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REVIEW

Molecular architecture of glycinergic synapses

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Abstract Synapses can be considered chemical machines, which are optimized for fast and repeated exocytosis of neurotransmitters from presynaptic nerve terminals and the reliable electrical or chemical transduction of neurotransmitter binding to the appropriate receptors in the postsynaptic membrane. Therefore, synapses share a common repertoire of proteins like, e.g., the release machinery and certain cell adhesion molecules. This basic repertoire must be extended in order to generate specificity of neurotransmission and allow plastic changes, which are considered the basis of developmental and/or learning processes. Here, we focus on these complementary molecules located in the presynaptic terminal and postsynaptic membrane specializations of glycinergic synapses. Moreover, as specificity of neurotransmission in this system is established by the specific binding of the neurotransmitter to its receptor, we review the molecular properties of glycine receptor subunits and their assembly into functional glycine receptors with different functional characteristics. The past years have revealed that the molecular machinery underlying inhibitory and especially glycinergic postsynaptic membrane specializations is more complex and dynamic than previously anticipated from morphological studies. The emerging features include structural components as well as signaling modules, which could confer the plasticity required for the proper function of distinct motor and sensory functions.

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Presynaptic terminals

Fast chemical signaling between neurons of the central nervous system (CNS) requires that neurotransmitters, which are secreted in a highly regulated manner from presynaptic nerve terminals cross the synaptic cleft and bind to their receptors in the postsynaptic membrane (Fig. 1). While postsynaptic scaffolding molecules differ distinctly between excitatory and inhibitory synapses, the complement of presynaptic scaffolding molecules (Schoch and Gundelfinger 2006) seems to be highly similar at the two types of synapses. No obvious differences in the release machinery and the composition of the cytomatrix of active zones (CAZ) have been demonstrated. However, genetic deletion of such pan-synaptic scaffolding proteins often has different effects on excitatory than on inhibitory presynaptic terminals. This holds true, e.g., for Munc13s (Schoch et al. 2002; Varoqueaux et al. 2002), RIM1α (Schoch et al. 2002), and CASK (Atasoy et al. 2007). Likewise, triple knockout of the synaptic vesicle associated cytomatrix proteins Synapsin 1, 2 and 3 changes the kinetics of synaptic depression, but not basal transmission, at excitatory synapses, whereas the kinetics of depression are unchanged, but basal transmission is reduced, at inhibitory synapses (Gitler et al. 2004). These data suggest that synapse- or even neurotransmitter-specific effectors may exist, which could mediate differential effects at excitatory and inhibitory release sites. These yet unknown effectors could include novel proteins as well as differential posttranslational modifications of known molecules.

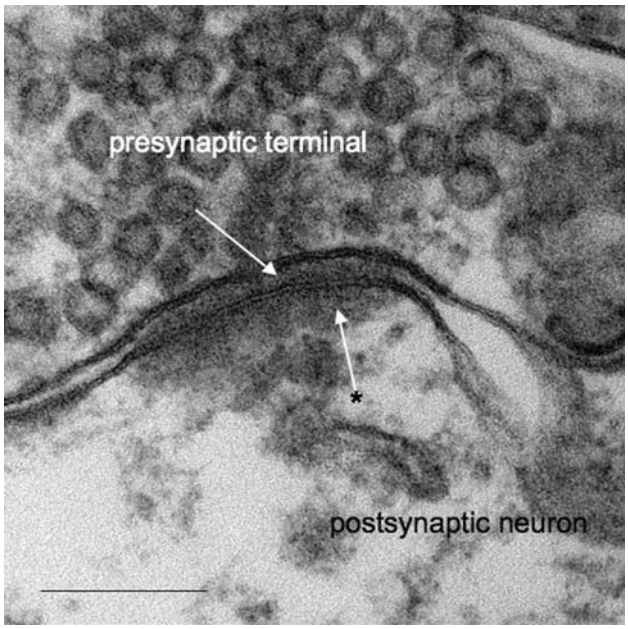

Fig. 1 Transmission electron microscopical image of a symmetrical synapse in rat spinal cord. Compared to excitatory synapses, only little electron dense material (*arrow with asterisk*) is present underneath the postsynaptic membrane of inhibitory synapses. The *white arrow* points at electron dense material in the synaptic cleft. *Bar* 100 nm

After membrane fusion and neurotransmitter release glycine is cleared from the synaptic cleft by two types of glycine transporters (GlyT), which belong to the family of Na^+/Cl^--dependent neurotransmitter transporters. GlyT1 and GlyT2 share an amino acid sequence identity of approximately 50%, but differ in their expression patterns, subcellular localization, and functional properties (Gomeza et al. 2003b).

GlyT1 is abundantly expressed in the CNS and transcripts are also found in liver. The transporter can be found in brain regions known to lack glycinergic transmission like the diencephalon, the olfactory bulb, and the cortex. GlyT1 expression is predominantly observed in glial cells and in neuronal elements throughout the brain, where it is closely associated with glutamatergic pathways. In glutamatergic neurons, it is found in the presynaptic bouton as well as a major component of the postsynaptic density (Cubelos et al. 2005). GlyT1 knock-out mice are anatomically normal but show severe motor and respiratory deficits, which lead to death during the first postnatal day (Gomeza et al. 2003a). The symptoms are very similar to those seen in human glycine encephalopathy, which is characterized by lethargy, hypotonia, myoclonic jerks, progressive apnea and often death. In brainstem slices of GlyT1-deficient mice the activity of the respiratory network is strikingly reduced but normalized by the addition of the competitive glycine receptor antagonist strychnine. Gomeza et al. (2003a) could show that the loss of GlyT1 results in an increase of the glycine concentration in the synaptic cleft of glycinergic synapses, due to impaired re-uptake into surrounding glial cells (Gomeza et al. 2003a). Thus, GlyT1 is essential for regulating glycine concentrations in the cleft of glycinergic synapses during early postnatal life.

It is believed, that the GlyT1 transport with a stoichiometry of $2Na^+/Cl^-$/glycine (Roux and Supplisson 2000) allows a bi-directional transport, which is appropriate for the regulation of glycine concentrations in the synaptic cleft. The GlyT1 expression in astrocytes near synaptic junctions and the synaptic localization in glutamatergic neurons are suggestive of its role as a modulator for glycine concentrations in the extracellular space. As glycine is also an essential co-agonist for the glutamatergic N-methyl D-aspartate (NMDA) receptors, GlyT1 might also effectively modulate NMDA receptor functions by regulating the availability of glycine in the synaptic cleft. This notion was confirmed by the demonstration of a modulating effect of GlyT1 at glutamatergic synapses, where a reduction of GlyT1 leads to an increase in NMDA receptor-evoked excitatory postsynaptic potentials (Yee et al. 2006).

GlyT2 expression is restricted to regions with mostly glycinergic transmission, e.g., in the cerebellum, brainstem and the spinal cord. In the aforementioned CNS regions GlyT2 is exclusively expressed by glycinergic neurons and localized in presynaptic terminals adjacent to the active zones and opposite glycinergic membrane specializations of the postsynaptic neuron (Gomeza et al. 2003c). Mice deficient in GlyT2 are normal at birth but develop a lethal motor deficiency during the second postnatal week reminiscent of severe forms of human hyperekplexia (hereditary startle disease). This rare disease is characterized by spasticity, tremor, and an inability to right. In hypoglossal motoneurons and dissociated spinal neurons from GlyT2-deficient mice the amplitudes of glycinergic miniature inhibitory currents (mIPSCs) were strikingly reduced (Gomeza et al. 2003c). Thus, postnatal GlyT2 function is crucial for efficient neurotransmitter reloading into synaptic vesicles of glycinergic nerve terminals.

Postsynaptic membrane specialization

The inhibitory glycine receptor

The amino acid glycine acts as co-agonist on excitatory NMDA receptors, and as the major of several agonists including β-alanin and taurin on glycine receptors (GlyRs). In the adult CNS, GlyR activation leads to an increased chloride conductance of the postsynaptic cell, resulting in hyperpolarization and inhibition of the postsynaptic neuron (Betz and Laube 2006; Kirsch 2006).

