Introduction

The mammalian brain has evolved to receive, process and store information. Later, stored information can be recalled allowing an animal to adjust its behavior based on past experience. The central unit of information processing is the nerve cell or neuron. The human central nervous system contains an estimated 10^{11} neurons, which transmit information coded in electrical currents. Neurons are connected in a highly organized but plastic network.

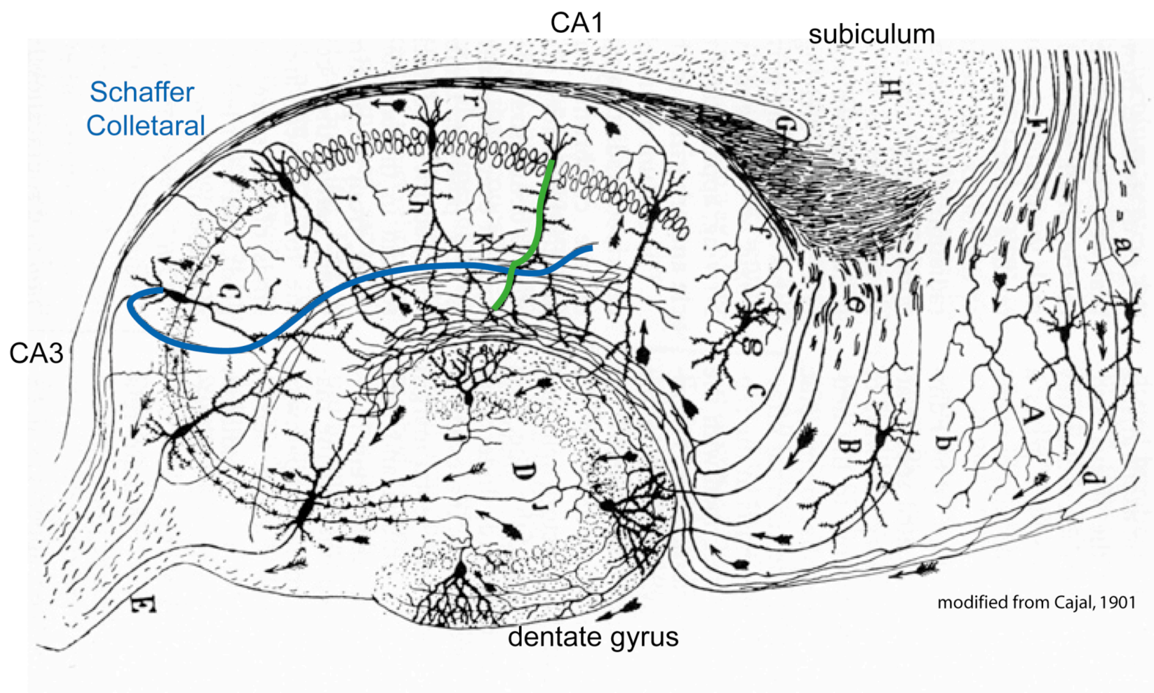


Figure 1 Illustration of the hippocampus. The hippocampus consists of two major pyramidal neuron populations in the CA1 and CA3 regions. The dentate gyrus, a neighboring region, relays input to CA3 pyramidal neurons (arrows indicate flow of signal). CA3 neurons in turn form projections, the Schaffer collaterals (example marked blue), that connect them to CA1 pyramidal neurons (green). (Cajal, 1901; modified)

During development, sensory inputs are wired to connective centers in the brain in a predetermined manner. Later this network is refined depending upon usage. This refinement is thought to be the basis for learning.

One well studied brain structure involved in episodal memory formation is the hippocampus. Clinical studies on epilepsy patients (1) showed that dissociation of the hippocampus from the temporal lobe prevents long term memory formation with no effect on short term memory. In rodent animal models the role of the hippocampus is most prominently studied in spatial memory formation. Introduced lesions of the hippocampus severely impair memory establishment and might also be involved in object recognition (1). Interestingly, pharmacological inhibition of plastic changes in transmission in the hippocampus weakens memory consolidation (1). The hippocampus is attractive to study as it is easily identified anatomically and mainly contains two uniform populations of pyramidal neurons. The Schaffer collaterals connect the two populations of pyramidal neurons of the CA3 and CA1 region (Figure 1). The synapses transmitting signals from CA3 to CA1 neurons use glutamate as the neurotransmitter and are excitatory in nature. This connection is the main connection studied under basal and plasticity inducing conditions in the hippocampus.

