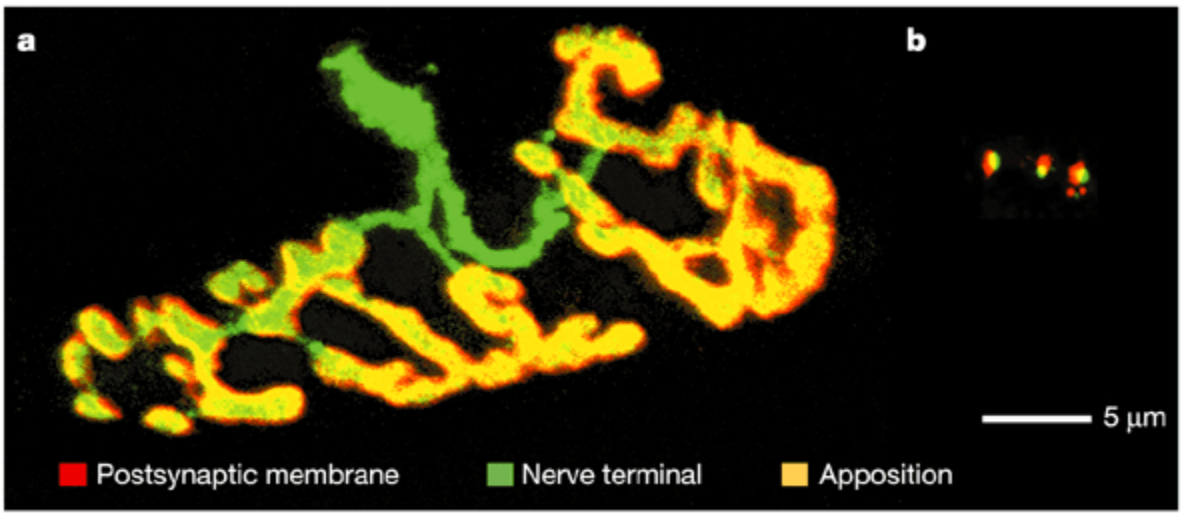
Neuromuscular junction

Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2001 Nov;2(11):791-805
Sanes and Lichtman (1999). Development of vertebrate NMJ. Annu Rev. Neurosci. 22: 389-442.



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Axons contact only a minute fraction of surface area of muscle fiber (NMJ).
Nicotinic AchRs concentrate at postsynaptic side of NMJ.

AChR 10,000 per square micron at NMJ versus 10 in extrasynaptic membranes
(any room for anything else? Yes…VG sodium channels; cell adhesion molecules such NCAM; integrins α7Aβ1, α7Bβ1; receptor tyrosine kinases erbB2, 3, 4 and MuSK; dystrophin-associated glycoproteins; etc)

Synaptic concentration of AchRs occurs via

- agrin-dependent (maintenance of) clustering of membrane receptors
- local synthesis of AchRs by subsynaptic nuclei stimulated by ARIA/neuregulin
- inhibition of extrasynaptic synthesis by activity (depolarization) and calcium

<u>Basal lamina</u>

Basal lamina is prominent in NMJ:
Highly infolded membranes, basal lamina, and specialized extracellular matrix (ECM) in synaptic cleft, in contrast to neuronal synapses

Synaptic BL is instructive:

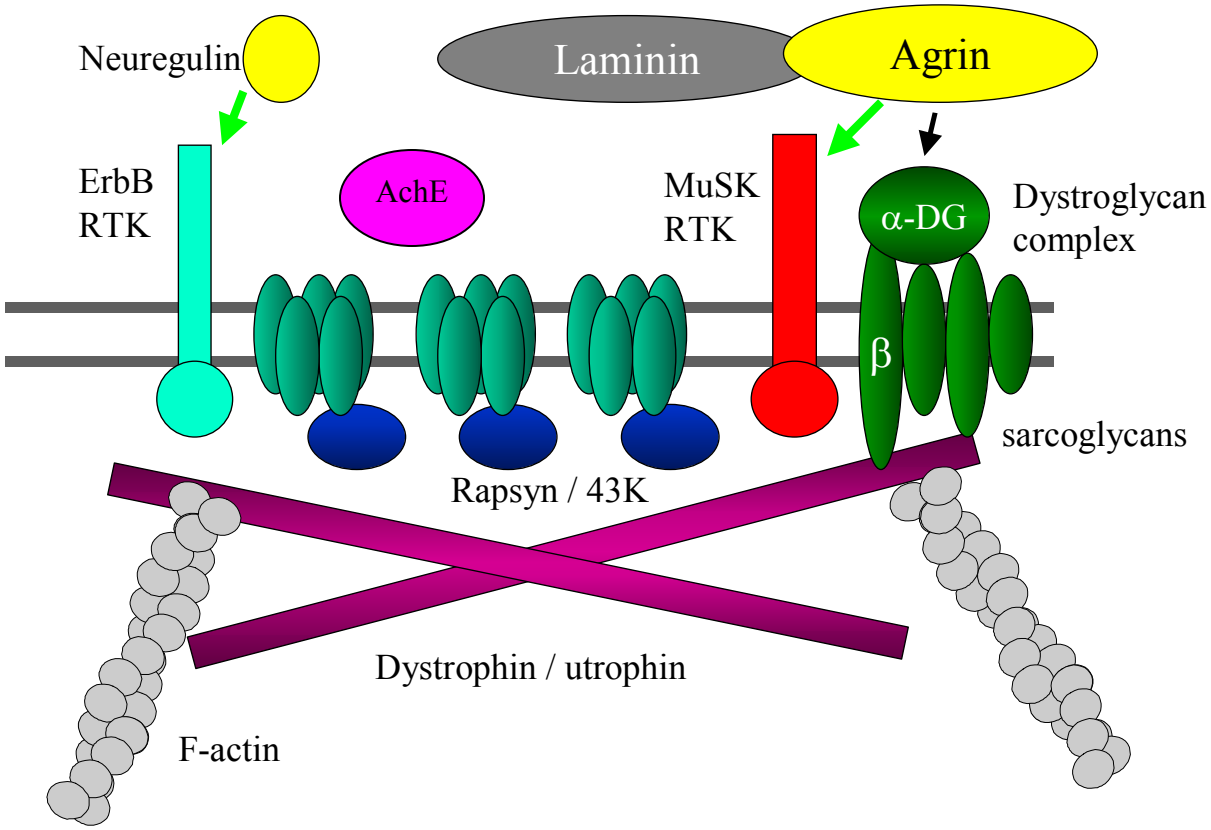
Regenerating motor axons form synapses specifically at those sites where a synapse had previously existed, even if the muscle fiber has degenerated (Sanes, Marshall, McMahan (1978) J.Cell Biol. 78: 176-198).
Transplanted patch of synaptic BL induces postsynaptic differentiation in muscle
Site of previous synapse *marked* by molecules in the basal lamina.
[What is the molecular determinant in BL for NMJ formation?]