Glycine receptors belong to the Cys-loop ion channel superfamily

The GlyR was the first neurotransmitter receptor to be isolated from the mammalian CNS. The use of amino-strychnine affinity chromatography allowed the purification of two kinds of subunits, named α (Mw: 48 kDa) and β (Mw: 58 kDa), together with a 93 kDa protein now called gephyrin (Pfeiffer et al. 1982). Each of these subunits contains a large extracellular N-terminal ligand-binding domain, followed by four α helical transmembrane segments (TM1–TM4). The TM2 segments contribute to the lining of the ion channel pore (Fig. 2a). A similar membrane topology is also found in other neurotransmitter receptors, including the nicotinic acetylcholine receptor (nAChR), the 5-hydroxytryptamine type-3 (5-HT_3R) receptor and the γ-aminobutyric acid (GABA) type A and C receptors. Along with these proteins, the GlyR makes up the superfamily of Cys-loop ligand-gated ion channel receptors. The term Cys-loop refers to a common disulfide bond-mediated extracellular loop, that was first identified in the synaptic protrusion of the *Torpedo* nAChR (Lester et al. 2004; Sine and Engel 2006).

Interestingly, mutations in the genes for Cys-loop receptors have been shown to alter their kinetic properties (the channels may open too slowly, or close too quickly or too slowly) and to cause neurological diseases. For example, mutations in the GlyR α subunits were found in patients suffering from hyperekplexia (hereditary startle disease). These patients show symptoms of reduced synaptic inhibition, such as elevated muscle tone or exaggerated responses to noise. Mouse mutants, such as the *spastic (spa)* mouse, are characterized by reduced levels of GlyR β subunits, owing to a LINE1 element insertion and exon skipping in the β subunit gene (Kingsmore et al. 1994; Mulhardt et al. 1994). Recently, compound heterozygote mutations in the GlyR β subunit were found in a patient with hyperekplexia. In heterologous systems, these mutant proteins exhibit reduced sensitivities to glycine-mediated activation (Rees et al. 2002). These studies and others on Cys-loop receptors (Sine and Engel 2006) greatly help to clarify, which kind of structural changes in these ion channels alter their functional properties.

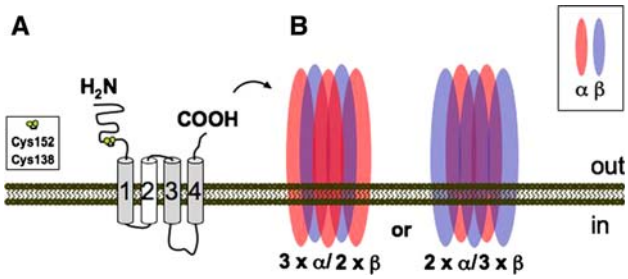

Fig. 2 Model of the structure of the strychnine-sensitive GlyR. **a** Schematic representation of the transmembrane topology of GlyR subunits (α and β subunits share about 50% identical amino acids and a very similar membrane topology). Following the amino-terminal ligand binding domain [which also includes the disulfide bond that gives Cys-loop receptors their name (see *inset*)], there are four transmembrane segments (1–4 for TM1-TM4), that are linked by one extracellular and two intracellular loops. The large loop between TM3 and TM4 of the β subunit contains the gephyrin-binding site. The carboxy-terminus of the polypeptide chain is localized to the extracellular space. **b** Schematic representation of pentameric GlyR heterodimers. Shown are two models, which differ in the stoichiometric ratios of their α and β subunits. Current data (see text) favor the presence of 2 α and 3 β subunits in a pentamer. Note, that there is also evidence for homo-oligomeric receptors built exclusively from α2 subunits (see also Fig. 2)

Heterogeneity of the GlyR α and β subunits

Molecular cloning approaches first discovered the existence of various isoforms of GlyR subunits (Kuhse et al. 1991a, b). At present, vertebrate GlyR subunits are known to be encoded by five separate, but related genes. There are four α subunits genes (GLRA1-4, encoding α1–α4) and a single one encoding the β protein (GLRB) (Laube et al. 2002; Lynch 2004). Alternative splicing and RNA editing can generate further heterogeneity among the subunits (Lynch 2004; Meier et al. 2005). In the α1, α2 and α3 subunits, the alternative exons modify the structures of the extracellular domain or of the intracellular loop regions. Little is known about the functional consequences of these modifications. An eight amino acid insertion into the large intracellular loop of the rat α1 subunit adds a consensus site for protein kinase A (Malosio et al. 1991) and thus, a potential element for regulating receptor function. RNA editing can generate α3 subunits, whose extracellular domain has a higher affinity to glycine than the unedited form (Meier et al. 2005). The edited subunit is suggested to confer high agonist affinities to extrasynaptically localized GlyRs.

Even less is known about sequence variations in the β subunit. Splice errors in the GLRB gene can lead to compound heterozygote mutations, as found in patients with hyperekplexia (Oertel et al. 2007; Rees et al. 2002). Recently, a novel splice variant lacking exon 7 (encoding TM 1 and 2) of the β transcript (βΔ7) has been described in glial and other non-neuronal cells (Oertel et al. 2007). Over-expression of this polypeptide in heterologous cells, either alone or in combination with the α1 subunit, shows that it integrates into the cell membrane and associates with the α1 subunit and gephyrin. These properties are interesting, given that this β splice variant lacks TM1 and TM2 due to the splicing event, while its physiological properties subunits remain unchanged upon co-expression with the α1 subunit (Oertel et al. 2007).

A revised stoichiometry of α and β subunits

Native adult GlyRs contain α1 and β subunits, while native neonatal GlyRs consist of α2 and β subunits (Lynch 2004; Malosio et al. 1991). As with other Cys-loop receptors, all subunits contribute to the assembly of the central pore (Fig. 2b). But what is the actual stoichiometry of the subunits in an individual receptor complex? Initial cross-linking studies using purified spinal cord GlyR suggested that the receptor forms a pentamer with a deduced stoichiometry of three α and two β subunits and that the β subunit was not involved in ligand binding (Langosch et al. 1988; Laube et al. 2002). Through its long intracellular loop between TM3 and TM4, the β subunit can bind to gephyrin (Kim et al. 2006; Meyer et al. 1995; Sola et al. 2004), thereby enabling the formation GlyR aggregations linked to subsynaptic protein scaffolds. However, the gephyrin scaffold (see below) is believed to provide three binding sites for GlyR β subunits (Schrader et al. 2004). Recent evidence provides a solution to this apparent inconsistency. Heterologous expression and metabolic labeling of wild-type α and β subunits and an α1β tandem construct in *Xenopus laevis* oocytes followed by affinity purification suggest that oocytes can assemble hetero-oligomeric GlyRs composed of two α and three β subunits (Grudzinska et al. 2005). Therefore, the revised stoichiometry would be consistent with the proposed binding of gephyrin trimers to three cytoplasmic loops of β subunits within one receptor complex.

Ligand binding sites

Inhibitory glycinergic transmission in spinal cord and brain stem is critical for the processing of motor and sensory information that controls activities such as movement, vision or audition as well as inflammatory pain sensitization (Harvey et al. 2004b; Legendre 2001). These properties can be influenced by a number of components. In addition to glycine, β-alanine and the sulfonic acid taurine, GlyRs bind with high affinity to (and can be selectively blocked with) the competitive antagonist strychnine (Betz and Laube 2006). Other components that modulate GlyR activity include zinc, alcohol and anesthetics, picrotoxin, cocaine and some anticonvulsants (Leite and Cascio 2001).

Excitatory GlyR function and cellular signaling

During neuronal maturation a switch from excitatory to inhibitory actions of GABA and glycine was observed (Rivera et al. 1999). In mature neurons, the neuronal K^+/Cl^--cotransporter 2 (KCC2) carries molar ratios of K^+ and Cl^--ions across the neuronal plasma membrane, leading to the extrusion of Cl^- from the cell interior and thus shifting the Cl^--equilibrium to more negative values. Low expression and/or reduced function of this cotransporter in young neurons are thought to be the reason for high intracellular Cl^- concentrations during embryonic and early postnatal time periods. Driven by the difference of the Cl^--equilibrium potential and the resting potential the outward directed Cl- flux may cause depolarization or shunting inhibition of the postsynaptic membrane.

