Cellular and Molecular Life Sciences

REVIEW

Molecular and functional heterogeneity of GABAergic synapses

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Abstract Knowledge of the functional organization of the GABAergic system, the main inhibitory neurotransmitter system, in the CNS has increased remarkably in recent years. In particular, substantial progress has been made in elucidating the molecular mechanisms underlying the formation and plasticity of GABAergic synapses. Evidence available ascribes a key role to the cytoplasmic protein gephyrin to form a postsynaptic scaffold anchoring $GABA_A$ receptors along with other transmembrane proteins and signaling molecules in the postsynaptic density. However, the mechanisms of gephyrin scaffolding remain elusive, notably because gephyrin can auto-aggregate spontaneously and lacks PDZ protein interaction domains found in a majority of scaffolding proteins. In addition, the structural diversity of $GABA_A$ receptors, which are pentameric channels encoded by a large family of subunits, has been largely overlooked in these studies. Finally, the role of the dystrophin-glycoprotein complex, present in a subset of GABAergic synapses in cortical structures, remains ill-defined. In this review, we discuss recent results derived mainly from the analysis of mutant mice lacking a specific $GABA_A$ receptor subtype or a core protein of the GABAergic postsynaptic density (neuroligin-2, collybistin), highlighting the molecular diversity of GABAergic synapses and its relevance for brain plasticity and function. In addition, we discuss the contribution of the dystrophin-glycoprotein complex to the molecular and functional heterogeneity of GABAergic synapses.

Keywords $GABA_A$ receptor · Gephyrin · Neuroligin, collybistin, dystrophin–glycoprotein complex · Synaptic plasticity

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Introduction

Early electron microscopy studies uncovered a fundamental dichotomy in the ultrastructure of synapses in vertebrate CNS, classified as type I (asymmetric) and type II (symmetric) synapses, based on the width of their electron-dense postsynaptic density (PSD) [1]. Subsequently, identification of glutamate (and aspartate) and GABA (and glycine) as neurotransmitters in the CNS, combined with the advent of immunohistochemistry, showed that this dichotomy corresponds to excitatory and inhibitory synapses, respectively. This structural and neurochemical distinction reflects major differences in biochemical composition of the PSD in both types of synapses. The PSD contains distinct sets of scaffolding proteins associated with neurotransmitter receptors and signaling molecules. Biochemical fractionation revealed hundreds of proteins in the PSD of glutamatergic synapses. These include numerous PDZ domain-containing scaffolding proteins, notably PSD-95 and other members of the family of membrane-associated guanylate kinase proteins (MAGUKs) [2]. The PDZ interaction motifs are a characteristic feature of many scaffolding proteins, allowing to build modular protein complexes [3], thereby ensuring rapid, efficient, and

site-specific propagation of incoming synaptic inputs [4]. The phototransduction complex assembled by INAD in *Drosophila* photoreceptors is a prototypical example of such signaling scaffold organized by PDZ motifs [5].

In glutamatergic synapses, MAGUKS play a key role in assembly and regulation of the PSD [6, 7]. MAGUKS are membrane-anchored by specific lipid modifications (e.g., palmitoylation) and/or interact with components of the cytoskeleton to ensure long-term structural stability of the PSD [8, 9]. In addition, they contribute to plasticity of synaptic transmission, mainly by binding to specific effector proteins, which induce posttranslational modifications regulating the trafficking and function of neurotransmitter receptors and other signaling molecules. Given the central role of PSD scaffolding molecules, even minor alterations in their expression, trafficking, regulation, or function have been associated with brain disease [7, 10–12]. Therefore, identifying and correcting these dysfunctions might provide promising new avenues for drug therapy.