Components of synaptic basal lamina:
e.g. synapse-specific laminins (eg laminin-β2), specific laminin and collagen heteromers, acetylcholinesterase, proteoglycans, **agrin**, **ARIA/neuregulins**; these proteins derive from both nerve and muscle and are stably retained in the synaptic cleft

Laminin-β2 selectively adhesive for motoneurons, stops laminin-1 (conventional laminin)-promoted outgrowth of motor axons, initiates differentiation of arrested axons into nerve terminal.
Mouse laminin-β2 KO (mild cf MuSK or Agrin) has aberrant differentation of nerve terminals at NMJ, and Schwann cells invade into synaptic cleft (Noakes et al (1995) Nature 374: 258-262).

Nerve-dependent and nerve-independent AchR clustering
α-bungarotoxin: high affinity, specific, "irreversible" ligand of muscle nAChR

After insertion into myotube surface membrane, some AchRs cluster into "hotspots" (in absence of nerve)
Axons contact myotubes more or less at random, inducing subsynaptic AchR aggregates with short-range signal (may also disperse non-synaptic hotspots by a long range signal)
→ secreted factor from nerve induces NMJ formation.
Central location of NMJs in muscle fibers more likely a consequence of symmetric muscle growth than specific targeting of synaptic sites.



Schematic diagram of major proteins at vertebrate NMJ
Nicotinic acetylcholine receptor is shown as pentamer.
RTK, receptor tyrosine kinase.
Mutations of dystrophin (Duchenne Muscular dystrophy) and many of the components of dystroglycan complex cause disease of muscle.

Postsynaptic proteins

Rapsyn/43K:
biochemically associated with AchR from torpedo;
apparently equimolar; colocalize with AchR throughout synaptogenesis;
removal of rapsyn increases the lateral and rotational mobilities of AchRs;
Rapsyn clusters AchR in heterologous cells; ?interact directly with AchR;

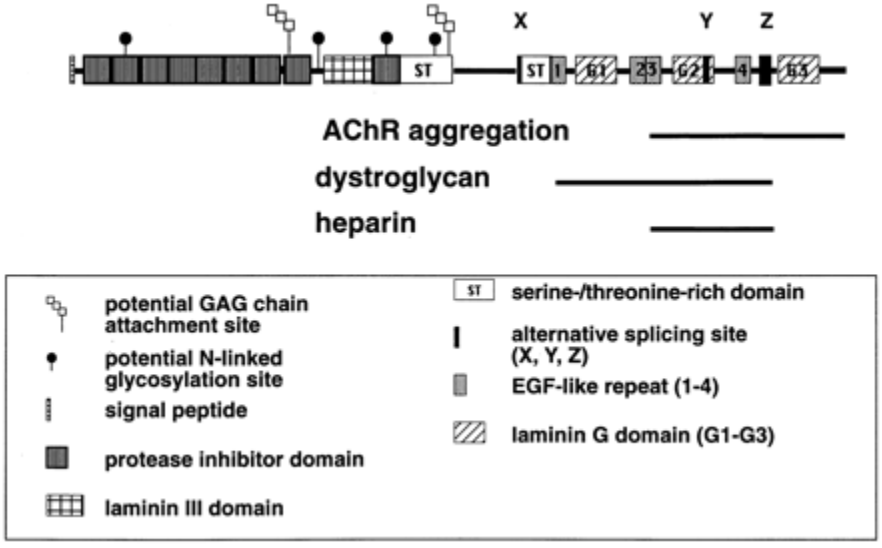
Rapsyn KO (Gautam et al (1995) Nature 377: 232-236): neonatal lethal;
Loss of AchR clusters; in addition, dispersal of other components of postsynaptic specialization (dystroglycan, ErbB3, utrophin, syntrophin)
→ rapsyn crucial for organization of the "entire" postsynaptic apparatus.

However, laminin-β2, acetylcholinesterase and MuSK are synaptic in rapsyn KO, thus synaptic BL differentiation relatively normal.
Presynaptic defects milder than MuSK or agrin KO.

Utrophin (NMJ), relative of **Dystrophin** (diffusely distributed in sarcolemma): both bind to **dystroglycan** which in turn binds to basal lamina and specifically to agrin; however, dystroglycan probably not "functional" agrin receptor.
Utrophin KOs are viable and have near-normal NMJ.

Agrin
Kleiman and Reichardt (1996) Cell 85: 461-464

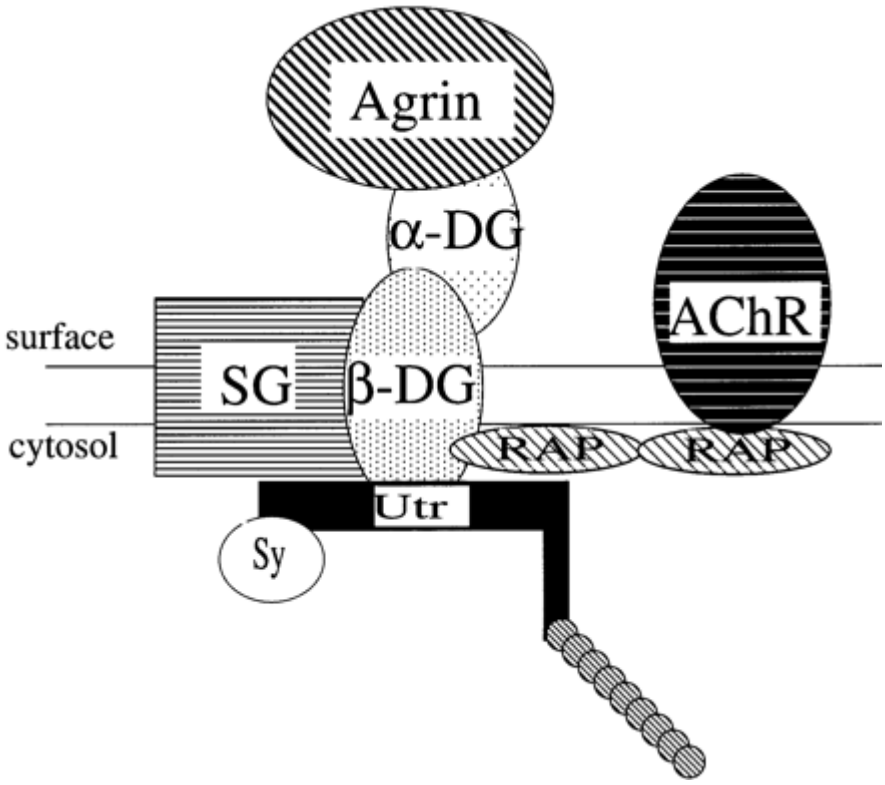
1. Nerve derived signals that induce AchR aggregation: FGF, HBGAM/pleiotropin, midkine, laminin, **agrin** (the only one implicated in vivo)
2. Cell free patches of synaptic basal lamina induced postsynaptic specialization in regenerating myotubes (Burden et al, (1979) JCB 82: 412-425)
3. Using formation of hotspots of AChR in cultured embryonic myotubes as assay, McMahon and colleages **purified agrin** from basal lamina extracts (native agrin is HSPG ~400 kDa).
4. Agrin (picomolar) induces clustering of AChRs plus associated cytoskeletal proteins such as rapsyn, cholinesterase; is present in and secreted by motoneurons, and stably associates with the basal lamina. (However, muscle also makes abundant agrin)
5. **Molecular cloning of agrin**: big protein (calculated 200 kD), multiple domains:
6. C-terminal region, which includes EGF repeats and laminin G-like domains, is sufficient for AchR clustering activity.
7. Alternative splicing at 3 sites in C-terminal region: active spliceforms for AchR clustering only made by nerve (8 amino acid insertion at the most C-terminal B/z site makes neuronal agrin 1000x more potent in AchR clustering in vitro.