Thus, in young neurons, GlyR or $GABA_A$ receptor activation may cause depolarization and thereby elicit Ca^{2+}-fluxes through voltage dependent Ca^{2+}-channels and consequently intracellular signaling. This signaling is essential for the formation or maintenance of postsynaptic clusters of GlyR in cultured spinal cord neurons (Kirsch and Betz 1998). Chronic inhibition of GlyR activity in these experiments resulted in the loss of GlyR clusters. Importantly, similar results were obtained with inhibition of L-typ Ca^{2+}-channels, suggesting that GlyR activity is coupled to Ca^{2+}-signaling in young neurons (Kirsch and Betz 1998). Consistently, GlyR function was shown to affect differentiation of retinal rod photoreceptors (Young and Cepko 2004). Similarly, GlyR function was shown to be crucially involved in interneuron differentiation in zebrafish (McDearmid et al. 2006). For $GABA_A$ receptors, GABA-induced excitation was shown to cause the expression of brain derived neurotrophic factor (Berninger et al. 1995) and NeuroD (Ge et al. 2007), however, much less is known about GlyR regulated gene and protein expression profiles. Interestingly, it was demonstrated recently, that the neonatal GlyR α2 subunit may bind different cellular signaling molecules like eEF1A, p70S6 kinase and calcineurin (Bluem et al. 2007), supporting the hypothesis that also the GlyR might be important for the specific regulation of neuronal protein expression (Fig. 3a). eEF1A is a multifunctional protein, which is important for protein synthesis and also actin bundling (Bunai et al. 2006; Gross and Kinzy 2005). Thus, it is well possible that α2 homo-oligomeric receptors might connect to actin-filament bundles through the interaction of α2 subunits with eEF1A (Fig. 3a). Recent data suggest that also GlyR isoforms, consisting from α and β subunits are involved in the regulation of cellular differentiation. Analyzing the adult *Spastic* mouse mutants, in which the OFF signal transmission in the rod pathway is largely blocked due to a reduction of GlyR-expression, Xu and Tian (2008) showed that these mice had altered retinal ganglion cell (RGC) light-evoked synaptic inputs from ON and OFF pathways. The *spastic* mutation also blocked the developmental redistribution of RGC dendrites from the center to sublamina of the inner plexiform layer, suggesting that visual stimulation regulates the maturation of RGC synaptic activity and connectivity primarily through GlyR-mediated synaptic transmission (Xu and Tian 2008). In

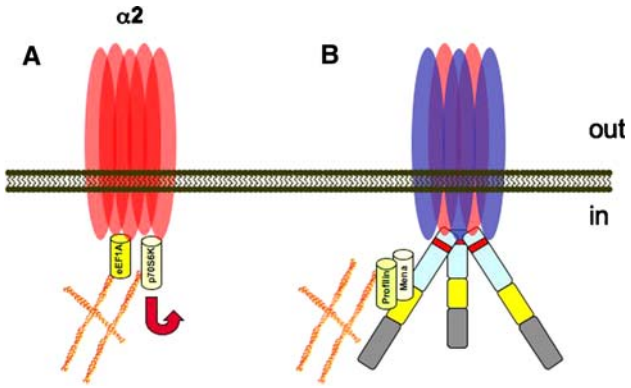

Fig. 3 Model of homo- and hetero-oligomeric GlyR anchoring and signaling. **a** α2 homo-oligomeric GlyRs are thought not to bind directly to gephyrin, instead the interaction with the actin-bundling protein eEF1A might be involved in binding to microfilaments. In addition, the association of p70S6 kinase with the GlyR α2 subunit might be involved in regulating cellular signaling important for receptor localization and/or protein synthesis. **b** α/β hetero-oligomeric receptors are bound to gephyrin by interacting with β subunits. Direct interaction of profilins and Mena/VASP might mediate the gephyrin-actin filament

addition, GlyR and $GABA_A$ receptors might act in concert, as it was shown that both agonist glycine and GABA contribute to the regulation of neurite outgrowth in developing spinal cord neurons (Tapia et al. 2001).

Subunit switch, assembly and function of GlyR isoforms

The switch from depolarization to hyperpolarization is thought to coincide in time with the replacement of the neonatal GlyR α2 subunit by the adult α1 and α3 subunits in most regions of the CNS, however, a detailed characterization of this process is still lacking. Moreover, the complete down-regulation seen for the expression of GlyR α2 subunits in, for example, spinal cord could not be observed in retina, in auditory brain stem (Piechotta et al. 2001) and hippocampus. In retina, dense immunoreactive puncta are detected with an α2 subunit-specific antibody in the inner plexiform layer of adult mouse tissue (Haverkamp et al. 2004). Thus, the mechanisms of the regulation of the GlyR α2 subunit might be more complex than that of α1 and α3 homologs.

The most abundant GlyR subunit in the retina is the α3 subunit, followed by GlyR α2 and GlyR α1 subunits. Approximately one-third of the α3 subunit puncta were colocalized with α2 puncta. It is unknown, however, whether these receptors subunits are forming α2-α3 hetero-oligomeric receptors in vivo. More likely, different receptor subunit isoforms are forming distinct pentameric receptors, as specific neurons express different GlyR α subunits and display a specific and distinct pattern of immunoreactivities for these subunits (Haverkamp et al. 2004). It is generally believed that α1, α3 and also α2 subunits are forming hetero-oligomeric receptors with the β subunit. The hetero-oligomeric GlyR 2xα/3xβ receptors are characterized by smaller channel conductance (about 50 pS) (Bormann et al. 1993), lower sensitivity to picrotoxin (Grenningloh et al. 1990) and β-carboline (Mangin et al. 2005). In contrast, α2 subunits are thought to form also homo-oligomeric receptors, characterized by larger channel conductivity (>50 pS) and slower kinetics of channel gating (Singer et al. 1998). Slow channel kinetics might impair synaptic transmission and therefore α2 homo-oligomeric receptors might be localized mostly extrasynaptically, where they may be involved in tonic inhibition (Mangin et al. 2003). Consistently, in hypothalamic supraoptic nuclei neurons, dispersed axonal GlyR immunoreactivity was reported (Deleuze et al. 2005). However, in cultured hippocampal neurons and retina, α2 subunits are clearly localized in large puncta. In the retina about 50% of these puncta are colocalized with gephyrin, suggesting the presence of α2/β-hetero-oligomeric receptors at postsynaptic membrane specializations. Moreover, also in hippocampal tissue and cultured neurons GlyR α2 subunits are found in clusters, both synaptically and extrasynaptically (Brackmann et al. 2004; Danglot et al. 2004). Pharmacological and single channel analysis showed that in acutely prepared hippocampal slices GlyRs in interneurons and CA1 pyramidal cells are mostly homo-oligomeric, picrotoxinin sensitive channels with larger single channel conduction states (>100 pS) whereas hetero-oligomeric, β subunit containing GlyRs were found with lower frequency (10–20%) (Chattipakorn and McMahon 2002). Also in cultured hippocampal neurons α2 homo-olgomeric receptors, which were replaced by β subunit containing GlyRs were observed (J. Kuhse, unpublished observation). Similar switches of GlyR subunit expression were also characterized pharmacologically in dopaminergic neurons in the *Substantia nigra* from rat (Mangin et al. 2002). In conclusion, at least three different α2 subunit localizations can be assumed; dispersed extrasynaptically, clustered extrasynaptically and clustered at synapses. Whether the synaptic localization of GlyR α2 subunit is due to co-assembly with β subunits or whether cluster mechanisms independent of the GlyR β subunit-gephyrin interaction exist, awaits further investigations.

In heterologous expression system, different α subunits are forming hetero-oligomeric receptors with variable stoichiometries, depending on the molar ratio of expressed subunit (Kuhse et al. 1993). In contrast β subunits co-assemble with GlyR α subunits with an invariant stoichiometry, which is determined by a number of crucial amino acid position in the N-terminal part of the extracellular domain (Griffon et al. 1999). Moreover, these sequences are crucial for the retardation of non-assembled β subunits within the ER, suggesting that only fully assembled pentamers that are

N-glycosylated can exit the ER and are transported to the cell surface (Griffon et al. 1999). In conclusion, the molecular mechanisms governing the assembly of GlyR subunits into distinct pentameric configurations, which are delivered to specific sites of postsynaptic membrane specializations, are still enigmatic, and mechanisms of receptor cluster formation might be different for distinct receptor complexes.