Pyramidal neurons of the hippocampus are large multipolar neurons with a triangular shaped cell body (Figure 2). They extend one axon and multiple dendrites. Presynaptic terminals of pyramidal neurons release glutamate. Their excitatory glutamatergic postsynaptic inputs are localized at dendritic spines –

mushroom shaped specializations that oppose the presynapse. Pyramidal neurons also receive inhibitory input directly onto their dendritic shafts.

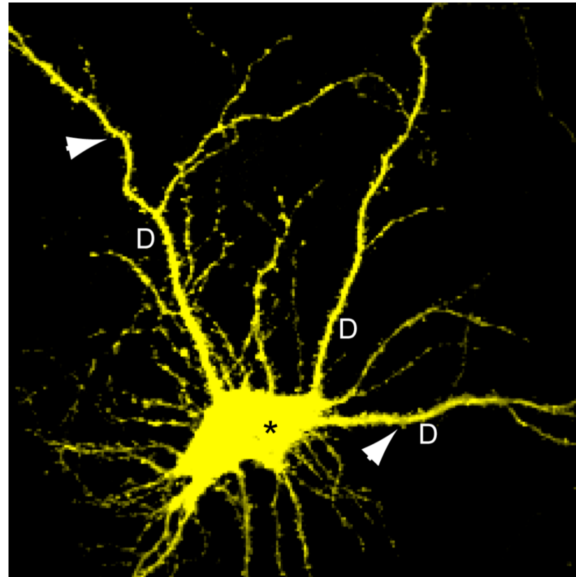


Figure 2 Lucifer Yellow filled hippocampal pyramidal neuron. Depicted is a pyramidal neuron in dissociated hippocampal culture at day in vitro 21 (DIV) filled with lucifer yellow. Spines (arrows) are visible on major dendritic processes (D) protruding form the cell body (asterix).

The glutamatergic synapse

Connections between neurons are established by synapses, highly specialized cellular compartments that transmit information uni-directionally from the presynaptic terminal, part of the axon, to the postsynaptic membrane on the receiving dendrite. To transmit the information from one neuron to another across the synaptic cleft, the signal has to be modified from an electrical to a chemical form. The incoming action potential moving along the axon causes the fusion of docked presynaptic vesicles, carrying glutamate in pyramidal neurons. Glutamate is released into the synaptic cleft and activates ligand gated ion channels enriched in the opposing postsynapse. Ion flow through the glutamate

receptors causes depolarization of the postsynaptic membrane. Multiple depolarization events sum up and propagate to the cell body where, if the threshold is reached, a new action potential is generated in proximity to the cell body at the axon hillock.

The postsynaptic density

Glutamatergic postsynapses of pyramidal neurons display two characteristic structures. On a light microscopic level postsynapses localize to mushroom shaped synaptic spines that protrude from the dendritic shaft (Figure 2).