Domain structure and functionalregions of the agrin protein. The domains required for aggregation of AChRs and for binding to heparin as well as α-dystroglycan are indicated.
From Hoch, Eur. J. Biochem. *265, 1-10 (1999)*

Agrin KO:

Die at birth, no movement.
Greatly reduced but not absent number, size, density of AchR aggregates (many not associated with nerve endings); normal overall level of AchR.
Few nerve-muscle contacts associated with AchR clusters or other postsynaptic specializations. → necessary for NMJ postsynaptic integrity
Moreover, most motor nerve terminals end blindly on mutant myotubes, and show aberrant excessive growth;
However, minority of nerve terminals opposed to specialized postsynapse enriched in AchRs and *still* associated with normal cytoskeletal/basal lamina components (suggesting a second agent capable of inducing postsynaptic differentiation to a limited degree, and/or assembly of postsynaptic complex?: ?laminin?).
Agrin -/- muscle transplanted to wildtype hosts is reinnervated by agrin expressing axons.
A more precise z-exon deletion with minor effects on muscle agrin expression had the same phenotype as first KO allele. → Muscle agrin, other splice variants, dispensable for NMJ development.
Agrin mechanisms: Tyrosine phophorylation: agrin induces tyrosine phophorylation of AchR; TK inhibitors prevent agrin-induced clustering; intracellular calcium may be important.



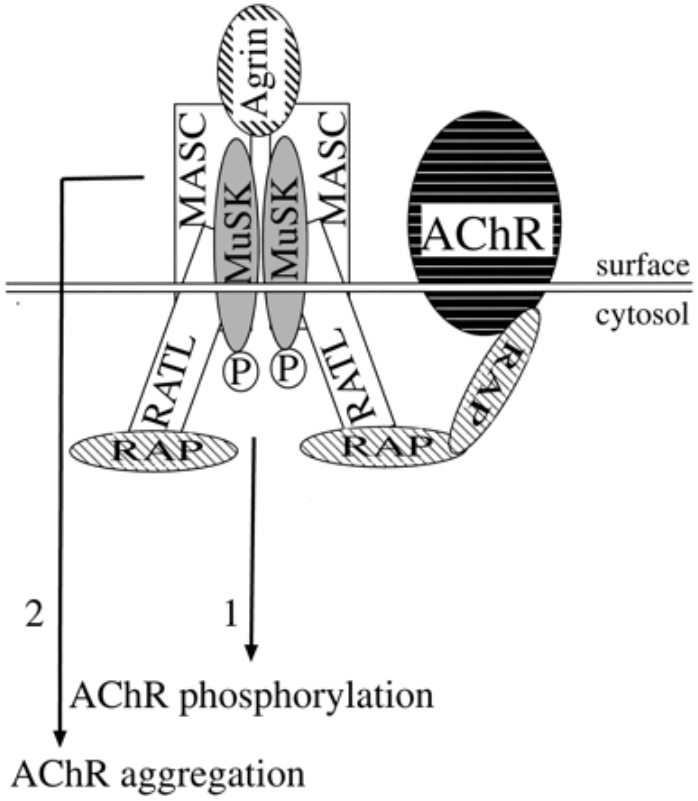
Interaction of agrin with the dystrophin-associated glycoprotein complex at the neuromuscular junction. Extracellular, transmembrane and cytoskeletal proteins together form this large complex which is represented by the following symbols: α, β-DG, αβ-dystroglycan; SG, sarcoglycan complex; Sy, Syntrophin. In its synaptic form, this complex is linked to utrophin (utr) rather than dystrophin. The striped circles on the bottom symbolize the actin cytoskeleton which is associated with utrophine. α-Dystroglycan is a high-affinity agrin-binding protein, whereas β-dystroglycan associates with rapsyn (Rap).
From Hoch, Eur. J. Biochem. *265, 1-10 (1999)*

Agrin receptor

Several proteins can interact with agrin in vitro, and are implicated in AchR clustering in culture, but none have been shown to be important for agrin action in vivo. The best candidate for agrin receptor (MuSK) was discovered in an unrelated screen, and does not even bind directly to agrin!

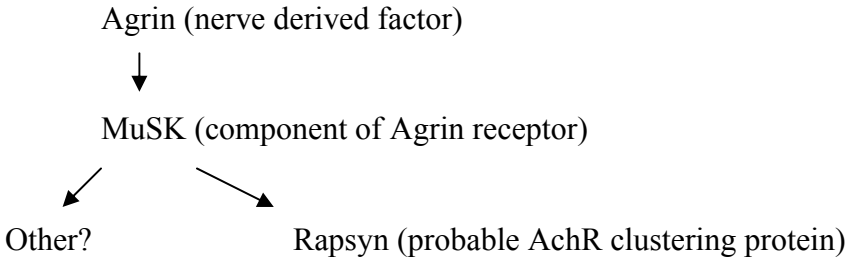
1. **α-dystroglycan**, a major agrin binding protein on muscle surface; mutations of several components of dystroglycan complex causes muscular dystrophy (Campbell (1995) Cell 80: 75-679); coclusters with rapsyn in heterologous cells. However, binding to splice forms of agrin does not correlate with active splice forms for AchR clustering [pharmacology, order of potency relationships]
2. **Heparan sulfate proteoglycans** (HSPGs): heparin blocks AchR clustering by neurons and by agrin (even recombinant agrin that contains no HS; [agrin is itself a HSPG]); ?HSPGs function as coreceptors for agrin, like for FGF??

3. **<u>MuSK</u>**: muscle specific receptor tyrosine kinase (first isolated from Torpedo electroplax membrane); induced by denervation; concentrated in postsynapse, selectively transcribed at synapse.
4. **MuSK KO**: grossly defective postsynaptic differentiation, similar or even worse than agrin Kos (e.g. have aberrant nerve branching patterns).
5. No AchR clusters, though AchR protein levels normal.
6. Myotubes from agrin -/- mice, but not MuSK-/- mice, can respond to exogenous agrin, suggesting MuSK acts downstream of agrin. AchR clustering in MuSK -/- myotubes rescued by MuSK transfection.
7. Dominant negative MuSK in WT muscle cells inhibits AchR clustering by agrin.
8. Agrin induces MuSK tyr phosphorylation in 1 minute; only clustering-active isoforms induce MuSK phosphorylation;
9. I^{125}-agrin (only active isoforms) can be cross-linked to MuSK on differentiated myotubes. However, NO direct binding of agrin to recombinant MuSK (in vitro).