In striking contrast with type I synapses, the PSD of GABAergic and glycinergic synapses contains only a few proteins with a PDZ domain. Rather, they are organized around a core scaffolding protein, gephyrin, which forms multimeric complexes by auto-aggregation and by interacting with other postsynaptic proteins, including $GABA_A$ receptors ($GABA_A$R), glycine receptors (GlyR), neuroligins (NL), and collybistin (CB) [13]. Models to explain the formation and maintenance of either glycinergic or GABAergic synapses are still fragmentary [14–16], notably because the biochemical composition of their PSD is poorly characterized and because gephyrin structure is only partially resolved. The paucity of PDZ domains in proteins forming GABAergic and glycinergic PSDs implies, however, that protein–protein interactions are based largely on other motifs. While the binding site of the GlyR β subunit intracellular loop to gephyrin has been well characterized [17, 18], much less is known about $GABA_A$R subunits, although information available suggests direct interactions with members of the α subunit family [19–21]. A major challenge for studying $GABA_A$R-gephyrin interactions is the presence of multiple receptor subtypes differing in subunit composition, each of them likely interacting differently with gephyrin and its partners.

PSDs typically are associated with specific subcellular compartments; glutamatergic synapses being formed on dendritic spines whereas GABAergic synapses occurring mainly on neuronal somata, dendritic shafts, and axon initial segments. Therefore, association with specific cytoskeletal elements and/or transmembrane proteins linked to extracellular matrix proteins likely is of primary relevance for the formation and maintenance of PSDs. Although gephyrin was characterized initially as a tubulin-binding protein, the role of the cytoskeleton for anchoring GABAergic and glycinergic PSDs remains largely obscure. Furthermore, PSDs interact closely with presynaptic terminals by means of trans-synaptic ligand/receptor protein complexes, such as neurexins/NLs, integrins, ephrins, cadherins, etc., raising the possibility that the subcellular localization of synapses is determined by specific molecular components of the PSD.

The purpose of this review is to highlight current knowledge of proteins interacting with gephyrin at GABAergic synapses. We will discuss how molecular and functional heterogeneity of GABAergic synapses arises from these differential interactions. We will also present evidence to reconsider the role of gephyrin as anchoring molecule in favor of gephyrin being the hub of a signaling scaffold organized in concert with specific $GABA_A$R subtypes to regulate GABAergic synapse function.

Overview of major PSD proteins at inhibitory synapses

Gephyrin

At the PSD of inhibitory synapses, anchoring $GABA_A$R and GlyR is ensured, at least in part, by a scaffold assembled upon self-oligomerization of gephyrin. These interactions will be discussed in more detail in the following sections. Additional well-characterized proteins associated with gephyrin include the transmembrane protein NL2 and CB, a member of the Dbl family of guanine nucleotide exchange factors (GEF), discovered upon its ability to translocate gephyrin to the cell surface in non-neuronal cells (reviewed in [13]). A number of additional gephyrin-interacting proteins have been reported, which are involved mainly in regulation of the cytoskeleton and gephyrin trafficking. So far, however, proteins directly regulating gephyrin auto-aggregation have not been described, despite the fact this aggregation exclusively occurs at postsynaptic sites.

Gephyrin is a highly conserved molecule, whose primary role in the living kingdom is to catalyze the synthesis of molybdenum cofactor (Moco) [22]. In vertebrates, gephyrin acts in addition as a postsynaptic scaffolding molecule, and current evidence from rodents indicates that Moco synthesis in brain is restricted to astrocytes [23]. Gephyrin has two major functional domains, E and G, linked by an unstructured C-domain, which contains most gephyrin regulatory sites and binding sites for interacting proteins. While the crystal structure of the G and E domains has been partially determined, the instability of full-length recombinant gephyrin in solution and the lack of information about the structure of the C-domain are major obstacles for elucidating gephyrin function and the

mechanism of gephyrin auto-aggregation [13]. Current models posit either formation of hexameric gephyrin scaffolds, with six gephyrin molecules binding to three GlyR [18], or aggregation of gephyrin trimers, each of which is bound to one GlyR [13].