Anchoring and trafficking of GlyR

Affinity purification of GlyR using immobilized aminostrychnine and SDS-PAGE (Pfeiffer et al. 1982) resulted in three proteins. In addition to the two membrane spanning α and β subunits a heterogeneous band of Mw 93 kDa was seen. This protein could be eluted from synaptic membranes by basic pH, and therefore was characterized as peripheral membrane protein (Schmitt et al. 1987). The 93 kDa protein was demonstrated to bind polymerized tubulin with similar affinity and stoichiometry as microtubule-associated protein 2 (Kirsch et al. 1991). It was hypothesized that this protein could form a bridge between the GlyR and the underlying microtubular cytoskeleton and therefore named gephyrin (γεφυρα; Greek: bridge) upon elucidation of its primary structure (Prior et al. 1992). Gephyrin can interact with a short sequence motif (18 amino acids) within the large cytoplasmic loop of the GlyR β subunit thereby establishing a direct, continuous molecular link between hetero-oligomeric GlyRs and gephyrin (Kirsch et al. 1995; Kneussel et al. 1999b; Meyer et al. 1995). The known GlyR α subunits and many $GABA_A$ receptors did not bind gephyrin in a cellular assay (Kirsch et al. 1995). Insertion of the GlyR β subunit binding-motif was shown to alter the subcellular distribution of an excitatory neurotransmitter receptor in transfected mammalian cells. Upon coexpression with gephyrin, a mutant N-methyl-D-aspartate (NMDA) receptor containing NMDA receptor 1 subunits, which harbored a gephyrin-binding motif within its cytoplasmic tail region, was targeted to intracellular gephyrin-rich domains, so called "blobs" (Kins et al. 1999). It is believed that the gephyrin-binding motif located in a cytoplasmic domain of an integral membrane protein suffices for routing to gephyrin-rich intracellular and/or submembranous compartments. Therefore, current research interest is focused on the molecular and cellular mechanisms underlying gephyrin cluster formation. By analogy, a 10 amino acid hydrophobic motif within the intracellular domain of the $GABA_A$ receptor α2 subunit has recently been identified, that can regulate the accumulation of $GABA_A$ receptors at inhibitory synapses in a gephyrin dependent manner (Tretter et al. 2008). Incorporation of this motif was sufficient to target cluster of differentiation molecule 4 (CD4) proteins to inhibitory synapses. The binding motif was also critical for direct binding of $GABA_A$ receptor α2 subunits to gephyrin in vitro. The identification of dominant binding motifs in inhibitory receptor subunits corroborates the notion that the synaptic accumulation of GlyRs and $GABA_A$ receptors depends on their ability to bind gephyrin.

Splice variants, expression and distribution

Three splice variants of gephyrin cDNAs were identified (originally named P1–P3), which differ by the insertion of polypeptide cassettes due to alternative splicing of the pre-mRNA and additional splice variants were identified later on (Hermann et al. 2001; Meier et al. 2000; Prior et al. 1992; Ramming et al. 2000). In the meantime, the nomenclature of gephyrin splice cassettes has been revised at a recent meeting exclusively devoted to the biology of gephyrin (Fritschy et al. 2008). Their respective names now reflect the position at which the exon is inserted (i.e., in the G, C or E domains, respectively). The precise functions of individual splice cassettes are not yet known. However, there are first indications that the insertion of certain cassettes may interfere with the formation of higher order GlyR structures (Saiyed et al. 2007) or the postsynaptic stabilization of GlyRs in cultures spinal cord neurons (Meier and Grantyn 2004). Moreover, gephyrin containing the C5 cassette has been shown to be unable to catalyze the final step in molybdenum cofactor (see below) biosynthesis in vitro (Smolinsky et al. 2008), indicating that alternative splicing may change different properties of gephyrin in a given cellular context.

Unexpectedly, Northern blots revealed, that gephyrin mRNAs are expressed in many organs except those of the lymphatic system (Prior et al. 1992). In brain and spinal cord, gephyrin expression is restricted to grey matter (Kirsch et al. 1993a). Although punctate gephyrin immunoreactivity (IR) co-distributes with GlyR IR in those regions of the CNS, where GlyRs are expressed, gephyrin expression was shown to exceed by far that of GlyRs in the CNS (Kirsch and Betz 1993). Detailed analyses of brain regions devoid of GlyR expression revealed co-localization of punctate gephyrin IR with $GABA_A$ receptors, namely those harboring γ2 and/or α2 subunits (Sassoe-Pognetto et al. 1995). These findings were impressively confirmed by analyzing $GABA_A$ receptor expression in gephyrin deficient mice (Fischer et al. 2000; Kneussel et al. 1999a, 2001).

Homologous proteins

Surprisingly, the analysis of gephyrin's primary structure revealed significant sequence homologies of the amino- and carboxy-terminal thirds to proteins involved in the biosynthesis of the molybdenum cofactor (moco) (Prior et al.

1992; Stallmeyer et al. 1999), a pterin cofactor required for the activity of molybdenum-dependent oxidoreductases, namely sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase all of which are predominantly expressed in liver but also in brain (Mendel and Schwarz 2002; Schwarz and Mendel 2006). The amino terminal part of gephyrin (amino acids 1–181), now called the G-domain, shows homologies to the MogA protein, whereas the carboxy terminal part (amino acids 318–736), now called E-domain, is homologous to the MoeA protein of *E. coli*. In plants and invertebrates these two polypeptides were fused to a single protein named cnx1 (*Arabidopsis thaliana*) and cinnamon (*Drosophila melanogaster*), respectively (Stallmeyer et al. 1999). In vertebrates the two domains with homology to the aforementioned proteins are connected by an extensive central region (C-domain), which does not display homologies to known proteins in the databases (Fig. 4). Indeed, molybdopterin (a precursor of moco) binding of gephyrin and its orthologs was demonstrated (Schwarz and Mendel 2006; Smolinsky et al. 2008) and its role in the moco biosynthetic pathway is well established (Reiss et al. 2001). Curiously, the G-domain of rat gephyrin could functionally replace the corresponding protein of *Arabidopsis* mutants lacking the cnx1 protein (Stallmeyer et al. 1999) and conversely, transgenic expression of cnx1 in gephyrin-deficient mice could partially restore the activity of moco-dependent enzymes (Grosskreutz et al. 2003).

Analysis of gephyrin deficient mice

As gephyrin can bind to microtubules, it was believed that this interaction with the cytoskeleton might be important for anchoring GlyR and $GABA_A$ receptors to postsynaptic membrane specializations. This notion was verified by attenuation of gephyrin expression using antisense oligonucleotides in cultured spinal neurons (Kirsch et al. 1993b) and targeted disruption of the gephyrin gene in mice (Feng et al. 1998). In the absence of gephyrin expression no postsynaptic GlyR and only few $GABA_A$ clusters were observed in spinal cord and higher brain regions. Careful analysis of retina, spinal cord and brain sections revealed, that only the punctate staining of $GABA_A$ receptor $\alpha 1$ and $\alpha 5$ subunits was unaltered in gephyrin knock-out mice, whereas the numbers of $\alpha 2$-, $\alpha 3$-, $\beta 2/3$-, and $\gamma 2$-subunit-immunoreactive postsynaptic sites were significantly or even strikingly reduced in the mutant animals (Fischer et al. 2000; Kneussel et al. 1999a, 2001). Thus, neuronal gephyrin expression is indispensable for the formation of most inhibitory postsynaptic membrane specializations. Gephyrin knockout mice die within one day after birth. The pups do not suckle and upon mild tactile stimuli they assume a hyperextended posture reminiscent of opisthotonus in humans (Feng et al. 1998). Interestingly, transgenic expression of the plant ortholog cnx1 in gephyrin knockout mice could partially restore the activity of moco-dependent enzymes, but did not rescue the lethal phenotype of these mice. Therefore, lethality of gephyrin-deficient mice is most likely due to the loss of most postsynaptic inhibitory neurotransmitter receptor clusters from postsynaptic membrane specializations but not due to the lack of moco-dependent enzyme activities (Grosskreutz et al. 2003).