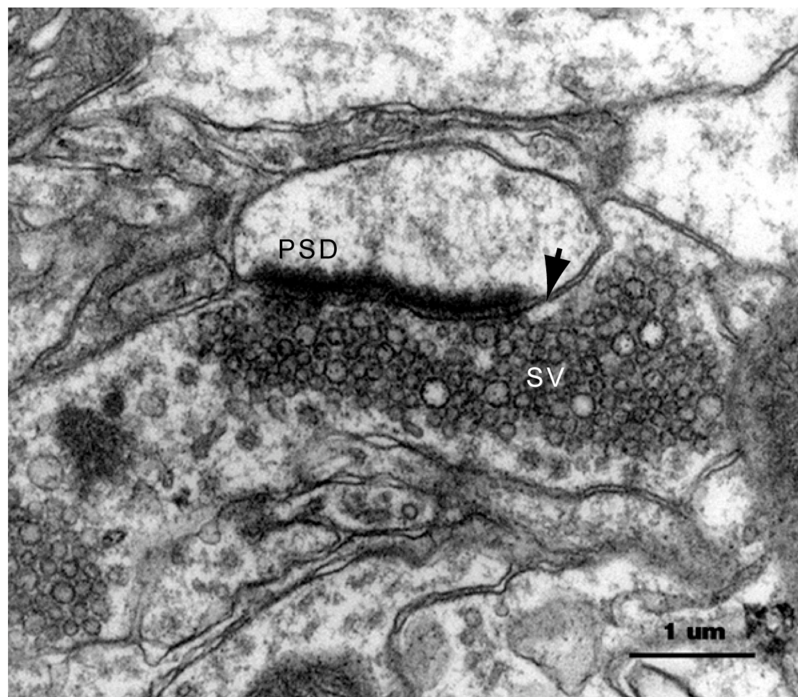


Figure 3 Electronmicrograph of excitatory hippocampal synapse. Shown are a presynaptic terminal (bottom) and its corresponding postsynaptic spine (center). Presynaptic vesicles (SV) concentrate in proximity to the plasma membrane. Arrowhead marks the synaptic cleft. In opposition to the presynaptic terminal the electron-dense PSD is visible. This dense protein assembly contains glutamate receptor and PSD-MAGUK scaffolding proteins among many others. (ITG at UIUC, 2005)

At the higher magnification of an electron microscope a postsynaptic density (PSD) is visible inside the spines (Figure 3). This electron-dense region directly aligns with the synaptic vesicle pool of the presynapse. The PSD contains the ligand gated glutamate receptors, a large variety of other signaling molecules (2) and groups of scaffolding proteins that connect the receptors to the underlying cytoskeleton. Prominent scaffolding proteins are the PSD-MAGUKs – PSD-95 (3), PSD-93 (4), SAP97 (5) and SAP102 (6).

Ionotrophic Glutamate Receptors subdivide into two families

Ionotrophic glutamate receptors separate into two subfamilies. The agonists α-amino-3-hydroxy-5-methylisoxazole-4-propionicacid (AMPA) and N-methyl-D-asparate (NMDA) activate AMPA and NMDARs respectively. Furthermore, NMDA and AMPA type glutamate receptors primarily conduct different ions. Their accumulative conductance is measured as the excitatory post synaptic current (EPSC).

NMDAR subfamily consists of NR1, NR2A through NR2D and NR3. NR1 is widely expressed throughout the brain. Of the other isoforms only NR2A and NR2B express significantly in the hippocampus (7, 8). A functional native NMDAR must contain the NR-1 subtype and at least one of the NR-2 subtypes. Mice lacking NR-1 die shortly after birth of a breathing defect, which underlines its essential importance (9). NMDARs conduct calcium ions but are generally blocked by magnesium ions. Depolarization of the postsynaptic membrane relieves the magnesium block. Coincidence of depolarization and glutamate

release allows NMDAR activation. Calcium influx through the NMDARs causes changes in synaptic strength that are believed to be the key to learning and memory (10, 11).

The AMPAR subfamily includes four members GluR1-4 (12). Splice variants and post-transcriptional editing diversify the subfamily further (13). Native AMPARs are believed to form tetramers of variable subtype combination although evidence is inconclusive. In adult hippocampus the subunit compositions GluR1/GluR2 and GluR2/GluR3 dominate (14). AMPARs generally conduct sodium ions, although subtype combinations lacking GluR2 conduct calcium (15). AMPARs conduct the majority of basal glutamatergic excitatory synaptic transmission. The number of AMPAR present at a given synapse determines its strength as they limit the total ion flux after each glutamate release. Mice lacking the GluR2 subunit show increased hippocampal plasticity and elevated lethality (15), whereas GluR1 knock-outs display an absence of plasticity but are otherwise normal (16). To ensure efficient synaptic transmission AMPARs are targeted and anchored at the postsynapse directly opposing the presynaptic bouton. The molecules and mechanisms involved in this process are poorly understood.

