type mice. The remaining synaptic transmission could have two explanations: One, shRNA mediated knock-down of SAP102 is not complete and the remaining protein targets the detected AMPAR to the synapse. Two, other compensatory mechanisms maintain minimal transmission. SAP97 might assume a role in targeting AMPAR in these cells devoid of other PSD-MAGUKS. A recent study on an independently developed PSD-95 knock-out mouse generally agrees with our findings. AMPARs are lost specifically from a subset of synapses (96). This increase in silent synapses is not accompanied by any morphological changes of the silenced spines (96). However, these deficits are observed without ablation of PSD-93. This difference might stem from the earlier developmental time point used in this study. I therefore conclude that PSD-95, PSD-93 and SAP102 are required for most if not all synaptic AMPAR targeting and that PSD-95 and PSD-93 serve similar function at mostly non-overlapping synapses.

What happens to AMPARs not targeted to the synapse?

My data suggest that loss of PSD-MAGUKs specifically leads to a loss of AMPARs from the synapse. The total amount of AMPARs in the double knock-out mice is not changed. I compared solubility of AMPARs in PSD-95/PSD-93 double knock-out mice to control littermates. AMPARs are redistributed from the

PSD enriched fraction to the soluble fraction. I conclude that PSD-MAGUKs specifically control the targeting of AMPARs to the synapse and play little or no role in control of their expression.

Developmental switch of PSD-MAGUKs

SAP102 enhances AMPAR mediated synaptic transmission when overexpressed. It is upregulated in the hippocampus of PSD-95/PSD-93 double knock-out animals and compensates for the loss of these two PSD-MAGUK isoforms. Nevertheless, its knock-down in wild type dissociated hippocampal neurons had no effect on surface GluR2 expression. AMPA EPSCs from shRNA SAP102 infected, mature slices display normal currents.

My analysis of the developmental expression profile of the three PSD-MAGUKs suggests that SAP102 is expressed early in development, when PSD-95 and PSD-93 are not detectable. At later stages, SAP102 is far less prominent and the other two isoforms are highly expressed. This developmental switch in expression coincides with the onset of a visible phenotype in PSD-95/PSD-93 double knock-out mice at about two to three weeks of age.

Indeed, when hippocampal slice cultures are infected at an earlier stage expression of SAP102 shRNA causes a reduction in AMPA mediated synaptic responses. The results show that targeting of AMPARs early in development depends on SAP102 with PSD-95 and PSD-93 dominating at mature synapse as PSD-95/PSD-93 double knock-out mice only show deficits after the first two weeks. Supporting evidence comes from another study in which AMPAR

mediated synaptic transmission in another PSD-95 knock-out line is normal at early postnatal development, but is reduced at around postnatal day 16 (P14) and recover slightly up to P24 (96). Our study at mature synapses is carried out using animals older than P30 at which point compensation by SAP102 has taken place. The underlying function of a switch from SAP102 to PSD-95 and PSD-93 is not yet know, but might resemble different affinities to other binding partners.

Loss of PSD-MAGUKs and NMDARs

NMDARs are another group of ionotrophic glutamate receptors present at excitatory synapses. They are crucial for induction of plastic changes at the synapse that are key to learning and memory. PSD-MAGUKs can bind to NMDAR subunits directly. They have been suggested to target NMDARs to the synapse. We did not find any effect on NMDA targeting in the PSD-95/PSD-93 double knock-out mice or in the shRNA mediated knock-down experiments. Only if SAP102 was knocked-down in the PSD-95/PSD-93 double kock-outs NMDARs were effected with a loss of only about 25%. This reduction seems not enough to conclude a major role for PSD-MAGUKs in synaptic NMDAR anchoring. Thus, the mechanism anchoring NMDARs remains unknown.