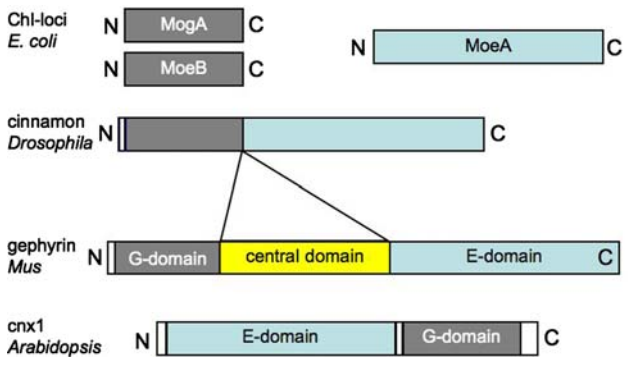

Fig. 4 Schematic representation of domain structures of gephyrin and other moco proteins. MogA and MoeA proteins of *E.coli* are homologous to cinnamon in *Drosophila melanogaster*, cnx1 in *Arabidopsis thaliana* and the G-, respectively E-domain of vertebrate gephyrin. Interestingly, G- and E-domains (*grey* and *light blue*, respectively) are swapped between gephyrin and cnx1. Note the extensive central (C-)-domain (*yellow*), which is exclusively present in gephyrin

Molecular basis of gephyrin scaffold formation

X-ray crystallography revealed that the gephyrin G-domain (amino acids 2–188) forms trimers in solution (Sola et al. 2001). A sequence motif thought to be involved in molybdopterin binding is highly conserved between gephyrin and its ortholog MogA of *E. coli* (Schwarz et al. 2001). Although the structure of the G-domain resembles that MogA, the path of its C-terminal ends suggests that the central and E- domains, which were both missing in the truncated polypeptide analyzed in the referring publication, should follow a similar 3-fold arrangement as the G-domain. Although full-length gephyrin was shown to form a trimer, in vitro proteolysis causes spontaneous dimerization of its E-domain (Lardi-Studler et al. 2007; Sola et al. 2004). As the G-domain forms trimers and the E-domain can form dimers, it is believed that gephyrin is able to form a hexagonal scaffold in vivo (Fig. 5). In fact, hexameric gephyrin was identified in blue native PAGE (Saiyed et al. 2007). It is believed that hexameric gephyrin represents an intermediate of a higher order gephyrin scaffold and that such a macromolecular scaffold could be underlying inhibitory postsynaptic membranes. The aforementioned properties are thought to provide the molecular basis of a mechanism, by which conformational transitions of

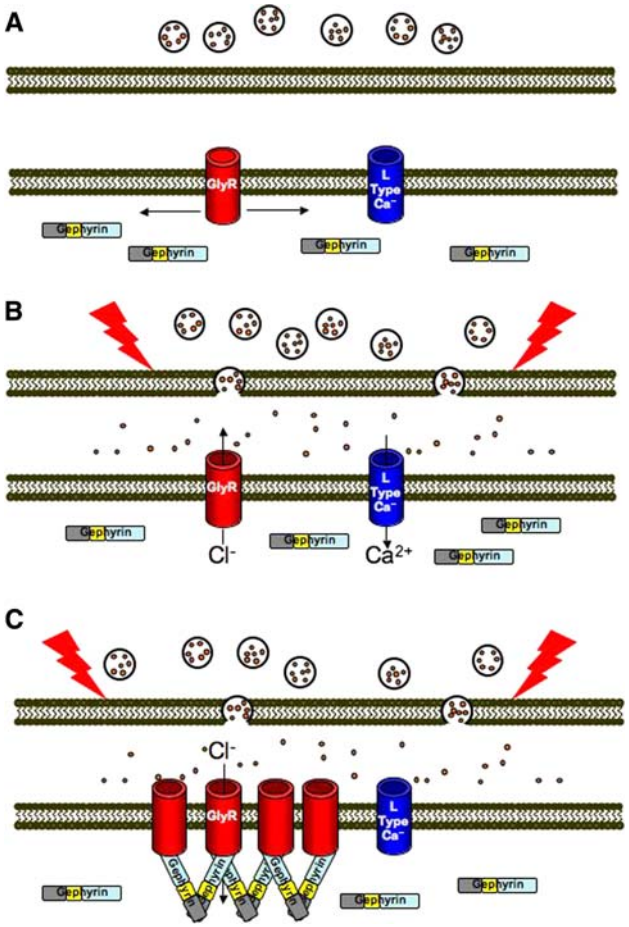

Fig. 5 Model for the activity-dependent aspect of glycinergic synapse formation. **a** Initially GlyR are freely diffusing (*arrows*) in the plasma membrane of a postsynaptic neuron. At this stage, gephyrin does not form aggregates. **b** Upon glycine release from a presynaptic terminal, GlyR activation in juvenile neurons leads to Cl^--efflux and depolarization of the postsynaptic cell followed by Ca^{2+}-influx through voltage-gated Ca^{2+}-channels (*blue*). **c** Ca^{2+}-influx can induce the aggregation of gephyrin. The gephyrin-aggregates can subsequently act as expression activation of GlyRs is now hyperpolarizing

trimeric gephyrin may generate a variable postsynaptic scaffold for GlyR recruitment and anchoring, which allows dynamic movement in and out of postsynaptic GlyR clusters and thereby generating plasticity of the postsynaptic response. This conception is in agreement with data from single particle tracking and of quantum dot-labeled GlyRs in the neuronal plasma membrane (Dahan et al. 2003; Meier et al. 2001). The high degree of sequence- and structural conservation between gephyrin G- and E-domains and the corresponding bacterial, respectively plant enzymes may reflect requirements common for both, moco biosynthesis and inhibitory neurotransmitter cluster formation. The nature behind this assumed common feature of both functions remains enigmatic, however, and therefore, it is a matter of debate, if gephyrin should be considered an ortholog or paralog to the respective moco synthesizing enzymes.

The E-domain can bind a peptide derived from the large cytoplasmic loop of the GlyR β subunit with high and low affinity. The central part of the GlyR β subunit large cytoplasmic loop is bound in a symmetric "key and lock" fashion to each E-domain monomer in a pocket adjacent to the dimer interface (Kim et al. 2006). Structure-deduced mutagenesis followed by in vitro binding and in vivo colocalization assays suggest a hydrophobic interaction between Phe 330 of the gephyrin E-domain and Phe 398 and Ile 400 of the GlyR β subunit as crucial for this interaction (Kim et al. 2006; Kneussel et al. 1999b; Meyer et al. 1995).

Interactions with cytoskeletal components

Treatment of primary spinal cord cultures with alkaloids affecting the integrity of microtubules or microfilaments, respectively, confirmed this notion and revealed that the size and packing density of gephyrin aggregates at postsynaptic membrane specializations is regulated in an antagonistic manner by these components of the neuronal cytoskeleton (Kirsch and Betz 1995). Whereas microtubules are important for aggregating gephyrin into clusters, microfilaments seem to disperse them. Thus, postsynaptic gephyrin clusters appear to be stabilized by a combination of tension and "pressure" (aggregation) elicited by both filament systems. The contribution of the cytoskeleton to the formation of postsynaptic gephyrin clusters can be considered a true "tensegrity" (condensed from "tension" and "integrity")—system, in which a structure is established and maintained by the finitely closed, comprehensively continuous, tensional behaviors of the system and not by the discontinuous and exclusively local compressional behavior of its components.

Closer insights into the nature of the interactions of gephyrin with the cytoskeleton were derived from a recent study using single particle tracking of GlyRs to assess the effects of a pharmacological dissolution of filamentous cytoskeletal elements (Charrier et al. 2006). In this study both F-actin and microtubule disruption were demonstrated to increase GlyR exchanges between synaptic and extrasynaptic membranes and simultaneously decreasing receptor dwell time at synapses. Lateral diffusion of GlyRs in the extrasynaptic membrane was predominantly controlled by microtubules, whereas the integrity of microfilaments affected both diffusion coefficients and confinement at synapses. Thus, the microtubular and actin based cytoskeleton can indeed regulate GlyR numbers at postsynaptic membrane specializations and thereby synaptic plasticity through the regulation of lateral diffusion in the plasma membrane and of receptor stabilization at synapses. It is

tempting to speculate, that cytoskeleton based mechanism could contribute to the recently discovered homeostatic regulation of GlyR surface distribution (Levi et al. 2008).