PSD-MAGUKs

Membrane associated guanylate kinases (MAGUKs) are a super family of proteins with a common domain structure. They contain at least one PSD-95, Dlg, ZO-1 (PDZ) protein-protein interaction domain followed by a catalytically

inactive guanylate kinase domain, which serves as an additional protein interaction module (17). Mammalian brain is the tissue expressing the greatest diversity of MAGUK proteins. Members from all groups in the MAGUK super family occur in brain, with particular high levels of the PSD-MAGUK family, homologues to Drosophila DLG, expressed. In fact, three of the four members of this family (PSD-95, PSD-93 and SAP102) occur almost exclusively at postsynaptic sites in brain. PSD-MAGUKs share the general domain structure of Drosophila's disks large (DLG) an important regulator of cell differentiation (18). Three PDZ domains are followed by a SH3GK domain that has no catalytical activity (19)(Figure 4).

PDZ domains occur in around 350 proteins encoded in the human genome (20). These proteins are found at sites of cell-cell contacts throughout an organism such as septate junctions, tight junctions, neuromuscular junctions and synapses (21-24).

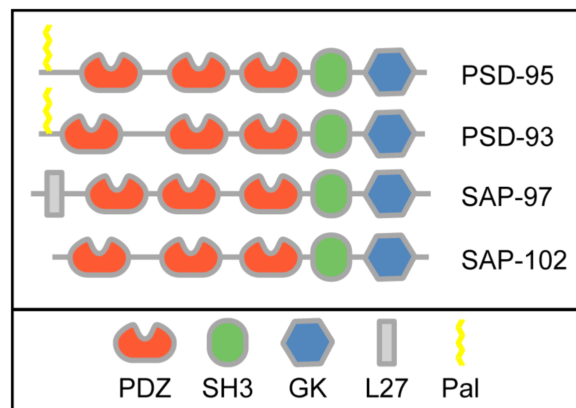


Figure 4 PSD-MAGUK domain structure. Cartoon depicting the domain structure of the dominant hippocampal splice variant of PSD-MAGUKs. As in drosophila DLG's three PDZ domains are followed by a SH3GK domain. The N-terminal region either contains a single L27 domain or a palmitoylated (Pal) cysteine motif. SAP102 has neither a L27 nor Pal N-terminal region but forms a zinc finger. Note that splice variants containing L27 domains have been identified for PSD-95 and PSD-93 as well as a Pal containing SAP97 variant.

PDZ domains typically bind to the last four amino acids of the target protein's C-terminus, but some have been shown to heterodimerize (25, 26). According to their target specificity, PDZ domains subdivide into class I and II. Class I PDZ domains bind to C-terminal peptides that have a threonine or serine at the –2 position from the C-terminus. A conserved histidine in the PDZ domain is necessary for this interaction. In class II PDZ domains this histidine is replaced by a valine. The replacement allows for interaction that display aromatic side chains like phenylalanine or tyrosine at the –2 position (27-29). Importantly, PDZ domains bind to a variety of C-terminal ligands with different amino acid combinations at the 0, -1 and –3 positions (30). To identify the native interactions careful analysis is necessary.

The SH3GK domain of PSD-MAGUKs is another protein-protein interaction module. This domain consists of the N-terminal SH3 domain and the C-terminal guanylate kinase domain (GK). The GK domain has 40% sequence identity to the *Saccharomyces cerevisiae* guanylate kinase (Guk1). It is catalytically inactive due to mutations in key residues involved in the nucleotide binding and transition state stabilization (19, 31). SH3 domains typically bind to proline rich stretches. In the SH3 domain of PSD-MAGUKs the Hinge/Hook region occupies the binding pocket of the SH3 domain (31). Multiple proteins like guanylate kinase-associated protein (GKAP)(32, 33) and microtubule-associated protein 1A (MAP1A) (34) bind to the SH3GK domain of PSD-MAGUKs

The PSD-MAGUKs N-terminus displays variations. Multiple splice variants have been identified for PSD-95 (35), PSD-93 and SAP97 (5, 36-39).

On a protein level these sequence variations result in either an L27 domain or a palmitoylation sequence upstream of the first PDZ domain.