Model of the agrin receptor complex. The scheme includes two hypothetical components which have been postulated according to their functional properties: (a) a myotube-associated specificity component (MASC) which binds to agrin with high affinity and mediates its association with MuSK; (b) a rapsyn-associated transmembrane linker (RATL) forming a bridge between the extracellular part of MuSK and rapsyn. In addition, rapsyn (Rap) is shown interacting with the AChR as well as with itself. MuSK is present in an activated and autophosphorylated homodimeric form. From its cytoplasmic domain, signal 1 is emerging which is sufficient for AChR phosphorylation. For AChR aggregation, both, signal 1 and a second signal originating from the extracellular domain of MuSK are required.
From Hoch, Eur. J. Biochem. *265, 1-10 (1999)*

"Genetic pathway"



Note:

MuSK is clustered at "synaptic sites" in rapsyn -/- muscle
MuSK is part of "primary synaptic scaffold" and rapsyn recruits other synaptic components(eg AchRs) to the scaffold.
The extracellular domain of MuSK required for MuSK-rapsyn interaction.
Probably intermediate protein kinases between MuSK and Rapsyn, AchRs
MuSK-independent AchR clustering pathways may exist

Recent studies of mutant mice rekindled the possibility that muscle may be patterned independent of motor innervation.

In mice KO for topoisomeriase 2 motor axons fail to grow into or branch within diaphragm and limb muscles, but AChRs are still clustered in the central region of muscle, in a zone that is only twofold wider than in normal mice (Yang et al., 2000 Science **287**, 131).
Subsequent analysis of additional mouse mutants that affect motor neuron generation, motor axon projections, and motor neuron-derived signals confirmed that *AChR* gene transcription and AchR clustering are patterned in developing skeletal muscle in the absence of motor neurons and motor axons
Thus neural signals maintain and refine, rather than initiate this pattern of AChR expression, to generate the mature NMJ.
Although the muscle AChR prepattern does not require Agrin, this prepattern is not observed in mice lacking MuSK
Thus MuSK plays an important early role in NMJ development, even before agrin is relevant.
Reviewed by Burden SJ. Building the vertebrate neuromuscular synapse. J Neurobiol. 2002;53:501-11.

Comparing neuronal synapses and NMJ

Are neuronal synapses induced by presynaptic nerve influence, as for NMJ?
Agrin is present in CNS but unlikely to be important for most central synapses.

NMJ is designed for faithful (unfailing) synaptic transmission (one axon to a set of muscle fibers [motor unit]);
NMJ is really a cluster of large synapses

Central synapse is small and shows high variable probability of failure; specialized for signal integration and plasticity

NMJ is much more stable in molecular organization (lifetime of Ach receptors in synapse is many hours to days);
central excitatory synapse shows great dynamism of proteins and function (plasticity);
NMDA receptors are relatively stable, but AMPA receptors cycle in and out of the postsynaptic membrane on a time scale of minutes to hours.

Central glutamatergic synapse

Review
Sheng M, Kim MJ. (2002) Postsynaptic Signaling and Plasticity Mechanisms. *Science.* 298:776-780.

1. Most abundant, probably most heterogeneous kind of synapse e.g. spine / shaft; differential presence of NMDA, AMPA, mGluR *[e.g. which major synapse lacks NMDA receptor? E.g. parallel fiber-Purkinje cell synapse]*
2. Evidence for subsynaptic and heterosynaptic segregation of different kinds of GluRs (NMDA and AMPA receptors in PSD, mGluRs in a peri-PSD distribution)
3. GluRs were cloned by functional expression and molecular biology, hence little biochemistry known. GluRs differ from the nAChR superfamily of ionotropic neurotransmitter receptors in that C-terminus of all GluRs is major intracellular domain.

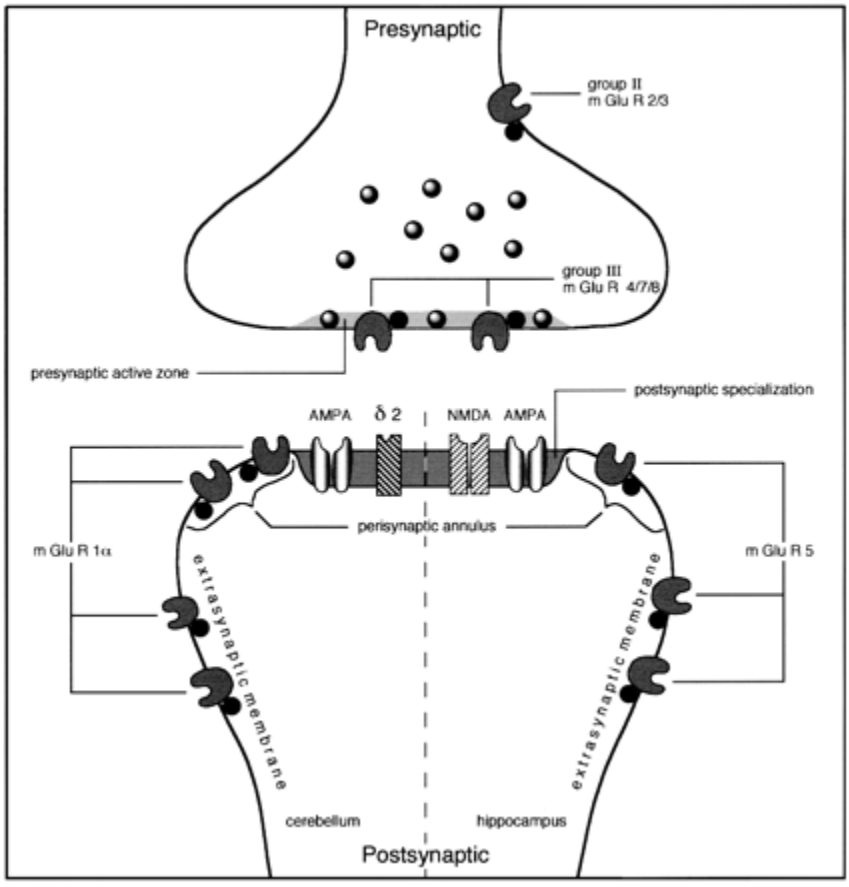


Diagram showing the arrangement of glutamate receptors at the synapse as revealed by high-resolution immunogold labeling. The drawing is not representative of all excitatory synapses since the indentities and subsynaptic localization of glutamate

receptors may vary considerably among the brain regions and cells, and even between different glutamate receptors on the same cell (see text). Most of the data on which the diagram was based were obtained in the hippocampus (presynaptic element and right half of spine) or cerebellum (parallel fiber-Purkinje cell synapses; left half of spine). NMDA, AMPA, and δ2 receptors are concentrated in the postsynaptic specialization, whereas metabotropic receptors occur peri- or extrasynaptically or in the presynaptic element. The differential postsynaptic distribution of the group I metabotropic receptors (mGluR1α and mGluR5) is according to Lujá *et al.* [2] Group III presynaptic mGluRs are found in the presynaptic active zone (shown in *gray*), while the group II presynaptic mGluRs are expressed in the preterminal axolemma. The *filled circles* associated with the mGluRs indicate G proteins. Kainate receptors and extrasynaptic ionotropic receptors are not included in the drawing.
Takumi Y, Matsubara A, Rinvik E, Ottersen OP. Ann N Y Acad Sci 1999 Apr 30;868:474-82.