The molecular basis of the interactions of gephyrin with microfilaments (Fig. 3b) is mediated indirectly by binding to key regulators of microfilament dynamics, namely profilin I and neuronal profilin IIa, and to microfilament adaptors of the mammalian enabled (Mena)/vasodilator stimulated phosphoprotein (VASP) family, including neuronal Mena (Giesemann et al. 2003). Complex formation requires the E-domain of gephyrin, not as suspected the proline-rich central domain. Consequently, gephyrin is not a ligand for the proline-binding motif of profilins. Instead, it competes with G-actin and phospholipids for the same binding site on profilin. Gephyrin, profilin, and Mena/VASP colocalize at synapses of rat spinal cord and neurons. Thus, Mena/VASP and profilin can contribute to the postulated linkage between GlyRs, gephyrin scaffolds, and the microfilament system, which may regulate the microfilament-dependent receptor packing density and dynamics at inhibitory synapses.

Synapse formation and plasticity

The observation, that the formation of submembranous gephyrin clusters precedes the appearance of postsynaptic GlyR clusters in cultured spinal cord neurons by approximately 1 day (Kirsch et al. 1993b) was surprising, since the signals instructive for gephyrin cluster formation are likely to come from the outside. As no other molecules responsive for glycine release were known, it was hypothesized that the activation of unclustered GlyR diffusing in the plasma membrane of a postsynaptic cell could provide the basis for gephyrin scaffold formation. This hypothesis was tested by blocking GlyR activation by the addition of its competitive antagonist strychnine to the culture medium of primary spinal cord neurons (Kirsch and Betz 1998). Under these experimental conditions neither gephyrin nor GlyR cluster formation was observed. Instead GlyR IR was seen in large intracellular vesicles, most likely endosomes (Kirsch and Betz 1998; Levi et al. 1998). As the activation of juvenile GlyR is excitatory due to the high intracellular Cl^- concentration of juvenile neurons, it was assumed that GlyR-dependent depolarization could open voltage-gated Ca^{2+} channels and that Ca^{2+} influx through these channels would trigger the formation of gephyrin clusters. Gephyrin aggregates would subsequently trap GlyRs diffusing in the plane of the plasma membrane by decreasing their lateral mobility. This prediction was impressively confirmed by Meier et al. (2001) and very elegantly refined by Dahan et al. (2003), who studied individual respectively quantum dot-conjugated GlyRs to follow their movements on the neuronal cell surface in real time. In membrane areas devoid of gephyrin clusters, GlyRs were mostly freely diffusing. Gephyrin induced long confinement periods spatially associated with submembranous clusters of gephyrin. Surprisingly, even when most receptors were stabilized, frequent transitions through the diffusive state were observed. These data indicate that postsynaptic GlyR clusters are highly dynamic structures and number of GlyRs recruited into a cluster results from a dynamic equilibrium between the pools of freely mobile and stabilized receptor complexes. Moreover, culture of motoneurons with or without spinal inhibitory interneurons revealed that the transmitter phenotype of the presynaptic element is sufficient to determine accumulation of specific receptors but not of gephyrin in or underneath the postsynaptic membrane (Levi et al. 1999). A similar concept but to some extent contradicting the latter was forwarded for gephyrin by demonstrating that "packaging units" containing gephyrin and GlyRs can enter and exit active synapses within several minutes (Rosenberg et al. 2001). Interestingly dynein-dependent co-transportation of both proteins was observed, which is likely to be mediated by the interaction of gephyrin with the dynein light chain (Fuhrmann et al. 2002).

The role of GlyR activation dependent Ca^{2+}-influx for gephyrin cluster formation was corroborated by pharmacological experiments, which demonstrated that blockade of voltage-gated Ca^{2+} channels by nifedipine elicited a similar effect on gephyrin cluster formation as strychnine (Kirsch and Betz 1998) (Fig. 5). As gephyrin is not known to be a Ca^{2+}-binding protein it is still unclear, how Ca^{2+} influx can induce gephyrin clustering. In fact, the experiments described indicate that GlyR activation is necessary for gephyrin clustering but is it sufficient? It can be predicted, that a second signal must provide the spatial information required for gephyrin cluster formation. In this context (Kneussel and Betz 2000) suggested that the then newly discovered guanine exchange factor collybistin might play an important role in this process. Collybistin, named after the Greek word for "money exchanger", had been identified as gephyrin-binding guanine exchange factor for monomeric GTPases of the Rho-family, which is exclusively expressed in neurons (Kins et al. 2000). The hallmark of this class of exchange factors is the occurrence of a tandem-domain composed of a dbl-homology (DH) and a pleckstrin homology (PH) domain. Initially two splice variants were identified with differed by the presence, respectively absence of an SH3-domain located amino-terminal of the tandem-domain and a carboxy-terminal region likely to form a coiled coil structure. In the meantime it was shown that collybistins harboring the SH3 domain represent the predominant isoform and in addition, three different carboxy-terminal ends have been identified (Harvey et al. 2004a). Gephyrin-binding occurs in small region between the SH3- and DH domains (Grosskreutz et al. 2001). Upon

heterologous expression of gephyrin and collybistin in mammalian cells, both proteins accumulate in punctate membrane patches near the cell membrane (Kins et al. 2000). The morphology of the gephyrin- and collybistin-rich membrane patches is reminiscent of postsynaptic gephyrin aggregates, moreover these microdomains were shown to accumulate GlyRs (Kins et al. 2000). The "membrane-activation-model" suggested (Kneussel and Betz 2000), that GlyR activity drives activation of phosphatidylinositol 3-kinase, which would generate membrane patches enriched in phosphatidylinositol bis- and/or trisphosphate, that could recruit collybistin to membranes via binding to its PH domain.

Although intriguing, this model has to be modified for two reasons: it was demonstrated that the PH domain of collybistin binds phosphatidylinositol-3-phosphate rather than phosphatidylinositol-bis- and/or trisphosphate (Kalscheuer et al. 2008) and more importantly, the analysis of collybistin knock-out mice revealed, that collybistin is essential for the formation of gephyrin clusters underlying GABAergic but not glycinergic postsynaptic membranes (Papadopoulos et al. 2007). Consequently, collybistin knock-out mice display a region-specific loss of postsynaptic gephyrin and $GABA_A$ receptor clusters in the hippocampus and the basolateral amygdala but not in the spinal cord. On the functional level, collybistin deficiency leads to significant changes in hippocampal synaptic plasticity, due to reduced dendritic GABAergic inhibition. In particular, long-term potentiation is enhanced, while long-term depression reduced, in hippocampal slices from collybistin deficient mice. The animals show increased levels of anxiety and impaired spatial learning. Recent analysis of conditional collybistin knock-down mice revealed that collybistin is required for both the initial localization and maintenance of gephyrin and gephyrin-dependent $GABA_A$ receptors at inhibitory postsynaptic membrane specializations in the hippocampus (Papadopoulos et al. 2008).

Consistent with the phenotype of collybistin deficient mice, a female patient with a balanced chromosomal translocation disrupting the human collybistin gene (ARHGEF9) presented with a disturbed sleep-wake cycle, late-onset epileptic seizures, increased anxiety, aggressive behavior, and mental retardation, but not hyperekplexia as would be expected, if glycinergic transmission was impaired (Kalscheuer et al. 2008).

In addition to intracellular protein interactions, cell-adhesion molecules contribute significantly to the formation and maintenance of postsynaptic membrane specializations. Unfortunately, the molecular determinants linking receptor-associated and regulatory proteins to cell-adhesion molecules remain to be identified. Moreover, most of the work on cell adhesion molecules at inhibitory synapses focuses on GABAergic synapses. Considering the facts that significant dissimilarities in cell-adhesion molecules exist between excitatory and inhibitory synapses and that both types of inhibitory synapses rely on shared subsynaptic proteins, it appears justified to review the recent advances in this field.