L27 domains are named after the *C. elegans* proteins Lin-2 and Lin-7 that both contain this motif through which they interact (40). L27 domains are unfolded when not bound to one another. Upon heterodimerizing two L27 domains assume a tightly packed bundle of three α-helices each. Two of these heterodimers bind to form a dimer of heterodimers (41). L27 domain containing splice variants have been reported for PSD-95, PSD-93 and SAP97 (5, 35, 42, 43).

Palmitoylation motifs consist of specific cysteine residues that get modified with the fatty acid palmitate (44-47) by palmitoyl transferases (48). Palmitoylation of PSD-95 in neurons is necessary for synaptic targeting and receptor clustering (49-51). N-terminal splice variants containing suitable cysteine residues occur for each PSD-MAGUK (6, 42, 43, 52). PSD-95 and PSD-93 are palmitoylated in vivo whereas the N-terminal region of SAP102 forms a zinc finger.

Regulating Synaptogenesis

Roles for MAGUKs in synapse formation derived first from studies of *Drosophila melanogaster* disk large (*dlg*) (53, 54). In mammalian brain, PSD-95 and related PSD-MAGUKs are also suggested to play roles in postsynaptic development. PSD-95 constitutes a major protein of the PSD and associates with neurotransmitter receptors, such as the NMDAR (Figure 5A); adhesion

molecules, such as neuroligin and signaling enzymes, like nNOS (21-24). A synaptogenic role for PSD-95 was suggested, as it is believed to cluster at synapses prior to any of its associated partners, suggesting a developmental role (55). Furthermore, overexpression of PSD-95 accelerates development and increases the size of neuronal synapses (56). This overexpression enhances the postsynaptic accumulation of certain PSD-95 binding proteins such as GKAP, and also promotes development of the presynaptic terminals that innervate PSD-95 expressing cells (56). This triggering of presynaptic differentiation implies that PSD-95 induces a retrograde signal. The retrograde signal is likely mediated by neuroligin which binds to PSD-95 and by itself can trigger presynaptic differentiation via binding to neurexin on nerve terminals (57, 58).

Genetic evidence that MAGUKs participate in synapse development remains elusive. Targeted disruption of PSD-95 does not grossly alter synaptic structure nor does it change synaptic transmission (59). Normalcy of synapses was also found in mice lacking PSD-93 (4). Whereas both PSD-95 and PSD-93 knockout mice are viable and fertile, mice lacking full-length SAP97 die at birth and have a cleft palate and other mid-line defects (60). Neurons cultured from these SAP97 mutant mice showed normal synaptic function (61). This normalcy of synaptic development and function in mice lacking individual MAGUKs may be explained by molecular redundancy. Breeding mice lacking multiple neuronal MAGUKs might identify the essential roles for these proteins in synapse formation and function.

Organizing Signal Transduction Pathways

In addition to scaffolding synaptic receptors, MAGUKs can organize signal transduction pathways at synapses. The best-studied example in mammals is the organization of the NMDAR complex by PSD-95 at excitatory synapses (21). Although PSD-95 is not essential for synaptic trafficking of NMDARs, numerous studies identify a crucial role for PSD-95 and associated proteins in accelerating and specifying signaling downstream of the NMDAR. Calcium influx through NMDARs plays a central role in synaptic plasticity (62).

An important downstream effector of NMDAR signaling is activation of the neuronal isoform of nitric oxide synthase (nNOS). PSD-95 PDZ domains bind to both the C-terminus of the NMDAR and to an N-terminal PDZ domain from nNOS (63). This ternary complex ensures that calcium influx through the NMDAR activates calcium/calmodulin regulated nNOS.

Another enzyme associated with PSD-95 is the synaptic ras GTPase-activating protein, SynGAP (64, 65). The third PDZ domain from PSD-95 binds to a C-terminal site in SynGAP and concentrates this enzyme at synaptic sites. MAP kinases, which play roles in synaptic plasticity, are negatively regulated by the rasGAP activity of SynGAP, which is inhibited by CaMKII, and prominent target of calcium entering through the NMDAR. PSD-95 controls a signaling pathway whereby activation of NMDARs can inhibit SynGAP and thereby increase activity of MAP kinase to modulate synaptic plasticity.