The postsynaptic density (PSD)

The ***PSD*** – a morphologically defined, biochemically purifiable "organelle" resistant to dissolution by non-ionic detergents
Glutamate receptors (especially NMDA receptors) are located in the PSD and physically associated with PSD.

Signaling complexes in the postsynaptic density (PSD)

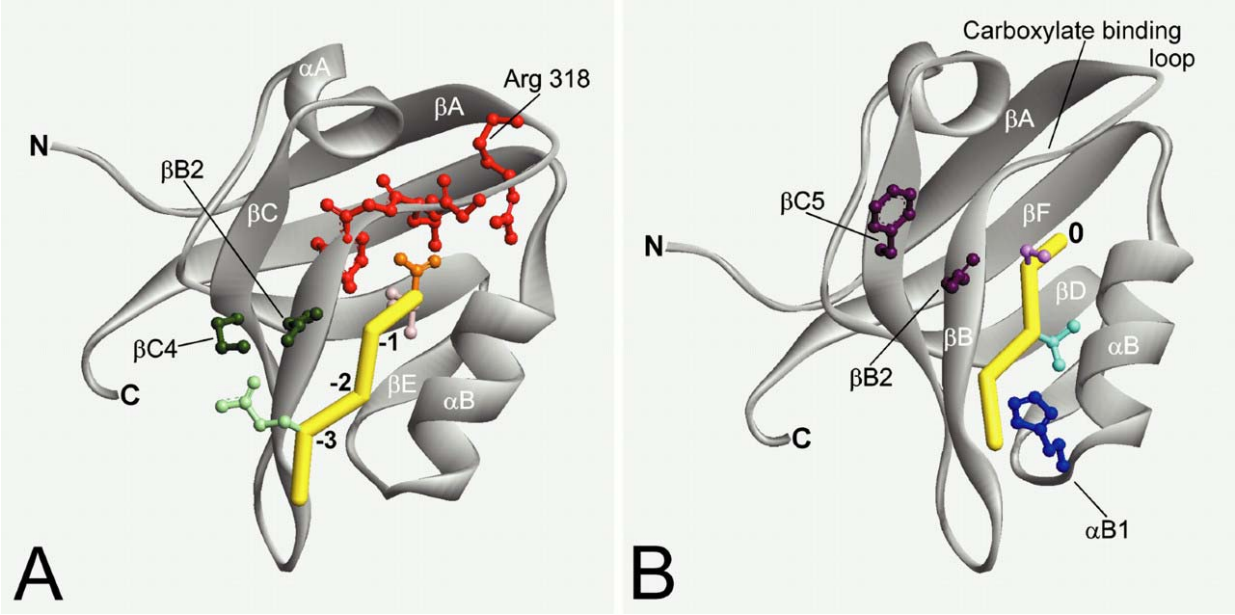
NMDA receptor-PSD-95 interaction

1. NR2 subunits C-termini bind to PSD-95 through PDZ domains (PSD-95, Discs large, ZO-1 domains) – yeast two hybrid screens

PDZ domains are modular protein interaction domains: bind to specific C-termini (approx 4 amino acids) e.g. NR2 –ESDV, Shaker K channels –ETDV

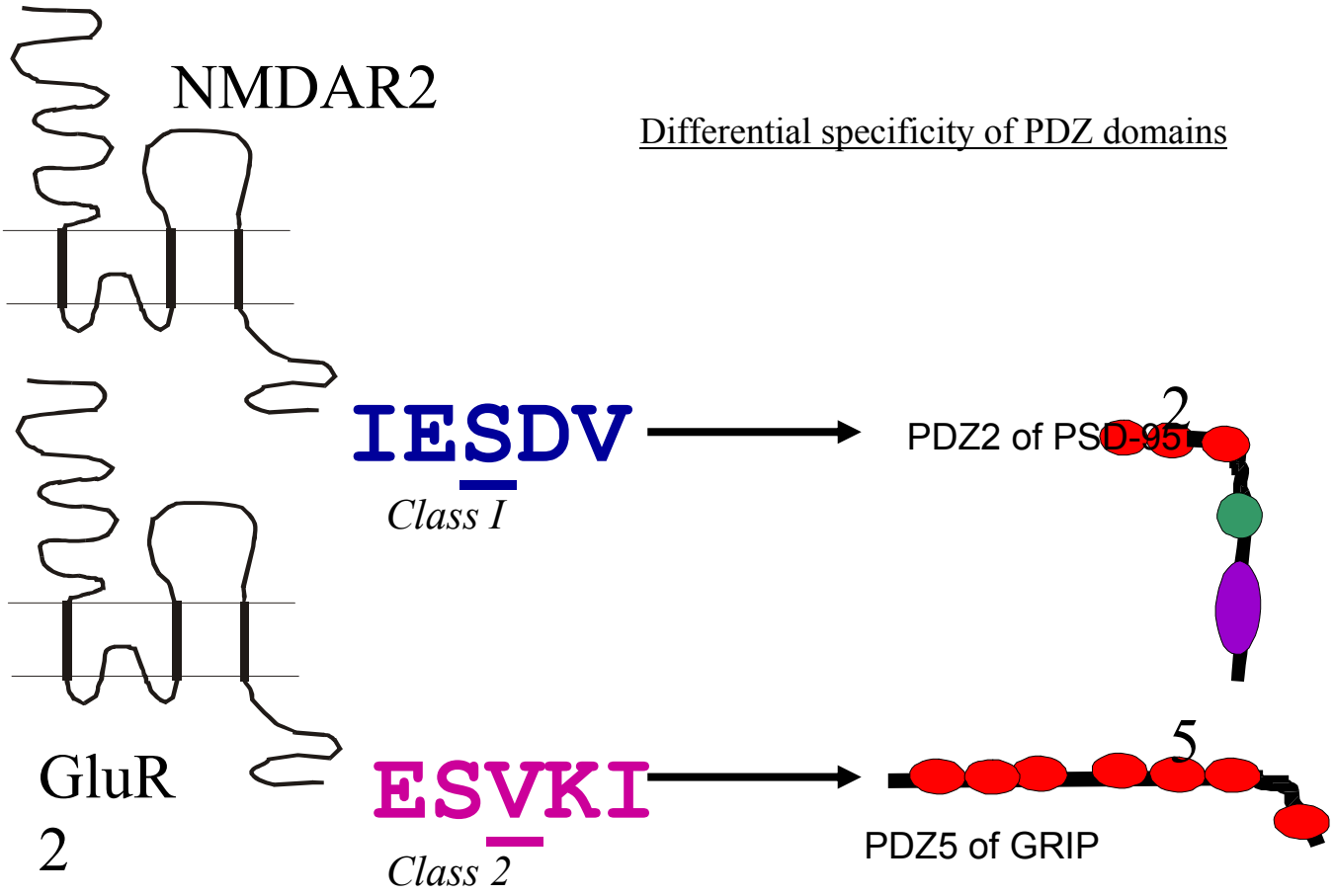
PSD-95 family of proteins: originally identified as abundant components of PSD by biochemistry
Colocalize with NMDARs in excitatory synapses (in PSD); in a coimmunoprecipitable complex
Cluster NMDARs and Shaker in heterologous cells (cf rapsyn and AchR)
Dlg is Drosophila homolog of PSD-95, binds and colocalizes with Shaker K+ channel (-ETDV) in larval NMJ (glutamate synapse)
Genetics: Dlg loss of function results in abnormal synaptic morphology and loss of Shaker clustering; C-terminus of Shaker directs heterologous protein to NMJ (in Dlg WT flies)
PSD-95 binds to cytoplasmic proteins as well as transmembrane proteins, e.g. nNOS and SynGAP (a ras GAP). PSD-95/Dlg not only involved in synaptic targeting of ion