The neurexin–neuroligin adhesion system in GABAergic synapse organization

Coordinating the assembly and function of pre-and postsynaptic specializations requires the exchange of anterograde and retrograde signals across the synaptic cleft. Probably the most direct way for the exchange of such signals might be represented by the action of cell-adhesion molecules that interact in the synaptic cleft to bridge the distance between the two cells physically and initiate or regulate signaling events in the partner cells. Recent reports implicate the neurexin–neuroligin adhesion system in assembly and function of GABAergic synapses.

The neurexin/neuroligin protein families

Both neurexins and neuroligins are transmembrane proteins. Current models hold that typically neuroligins act as postsynaptic, neurexins as presynaptic partners in the trans-synaptic interaction (Dean and Dresbach 2006). In mammals, neurexins are encoded by three genes, in which two promoters give rise to longer α-neurexins (termed α-neurexin 1–3) and shorter β-neurexins (termed β-neurexin 1–3). The gene structure, alternative splicing at five sites, and varying glycosylation patterns account together for the existence of potentially thousands of neurexin isoforms. In rodents, neuroligins are encoded by four genes (termed neuroligin 1–4), and five genes exist in humans. Alternative splicing at two sites, termed A and B, potentially allows for the generation of four isoforms per gene. Splicing in both protein families affects the features of the respective isoforms with respect to their interactions and their localization to excitatory versus inhibitory synapses (Boucard et al. 2005; Chih et al. 2006; Craig and Kang 2007; Graf et al. 2006; Missler et al. 1998). In this review, we will use the terms "neurexin" and "neuroligin" to refer to the isoforms that include all splice inserts.

Role of neuroligins in GABAergic synaptic transmission

Neuroligin 2 appears to be exclusively localized to inhibitory synapses (Graf et al. 2004; Varoqueaux et al. 2004). By contrast, neuroligin 1 appears to be specific for excitatory synapses (Dresbach et al. 2004; Song et al. 1999), and neuroligin 3 was found at both types of synapses (Budreck and Scheiffele 2007). Expressing recombinant neuroligin 1

or 2 in non-neuronal cells or in neurons triggers the formation of excitatory and inhibitory presynaptic specializations in axons. This action of neuroligins is blocked by the addition of a soluble β-neurexin to the culture medium to prevent binding between neuroligins and β-neurexins. Moreover, adding soluble β-neurexin to cultures or "knocking down" endogenous neuroligins reduces the number of excitatory and inhibitory synapses, suggesting that transsynaptic interactions between neurexins and neuroligins are important for synapse formation or stability (Dean and Dresbach 2006; Lise and El-Husseini 2006). Triple-knockout of neuroligins 1,2 and 3 reduces the number of synapses in the brain stem by only 20%, suggesting that these three neuroligins are not essential for synapse formation per se (Varoqueaux et al. 2006). However, in both triple knockouts and triple knockdown cultures inhibitory synaptic transmission is impaired, and in fact is more severely affected than excitatory transmission (Chih et al. 2005; Varoqueaux et al. 2006). Neuroligin triple knockout mice show a 30% reduction in the number and staining intensity of postsynaptic clusters containing $GABA_A$ receptor α1 subunits, while the number of clusters containing either PSD95, gephyrin or GlyR α subunits is unchanged (Varoqueaux et al. 2006). This suggests an important role of neuroligins in GABA receptor clustering and GABAergic transmission.

Particular role of α-neurexins and neuroligin 2 in GABAergic synapse organization

While the molecular details underlying the action of neuroligins in GABAergic synapse organization are not yet clear, several pieces of evidence suggest that neuroligin 2 might play a major role in these events. First, analysis of acute cortical slices from neuroligin 2 single knockout mice reveals a selective impairment of GABAergic transmission, while glutamatergic transmission is unaffected. Specifically, the amplitude of evoked inhibitory postsynaptic currents was reduced by 50%. On the contrary, genetic deletion of neuroligin 1 only affects excitatory transmission (Chubykin et al. 2007). Second, experimentally induced aggregation of recombinant neuroligin 2 in dendritic membranes results in the recruitment of gephyrin and PSD95 to sites of neuroligin aggregation, whereas aggregation of recombinant neuroligin 1 only recruits PSD95. Thus, neuroligin 2, but not neuroligin 1, seems to have a potential for recruiting components of inhibitory synapses in addition to recruiting proteins of excitatory synapses. Local dendritic accumulations of neuroligins can be induced by the addition of neurexin-expressing non-neuronal cells to neuronal cultures. In this assay, β-neurexins induce the local accumulation of neuroligins 1 and 2 as well as NMDA-receptor subunits, PSD95, GABA-receptor subunits and gephyrin in dendrites contacting the neurexin-expressing cells (Graf et al. 2006; Nam and Chen 2005). In the same assay, α-neurexins selectively recruit neuroligin 2, gephyrin and GABA-receptor subunits into local accumulations (Kang et al. 2008). Thus, all neurexins are able to induce the local recruitment of neuroligin 2 in dendrites, and α-neurexins have a selective capability for recruiting neuroligin 2 and additional components of GABAergic synapses. Conversely, over-expression of neuroligin 2 in cultured neurons has a stronger effect on inducing the formation of inhibitory than excitatory presynaptic terminals on the transfected neurons (Chih et al. 2005; Graf et al. 2006; Levinson et al. 2005). A recent study using over-expression of neuroligin 2 reported a selective increase in the amplitude of evoked inhibitory but not excitatory postsynaptic currents, suggesting that on this functional level neuroligin 2 acts selectively on inhibitory synapses, consistent with the selective effects of neuroligin 2 deletion and with its localization at inhibitory synapses (Chubykin et al. 2007). By the same token, α-neurexin triple knockout mice, in addition to showing a general impairment of calcium channel function at all synapses, show a 50% percent reduction in the number of symmetric (GABAergic), but no change in the number of asymmetric (likely glutamatergic) synapses in the brain stem, as analyzed by electron microscopy (Missler et al. 2003). Together, these data suggest that a transsynaptic interaction between α-neurexins and neuroligin 2 is of particular importance for the formation or stabilization and for the function of GABAergic synapses, although further interactions of the neurexin–neuroligin system might play additional roles in the organization of inhibitory and excitatory synapses.

Molecular models for neurexin/neuroligin 2 interactions in GABAergic synapse organization

A recent model for GABAergic synapse development put forward by (Huang and Scheiffele 2008) has suggested a sequence of events in which GABA receptor subunits diffusing in the membrane and neuroligin 2 stabilize each other at synaptic sites. This stabilization and immobilization might be further promoted by GABA receptor activity. In this case, synaptic transmission and transsynaptic interactions between neuroligin 2 and neurexins could promote each other in a fashion that would lead to stabilization and maintenance of functional synaptic connections. This model takes into account that GABA release promotes the stable formation of presynaptic axon branches, that postsynaptic GABA receptors control the maintenance of stellate cell to Purkinje cell synaptic connections in the cerebellum, and that impaired GABA receptor clustering in a subset of cells reduces GABAergic innervation of these cells. Together, these data argue for a role of GABAergic

transmission and a retrograde signal in the maintenance of GABAergic synapses (Huang and Scheiffele 2008). Given its properties, a neurexin–neuroligin 2 interaction appears to be perfectly suited to act in this transsynaptic signaling. This is further corroborated by the observation that the synapse enhancing action of overexpressed neuroligin 1 and 2 requires synaptic activity (Chubykin et al. 2007). Furthermore, coexpression of neuroligin 2 with GABA receptors in non-neuronal cells is sufficient to reconstitute GABAergic transmission when these cells are innervated by hypothalamic axons (Dong et al. 2007). In these heterologous expression experiments, neuroligin 2 can coaggregate GABA receptors, suggesting a physical link between them, which might represent a direct interaction or involve further bridging partners, possibly low levels of endogenous gephyrin expressed in HEK293 cells.