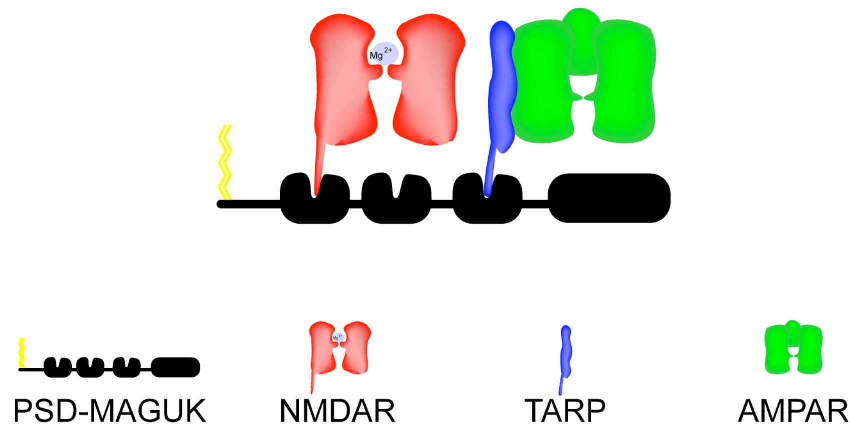
Although PSD-95 is the most prominently studied of the PSD-MAGUKs, some studies have focused on specific properties of other PSD-MAGUKs. For

example, the Src family kinase regulator Csk binds to a PSD-93 specific peptide sequence inserted between PDZ domains two and three (66). The binding depends upon phosphorylation of this sequence by Fyn, a kinase implicated in long term potentiation (LTP) and spatial learning (67, 68). SAP102 binds to the NR2B subunit. This interaction can be mediated by any of its three PDZ domains (69). SAP102 can also bind to mPins (mammalian partner of inscruteable) through its SH3GK domain. In dissociated neurons knock-down of mPins expression through RNAi reduces NMDA receptor surface expression (70). SAP97 is the only PSD-MAGUK reported to directly bind to AMPAR subunit GluR1 (71). A functional relationship for this interaction is not identified. In fact, SAP97 overexpression does not enhance AMPAR mediated transmission whereas overexpression of other PSD-MAGUKs causes a strong enhancement (72). However, overexpression of SAP97 most potently of all PSD-MAGUKs increases presynaptic protein content and active zone size. This function is mediated by cell-cell adhesion molecules such as cadherins, integrins and ephrins. SAP97 might thus play a role in trans-synaptic adhesion (73).

Clustering Receptors at Synapses

A crucial function for neuronal MAGUKs at the PSD involves their clustering of ion channels. The PDZ domains from PSD-95 and the other neuronal MAGUKs bind potently to the C-termini of numerous ion channels including potassium ion channel Kv1.4 and the NMDARs (Figure 5)(23, 24). The enlarged synapses in neurons over-expressing PSD-95 do not, however, show

augmented accumulation of these receptors (56). Surprisingly, synapses overexpressing PSD-95 show enhanced clustering of the AMPA-type glutamate receptors, which do not directly bind to PSD-95. Furthermore, the synaptic function of AMPA - but not NMDA – receptors is enhanced by overexpression of PSD-95 (23, 24). The resolution of this paradox came from the discovery that stargazin, the protein mutated in stargazer mice, links AMPARs to PSD-95 (Figure 5)(72, 74).



PSD-MAGUK NMDAR TARP AMPAR

Figure 5. PSD-MAGUK complex. The PDZ domains of PSD-MAGUKs (black) directly bind to the C-terminal PDZ ligands of NMDARs (red) and indirectly to AMPAR (green) through a group of AMPAR auxiliary subunits called TARPs (blue). (Note that, for clarity, numerous other interactions with other proteins are not depicted.)

Stargazin is a four-pass transmembrane protein, and mice lacking stargazin show a complete absence of AMPAR currents in cerebella granule cells (74, 75). Stargazin modifies AMPAR gating and kinetics and the larger group of transmembrane AMPA receptor regulatory proteins (TARPs) is now accepted as an auxiliary subunit of AMPAR. Stargazin promotes the synaptic trafficking of AMPARs by two distinct mechanisms. First, the body of stargazin

interacts with AMPARs and promotes their trafficking to the neuronal plasma membrane. Second, the cytosolic tail of stargazin contains a consensus PDZ binding site that interacts with all three PDZ domains of PSD-95 (Figure 5)(74, 76, 77). The density of the PSD-95/stargazin complexes at synapses thereby determines the strength of AMPAR mediated currents (Figure 6B) (72).