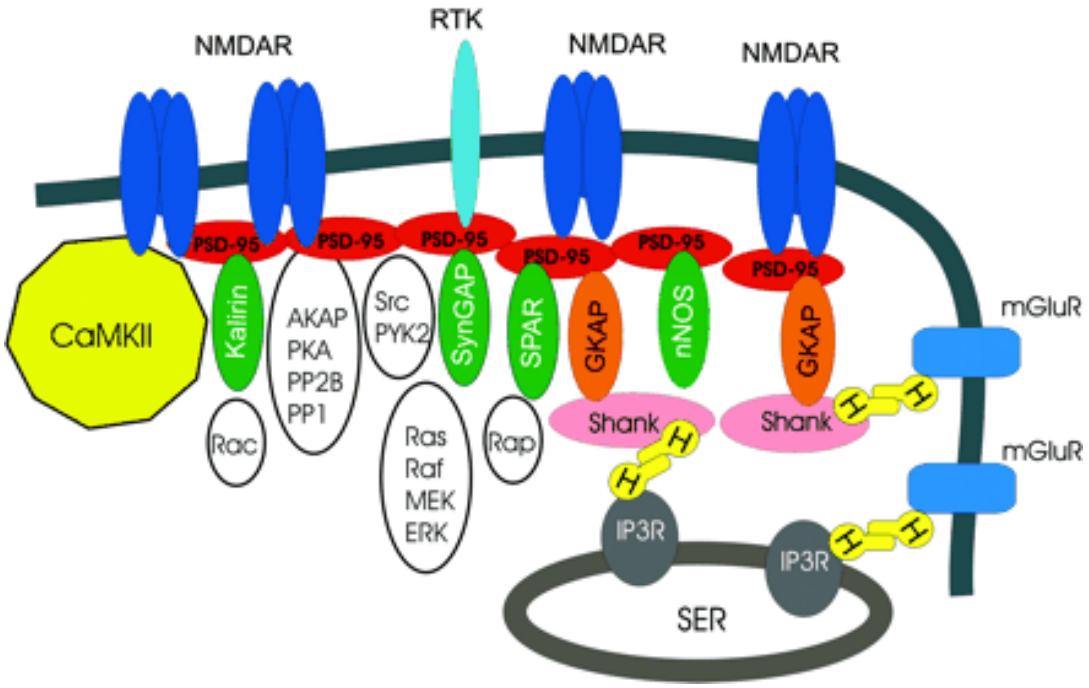
channels and receptors, but also links them to functionally coupled proteins e.g. nNOS coupled to NMDA receptors through PSD-95
Other membrane proteins that might be clustered at synapses through binding to PSD-95 (neuroligin, erbB4, PMCA4b); multi-PDZ scaffolds can organize a cluster containing several different proteins with the appropriate C-termini (heterogeneous clusters of functionally related proteins)
Dlg/PSD-95 proteins may link to cytoskeleton via 4.1/ERM actin binding proteins, microtubule binding proteins
Dlg/PSD-95 protein complex is very complicated and likely to be heterogeneous and dynamic (regulated in space and time)



Structure of a PDZ domain complexed with a C-terminal peptide ligand, based on PDZ3 of PSD-95 complexed with CRIPT (Doyle, 1996; Niethammer, 1998). The ribbon diagram of the PDZ domain (gray) is shown bound to the peptide ligand (main chain represented in yellow). The structures in A and B are slightly rotated relative to each other to better show particular sets of interactions. A. The free carboxylate group (orange) of the C-terminal residue (0 position) of the peptide interacts with the conserved amino acids (Arg-318 and Gly-Leu-Gly-Phe) of the carboxylate binding loop (red). The side chain of the -3 residue (glutamine; light green) interacts with ØB2 (asparagine) and ØC4 (serine; dark green). B. The hydroxyl group of the -2 residue (threonine; light blue) interacts with the sidechain of ÆB1 (histidine; dark blue). The side chain of the -1 residue (serine; light purple) of the CRIPT peptide shows no interactions with the PDZ domain. However, ØB2 and ØC5 residues (dark purple) are likely to influence selectivity at the -1 position of the peptide ligand.
From Sheng and Sala, PDZ domains and the organization of supramolecular complexes. Annu Rev Neurosci. 2001;24:1-29. Review.

Differential specificity of PDZ domains





A schematic of the NMDAR-associated protein complex. Major individual proteins of the PSD are shown as colored shapes, and their interactions are indicated by overlapping shapes (see text for details). Some specific sets of interacting proteins (e.g., the Ras-MAPK pathway) are grouped together for simplicity. The proteins GKAP, Shank, and Homer (H) link together the NMDAR complex, metabotropic glutamate receptors (mGluR), and IP_3 receptors (IP3Rs). It should be emphasized that the PSD is a dynamic structure, in which protein interactions may be transient and stoichiometries variable. nNOS, neuronal nitric oxide synthase; RTK, receptor tyrosine kinase; SER, smooth endoplasmic reticulum.

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The PSD can be considered a huge signaling complex organized around a set of scaffold proteins such as PSD-95 and coupled to postsynaptic glutamate receptors and other receptors (e.g. receptor tyrosine kinases and cell adhesion molecules).