These models of gephyrin aggregation are based on partial structural information, localization of the GlyR binding site in the E-domain, and identification of surface-exposed loops regulating gephyrin postsynaptic aggregation [13, 18, 24]. Although available experimental evidence does not allow distinguishing between them, an important outcome of gephyrin structure is its enzymatic activity, which requires the active sites on the G and the E domains to be in close spatial proximity. The model of gephyrin trimers takes this requirement into consideration [13]. Data from GlyR single-particle tracking experiments and gephyrin fluorescence recovery after photobleaching revealed that gephyrin modulates synaptic retention time and lateral mobility of these receptors, and that GlyR diffusion properties modulate gephyrin clustering [25, 26]. Therefore, a dynamic exchange of gephyrin and receptors occurs in apparently stable postsynaptic structures. Furthermore, time-lapse recordings from neurons expressing fluorescent-tagged gephyrin revealed that gephyrin clusters are constantly remodeled and can move in dendrites over distances of several micrometers in concert with apposed presynaptic terminals [27]. Collectively, these observations are consistent with both views that gephyrin units (e.g., trimer) can be added and removed from the scaffold and that the gephyrin scaffold acts as a stable but mobile entity in dendrites.

There are multiple splice-variants of gephyrin generated by alternative splicing of cassettes localized mainly in the G- and C-domain (see [13] for nomenclature). The splice variants capable of Moco synthesis have been identified, but they do not necessarily differ from those forming postsynaptic clusters in neurons; further, insertion of the G2 splice cassette, a gephyrin isoform expressed in both neuronal and non-neuronal tissues, prevents gephyrin aggregation and Moco synthesis, and has been proposed to regulate gephyrin function, synaptic localization, and clustering [23, 25, 28]. Further characterization of gephyrin splice variants, notably in the C-domain, will be necessary to better understand the regulation of gephyrin enzymatic activity and clustering in different types of synapses.

Recent evidence points to gephyrin posttranslational modification by phosphorylation as a mechanism regulating its postsynaptic function. Although gephyrin is a known phospho-protein [29], it is only in 2007 that a first report suggested that phosphorylation-dependent recruitment of the peptidyl-prolyl isomerase Pin1 to a site located in the C-domain regulates GlyR binding [30]. We have identified a novel phosphorylation site on gephyrin, Ser270, and shown that it selectively regulates formation of postsynaptic gephyrin clusters in vitro and in vivo under the control of GSK3β [31]. These findings provide a novel mechanism to regulate GABAergic synapse formation and function by a phosphorylation cascade activated by a plethora of extra- and intra-cellular signals and controlling basic cellular functions.

Neuroligin-2

Neurexins and NLs gained major interest upon discovery that they induce formation of synapses in recombinant non-neuronal cell systems. Reports of preferential distribution of NL1 in glutamatergic synapses and NL2 in GABAergic synapses [32–34] fuelled considerable efforts to elucidate their role in synapse formation and specification in the CNS. Importantly, in the context of the present review, NL2 has been shown to interact directly with gephyrin and this interaction is necessary for postsynaptic localization of gephyrin clusters at GABAergic sites [35]. Apparently, NL2 is absent from glycinergic synapses, whereas NL3 and NL4 have been reported to be present in a subset of GABAergic and glycinergic synapses, respectively [36, 37]. The significance of a segregated distribution of NLs to different types of synapses is not fully understood [38], because this apparent selectively is lost upon overexpression and because NL3 has been reported to be present in both glutamatergic and GABAergic synapses in vivo. It is also likely that NLs are functionally interchangeable to some degree, as seen in targeted gene deletion experiments, in which the loss of a particular NL isoform appears to be compensated in part by another isoform [39]. Nevertheless, the significance of the NL2-gephyrin interaction was highlighted by a study showing that NL2 also interacts with CB, in a manner that favors formation and regulation of gephyrin clusters at postsynaptic sites (see below). Therefore, NL2 is widely considered to play a crucial role to initiate the formation of a GABAergic PSD.