Modulating Synaptic Plasticity

Synaptic plasticity in brain reflects activity-dependent changes in the strength of synaptic connections, and this process represents a mechanism for information storage in brain and learning and memory (78). Whereas calcium influx through the NMDAR induces plasticity, the change in synaptic strength is manifested by alterations in the density of synaptic AMPARs (Figure 6C) (79, 80). As PSD-95, through its association with stargazin, directly determines the density of AMPARs at synapses (72), this MAGUK complex can directly participate in controlling synaptic plasticity.

Overexpression of PSD-95 and PSD-93 dramatically increases synaptic density of AMPARs (56), and this overexpression prevents the recruitment of new AMPARs that normally occurs during long-term potentiation (LTP) (Figure 6C) (81, 82), a well-studied model of synaptic plasticity. This occlusion of LTP is traditionally interpreted to mean that overexpression of PSD-95 and endogenous LTP occur through similar mechanisms.

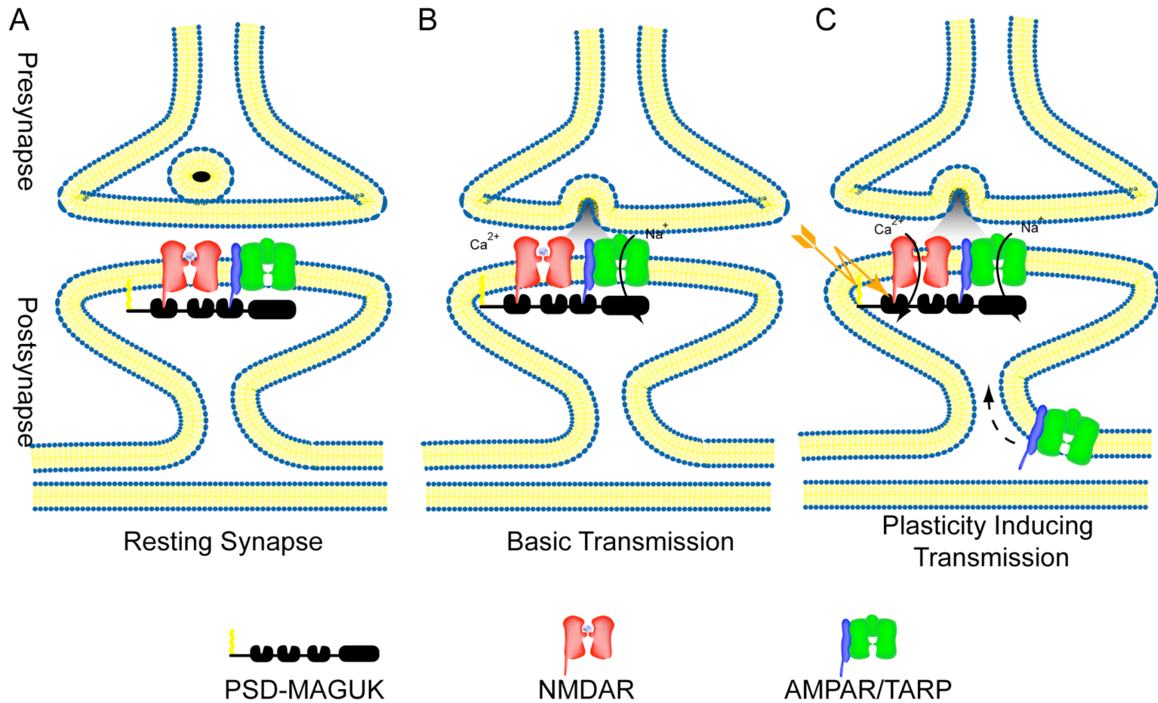


Figure 6 Schematic of excitatory synapse A) Resting excitatory synapse with a docked synaptic vesicle in the presynaptic terminal (top) and a basic PSD-MAGUK complex in the opposing postsynaptic spine (bottom). Depicted are, a palmitoylated isoform of PSD-MAGUK, a NMDA receptor with magnesium ion block (red), a TARP/AMPAR complex (blue/green). B) Upon presynaptic glutamate release (grey), glutamate activates AMPAR and sodium ions depolarize the synaptic spine. C) When glutamate and postsynaptic membrane depolarization (orange arrow) co-incite, the magnesium block is release from NMDAR. Calcium ion flux through the NMDA receptor causes changes in AMPAR number at the postsynaptic membrane (dashed arrow).