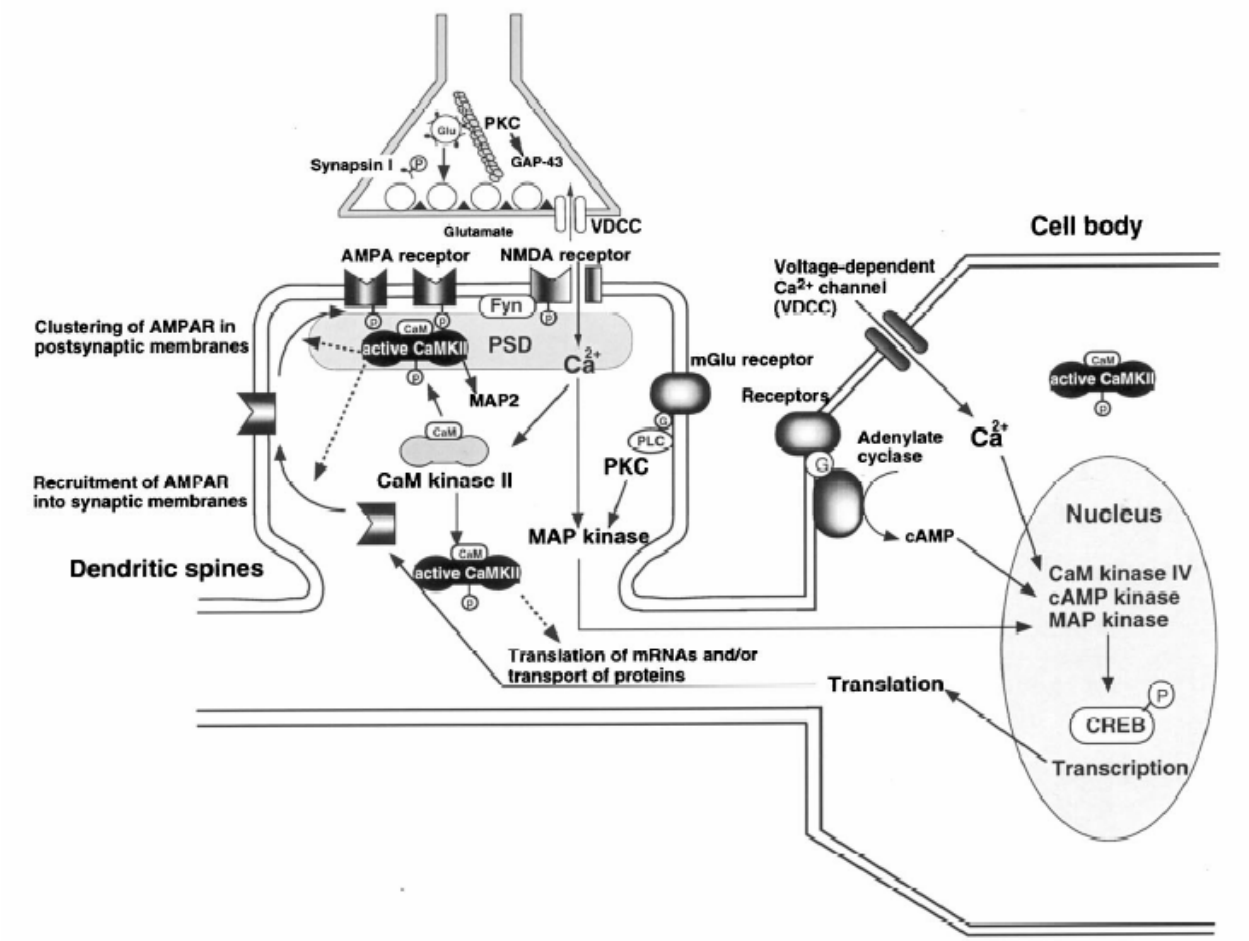


Diagram of major postsynaptic signaling pathways

Activation of NMDA receptors leads to calcium influx into dendritic spines.
Ca2+ is the central second messenger in postsynaptic signaling
Calcium binds to calmodulin (CaM, a ubiquitous calcium binding protein and calcium sensor).
Ca/CaM binds to and activates downstream enzymes such as:

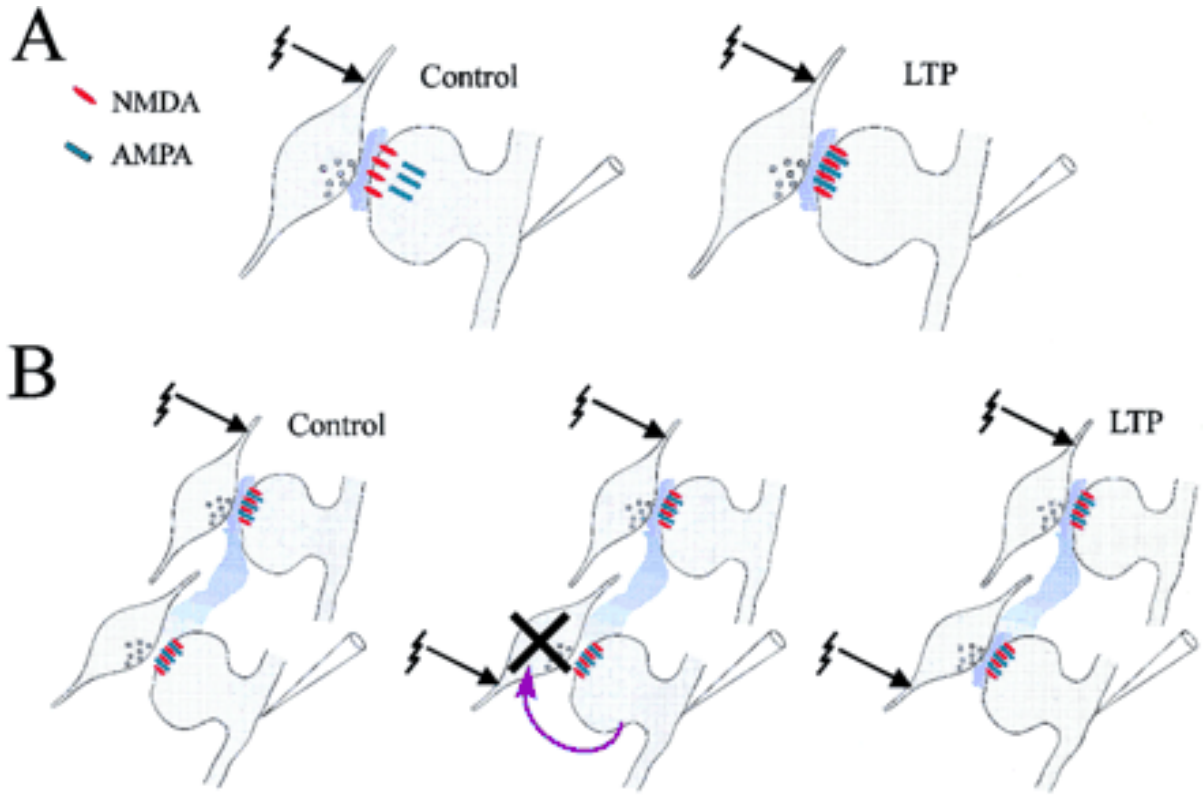
<u>calcium/calmodulin-dependent protein kinase II (CaMKII)</u>
a highly abundant enzyme of the PSD that is additionally recruited to PSD by translocation after activation
activation of CaMKII involves autophosphorylation that renders it Ca/CaM independent (constitutively active)
CaMKII necessary and sufficient for long term potentiation (LTP)
CaMKII phosphorylates AMPA receptors directly causing increased unitary conductance
CaMKII induces the synaptic delivery of more AMPA receptors (particularly GluR1 containing AMPA receptors)
CaMKII activates translation of dendritic mRNAs
CaMKII activates the Ras-MAPK pathway via unknown mechanism

calcineurin (Ca/CaM-activated protein phosphatase)
leads to activation of protein phosphatase-1 (PP1)
both calcineurin and PP1 are required for long term depression (LTD)
both calcineurin and PP1 dephosphorylates important synaptic proteins such as AMPA receptors, CaMKII, etc

Postynaptic group I mGluRs (mGluR1, mGluR5)

can cooperate with NMDA receptors to raise intracellular calcium by activating Gq proteins, and subsequently PLC (to generate DAG and IP3) and IP3 receptor and release of calcium from intracellular stores (SER).