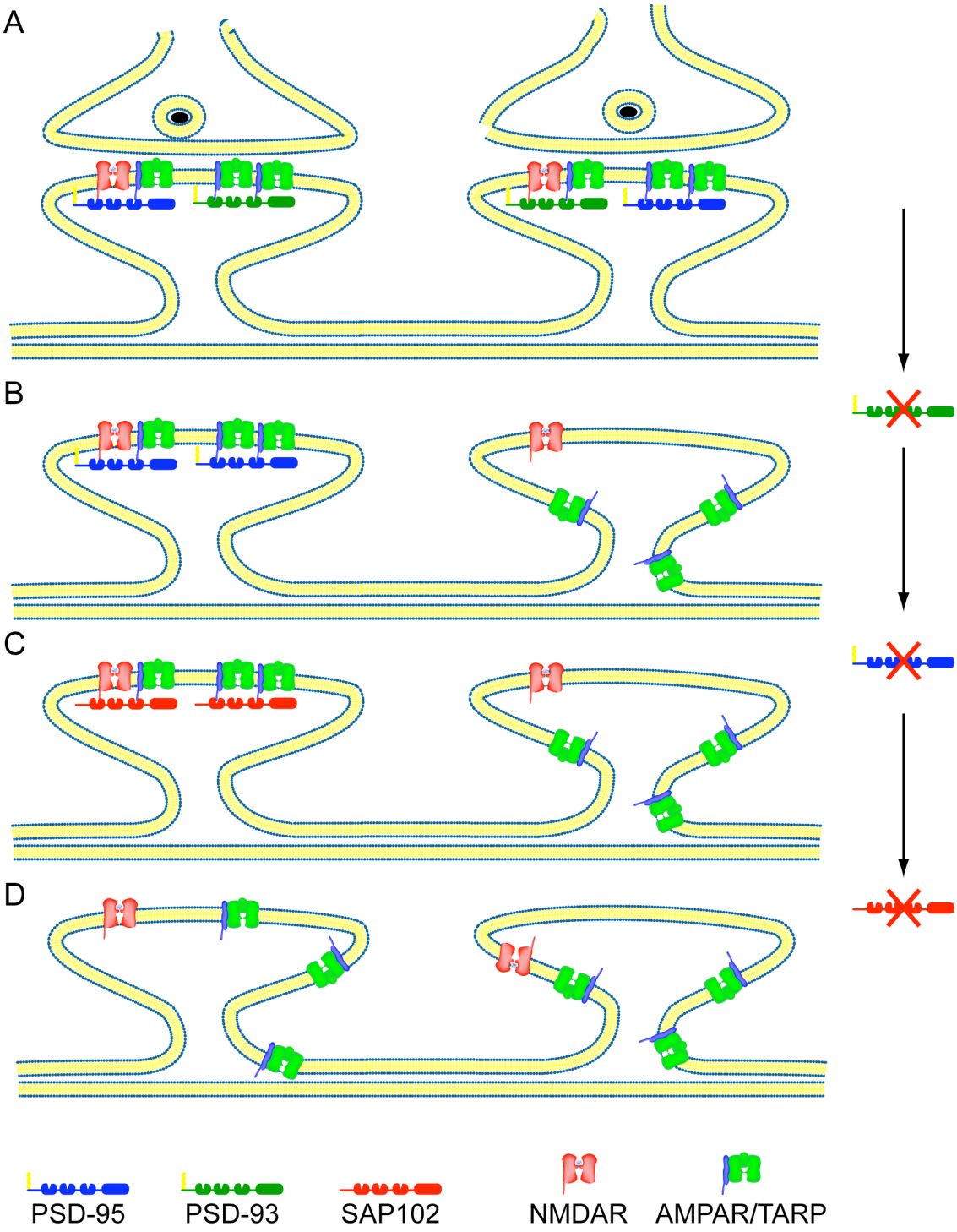

Figure 21 Schematic representation of PSD-MAGUK function. A) Two neighboring dendritic spines with their corresponding presynaptic terminals. PSD-95 and PSD-93 bind NMDARs and the AMPAR/TARP complexes and anchor them at the synapse. B) Loss of PSD-93 (or PSD-95; not shown) results in loss of AMPAR from about 50% of synapses (right spine) as demonstrated in knock-down experiments using shRNAs. C) Additional loss of PSD-95 causes compensation through SAP102 as demonstrated in experiments using double knock-out animals. D) Knock-down of SAP102 in cells from PSD-95/PSD-93 double knock-out animals causes loss of most all synaptic AMPAR with accompanying modest reduction in synaptic NMDAR.

Conclusion

The results presented in this study show that PSD-95 and PSD-93 target AMPARs to mature excitatory synapses. Chronic loss of either isoform in gene targeted mice is compensated by the other. Loss of both isoforms results in a 50% loss of AMPARs from PSD enriched fractions. This likely under represents functions of PSD95 and PSD-93, as SAP102 is up regulated and compensates for the loss of the other isoforms. Knock-down of SAP102 in these mice diminishes the remaining AMPA currents to about 15% of wild type control. Acute knock-down of PSD-95 or PSD-93 using RNAi resulted in a 50% loss of GluR2 synaptic and surface expression. This likely represents the actual contributions PSD-95 and PSD-93 have in targeting AMPARs at mature synapses.

Knock-down of SAP102 in mature wild type neurons has no effect. During early postnatal development, the same experiments result in depression of AMPA EPSCs. The major PSD-MAGUK isoform switches during development from SAP102 to PSD-95 and PSD-93.

These results establish the three PSD-MAGUKs, PSD-95, PSD-93 and SAP102 as major factors determining AMPAR number at excitatory synapses of the hippocampus. Synaptic strength directly correlates with AMPAR receptor number and changes in synaptic strength are thought to be the basis for learning and memory formation.

Three intriguing questions remain with regard to glutamate receptor synaptic targeting. One, what is the molecular basis for the apparent distinct subpopulations defined by PSD-93 and PSD-95? To answer this question I performed preparative immunoprecipitations, but no differential binding partners of PSD-95 or PSD-93 were identified due to cross reactivity of the available antibodies. Antibodies specific only to PSD-95 and PSD-93 might answer this question.

Two, how is the number of PSD-MAGUKs at synapses regulated. May PSD-MAGUKs and AMPAR be delivered together? Co-localization studies in dissociated neurons suggest that these groups of proteins exclusively interact at synaptic sites. Might regulation of TARP PSD-MAGUK interaction be the major determinant of synaptic AMPAR number as shown by Tomita et al, 2005. Nevertheless, overexpression of PSD-MAGUKs alone increases synaptic AMPAR number.

Three, what delivers and anchors NMDARs to the synapse? PSD-MAGUKs have been shown to bind NMDAR subunits but loss of three of these scaffolding proteins has little or no effect on NMDAR targeting. Interestingly, it has been suggested that PSD-95 is involved in regulating NMDAR current decay kinetics (96, 97); the underlying mechanism however is controversial. My data indicate that PSD-MAGUKs might bind to NMDARs but are not essential for their anchoring at the synapse. The molecular basis for synaptic NMDAR targeting remains an intriguing question.