To be an effective learning mechanism, synaptic plasticity must be reversible. Indeed, prolonged low intensity activation of NMDARs can decrease AMPAR density, a process known as long-term depression (LTD) (83). Interestingly, overexpression of PSD-95 augments the magnitude of LTD induction suggesting a role for PSD-95 in this process (81, 84). The mechanism whereby PSD-95 might control LTD remains uncertain. One possibility is that calcium influx through the NMDAR can enhance the depalmitoylation of PSD-95 (85). Because palmitoylation is required for synaptic clustering of PSD-95, this

depalmitoylation disperses PSD-95 and diminishes synaptic AMPAR density. Whether the NMDAR stimulation that occurs during LTD can modulate PSD-95 palmitoylation or whether this participates in other aspects of synaptic scaling or homeostasis remains uncertain. PSD-MAGUKs PSD-95, PSD-93 and SAP97 express distinct N-terminal splice variants that result in α- and β-isoforms. The α-isoform contains characteristic cysteins, that are palmitoylated and necessary for synaptic targeting, whereas the β-isoforms contain L27 protein-protein interaction domains. A recent study suggests that the α-isoforms target AMPAR to synapses in an activity independent manner. Delivery through β-isoforms is activity dependent and involves NMDAR and CamKII activity (42). Regulated degradation of PSD-95 represents another possible mechanism for linking NMDAR activity with LTD, e.g. stimulation of the NMDAR activates the ubiquitin-proteasome pathway to degrade PSD-95, and this results in AMPARs endocytosis. Importantly, proteasome inhibitors prevent NMDAR induced LTD (86).

Targeted disruption of PSD-95 produces synaptic defects that partially fit with the proposed role for this protein in regulating synaptic AMPARs (59); however, important issues remain unresolved. Mice lacking PSD-95 show an absence of LTD as might be expected (59). On the other hand, these mice show enhanced LTP, which is not easily reconciled with the proposed roles for PSD-95 in controlling synaptic plasticity. Nevertheless, these mice show impairment in models of learning and memory, which likely reflect alterations in their activity-dependent control of AMPARs (59). An important unresolved issue is why basal

synaptic strength remains intact in mice lacking PSD-MAGUKs. No changes in baseline AMPAR function are noted in mice lacking functional PSD-95, PSD-93, or SAP97 (4, 59, 61). This seems inconsistent with the prevalent model that PSD-95, via its interaction with stargazin and other TARPs, control AMPAR density at synapses. This discrepancy may be explained by molecular redundancy. The neuronal MAGUKs are highly similar in sequence and the C-terminus of stargazin can bind to multiple members of this family (87). Defining the essential roles for PSD-MAGUKs in brain remains a critical challenge and may require inter-breeding of these mutant mice.

In this study, I focus on a comprehensive examination of the common and distinct functions of the four PSD-MAGUKs, PSD-95, PSD-93, SAP97 and SAP102 in AMPAR targeting and anchoring at the excitatory synapse. I find that, three of the PSD-MAGUKs, PSD-95, PSD-93 and SAP102 account for most if not all synaptic targeting of AMPARs. In mature animals PSD-95 and PSD-93 carry out this function in mostly non-overlapping subpopulation of synapses. The molecular basis for this phenomenon is intriguing but unknown. In early development SAP102 full fills the function of PSD-95 and PSD-93. During postnatal development the predominant PSD-MAGUK switches from SAP102 to PSD-95 and PSD-93. This study places PSD-MAGUKs as major determinants of synaptic strength, the key feature modulated during learning and memory formation.