Silent synapses



Models of LTP at Silent Synapses in the Hippocampus

(A) Synapses express functional NMDA receptors but not AMPA receptors (left). Following LTP (right), functional AMPA receptors are expressed.
(B) Glutamate can spill over from one synapse to another, resulting in the activation of NMDA receptors but not AMPA receptors, due to the higher affinity of NMDA receptors (left). Before LTP (middle), the synapse is silent because it cannot release neurotransmitter when activated. LTP results from the release of a retrograde messenger that turns on the presynaptic bouton, so that it now can release neurotransmitter (right).

Morphological (immunoEM and LM) evidence for silent synapses – labeling with NMDA receptor antibodies but not with AMPA receptor antibodies.

Silent synapse can also arise from presynaptic mechanisms –
slow rate of release of glutamate is sufficient to activate NMDA receptors (higher affinity for glutamate) but not to activate AMPA receptors (low affinity for glutamate)
[see Renger et al A developmental switch in neurotransmitter flux enhances synaptic efficacy by affecting AMPA receptor activation. Neuron. 2001 Feb;29(2):469-84.]

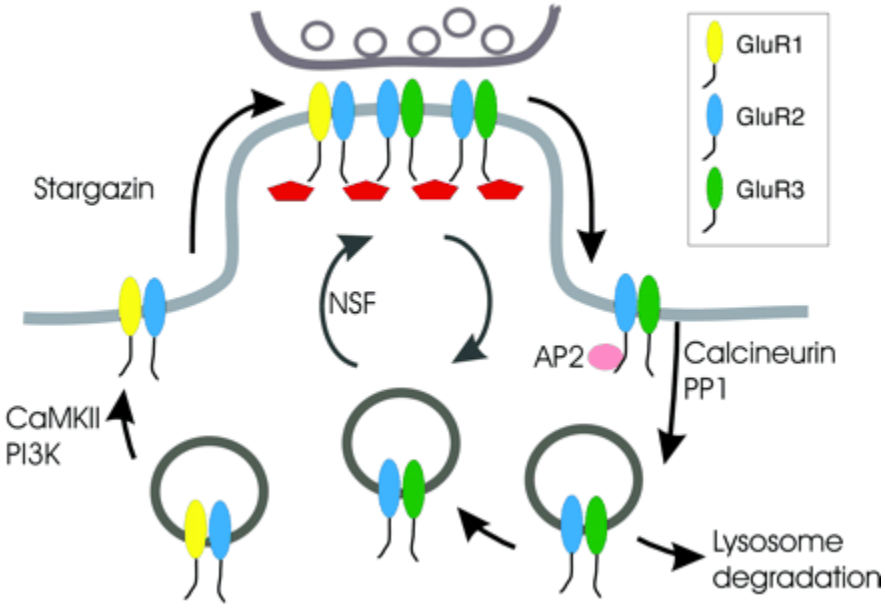
The dynamics of AMPA receptors

GluR2/3 (-ESKVI) cannot bind PSD-95 but instead bind to PDZ5 of GRIP (7 PDZs; no other recognizable domains) and PDZ domain of PICK-1; GluR1 C-terminal binds to SAP97, a close relative of PSD-95. These scaffold proteins are more implicated in trafficking rather than anchoring.

GluR2 C-terminal interfering peptide inhibits synaptic AMPAR synaptic clustering in cultured neurons

AMPA receptors and their associated proteins are “core” components of the PSD, but easily extracted from PSD by weak detergents (in contrast to NMDA receptors and PSD-95, which are highly insoluble components of the PSD)

AMPA receptors are much more dynamically regulated: evidence for rapid constitutive insertion and internalization (recycling) of AMPA receptors between postsynaptic membrane and intracellular pools. Culture studies suggest that even in basal conditions, AMPA receptors may turn over on the cell surface with a half life on the order of an hour or two. During synaptic activity, this can increase even further.



Model of AMPAR trafficking. AMPARs cycle between the postsynaptic membrane and intracellular compartments. Via a NSF-dependent mechanism, intracellular GluR2-GluR3 receptors exchange constantly with synaptic receptors. GluR1-GluR2 heteromeric receptors are "retained" in intracellular compartments but are delivered to the dendrite surface upon activation of NMDARs CaMKII and PI3K. Exocytosis of GluR1-GluR2 receptors occurs at extrasynaptic sites and is followed by lateral translocation into synapses (both these steps

requiring Stargazin). The number of postsynaptic AMPARs also depends on the availability of specific anchoring proteins (red pentagons) that bind to AMPAR subunits. The activation of calcineurin and PP1 leads to the recruitment of the AP2 clathrin adaptor complex to AMPARs, resulting in endocytosis. Internalized AMPARs can be recycled to the surface or sorted to lysosomes for degradation. Circles in presynaptic terminal represent synaptic vesicles.

Receptor / ion channel	**Interacting protein**	**Evidence for targeting function of interacting protein**
Nicotinic AChR	Rapsyn / 43K	Synaptic colocalization in NMJ; 1:1 association; loss of NMJ clustering of AChR in rapsyn KO mice
Glycine receptor β-subunit	Gephyrin	Synaptic colocalization; loss of GlyR synaptic clustering with gephyrin antisense and KO. Gephyrin may be important also for GlyR signaling
GABA(A) receptor γ2 subunit GABA(A) receptor	GABARAP Gephyrin	? No direct interaction, but gephyrin required for GABA(A)R clustering in GABAergic synapses
Shaker K+ channel	Dlg / PSD-95 family	Synaptic colocalization in Drosophila NMJ; loss of synaptic clustering of Shaker in Dlg mutants; Sh C-terminus confers synaptic localization
NMDA receptor NR2A-D	PSD-95 family	Colocalization in PSD; but synaptic clustering of NMDARs retained in PSD-95 KO [caveats?]; PSD-95 is important for NMDA receptor signaling
NMDA receptor NR1	Actinin, spectrin	Colocalization in dendritic spines, but localization can be dissociated by latrunculin B; actinin is important for NMDA receptor function/gating
AMPA receptor GluR2/3	GRIP / ABP NSF	Weak colocalization in synapses; may be more important in trafficking of AMPAR Prob involved in conformational changes / trafficking of AMPAR
mGluR1α/5	Homer	Colocalization in synapses; Homer probably more important for mGluR signaling
VGNaCh	Ankyrin G	Colocalization at nodes of Ranvier and initial axon segments; loss of initial axon clustering of VGNaCh in ankyrin G KO