Importance of neurexin/neuroligin 2 interactions in GABAergic synapse organization, network function and behavior

Irrespective of the underlying molecular interactions, neuroligins are clearly important to maintain a normal ratio of excitatory versus inhibitory synaptic transmission (E/I ratio) in neuronal networks, as this ratio is affected by the relative concentrations of neuroligins. In addition, it has been reported that neuroligin 2 can be made to change its localization from inhibitory synapses to excitatory synapses by over-expression of PSD95 (Graf et al. 2004; Levinson et al. 2005). This dynamic behavior could potentially represent an additional mode of regulating the E/I ratio. Alterations of the E/I ratio have been proposed to contribute to the behavioral aspects associated with autism (Rubenstein and Merzenich 2003) or developmental intellectual disability (Fernandez and Garner 2007). Strikingly, both neurexins and neuroligins have been genetically linked to cases of autism (Geschwind and Levitt 2007), and neuroligin 4 knockout mice display social interactions reminiscent of autistic behavior (Jamain et al. 2008). Moreover, both transgenic mice overexpressing neuroligin 2 and transgenic mice expressing a mutated neuroligin 3, which mimics a neuroligin 3 point mutation genetically linked to autism show increased inhibitory transmission and a reduced E/I ratio as well as behavioral abnormalities reminiscent of autism (Hines et al. 2008; Tabuchi et al. 2007). A major challenge for the future will be to determine how GABA receptor activity and the neurexin–neuroligin system contribute to GABAergic, respectively glycinergic synapse organization, network activity and potentially behavior. It will also be interesting to see whether glycinergic synapses involve the same or a different cell-adhesion system (Fig. 6).

The evidence for the contribution of other cell adhesion molecules (Fig. 7) to the formation and maintenance of glycinergic synapse is sparse. Neurofascin, a member of the L1-family of adhesion proteins, was demonstrated to be essential for the generation of gephyrin clusters, as well as for the targeting of these clusters and of inhibitory synapse formation to the axon initial segment in Purkinje cells (Ango et al. 2004; Burkarth et al. 2007).

SynCAMs induce synapse formation in cell culture assays. The isoforms SynCAM 1 and 2 have been detected at inhibitory and excitatory synapses and have been shown

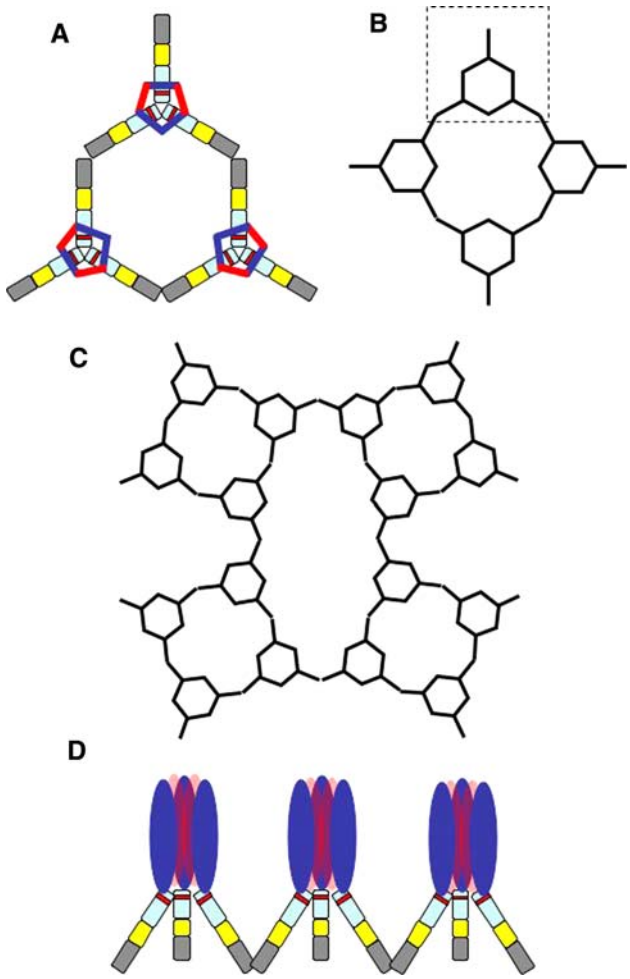

Fig. 6 Model of postsynaptic gephyrin scaffold formation. **a** Gephyrin molecules can form in dimers with their G-domains and trimers with their E-domains as deduced from crystallization studies. The resulting hexamers could form the basic element of a gephyrin scaffold (the complementary trimers extending from the hexagonal structure are not shown due to spatial restrictions). Gephyrin domains are coded as before; the binding sites for the GlyR β subunit are indicated by *red bands*. The suggested position for GlyRs is indicated by the *red* and *blue pentamers*. **b** Basic scaffold modules can aggregate to *square shaped* structures. **c** *Square shaped* structures can assemble into higher order structures, which could in principle be observed by advanced light microscopy techniques such as, "stimulated emission depletion" or, "total internal reflection" microscopy. **d** The postsynaptic gephyrin scaffold is predicted to be a three dimensional structure, that extends into the subsynaptic cytoplasm

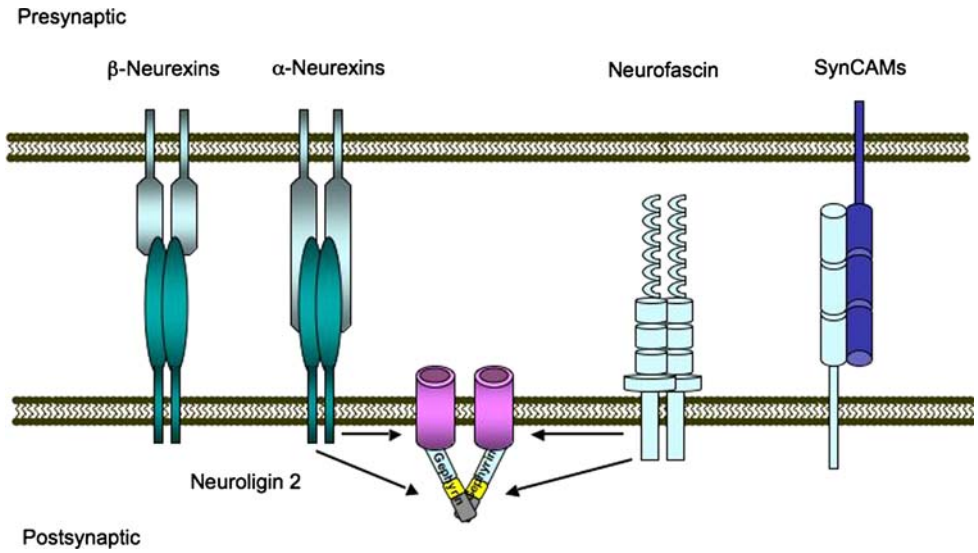

Fig. 7 Cell adhesion molecules detected at inhibitory synapses. α-neurexins specifically induce local accumulations of neuroligin 2, gephyrin and the $GABA_A$ receptor γ2-subunit, but not of components of excitatory synapses. Knockout of all three α-neurexins reduces the number of inhibitory synapses in the brain stem, and knockout of neuroligin 2 impairs inhibitory synaptic transmission. β-neurexins induce the accumulation of induce accumulation of essential components of both excitatory and inhibitory synapse components. Triple knockout of neuroligins 1, 2 and 3, impairs both excitatory and inhibitory synaptic transmission, and leads to a reduction in the number of inhibitory receptor clusters in the brain stem. Neurofascin, a member of the L1-family of adhesion proteins, is implicated in the generation of gephyrin clusters, as well as for the targeting these clusters and of inhibitory synapse formation to the axon initial segment in Purkinje cells. The isoforms SynCAM 1 and 2 have been detected at inhibitory and excitatory. Not shown is N-Cadherin, which is localized to excitatory and inhibitory synapses in immature neurons and later becomes restricted to excitatory synapses

to form preferentially heterophilic complexes, although homophilic interactions have also been reported (Biederer et al. 2002; Thomas et al. 2008).

Our conception of the molecular composition of glycinergic synapses is far from complete. Emerging fields of research are the interfaces of "receptor-associated proteins" and cell-adhesion molecules as well as signaling pathways originating underneath glycinergic synapses. Moreover, the elucidation of the molecular determinants of synapse formation, stabilization and adaptation in plastic processes will help to understand the role of synaptic inhibition under normal and pathological conditions.

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