Collybistin

CB is a neuron-specific Rho-GEF encoded by a single gene (ARHGEF9) subject to alternative splicing. In rat, there are three main CB variants, CB1–3, differing in the C-terminal region. They all possess three major functional domains: an N-terminal SH3 domain, encoded by a spliced exon (SH3+ or SH3−), a DH (or GEF) domain selectively activating the small GTPase Cdc42, and a PH domain (Fig. 1). Available literature is somewhat confusing about the existence and structure of CB variants in other species. In human, CB is also known as hPEM2 and only one sequence, with a C terminus identical to rat CB3 has been described [40]. In mouse, four transcripts have been identified, which are
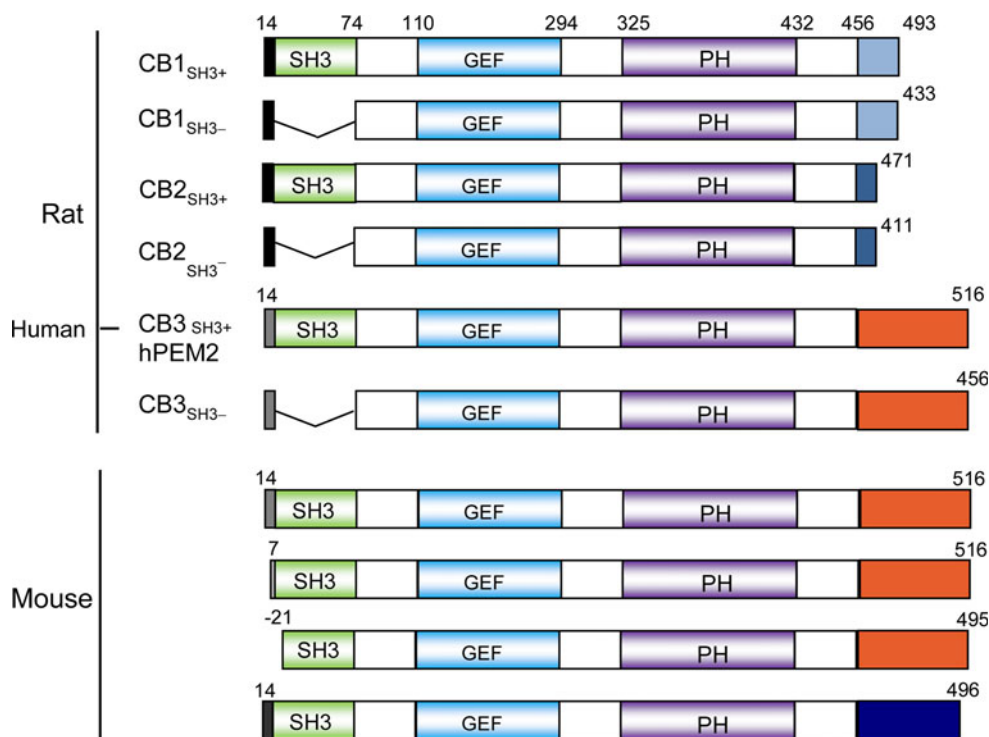
Fig. 1 Comparison of CB isoforms in rat, human, and mouse. The three main functional domains are indicated, *different colors* denote distinct sequences in the N- or C-termini. The number of residues present in each domain or in each isoform is given on top of each variant. CB isoforms are best characterized in rat, where the existence of three splice variants (CB1–CB3) differing in the C-terminal domain is well established. In addition, each of CB1–CB3 variants is believed to be N-terminally spliced to include or exclude the SH3 domain. The C-terminal region of CB3 is identical to hPEM2, the only CB isoform described in human. The N terminus of CB3 differs slightly from that of CB1/2. In mice, the CB1–CB3 nomenclature does not apply because the sequence closest to rat CB3 is encoded in three variants with different N-termini, whereas the sequence closest to either CB1 or CB2 has a unique C terminus

largely conserved compared to human and rat CB3, but differ in their N terminus (Fig. 1). The relative abundance of CB splice variants in the CNS and their expression pattern across different brain regions or developmental stages have not been investigated systematically. A recent report using antibodies recognizing the majority of CB isoforms (notably CB3) confirmed the localization of CB at GABAergic PSDs in most brain regions [41].

The significance of CB for glycinergic and GABAergic synaptic function is underscored by the discovery of several loss-of-function mutations in *ARGEF9* underlying severe forms of X-linked mental retardation and hyper-ekplexia [42, 43]. In recombinant expression systems, CB is essential for cell-surface translocation of gephyrin [44]. However, this function is only assumed by CB variants lacking the SH3 domain, mainly CB2$_{SH3-}$, suggesting that the SH3 domain somehow controls CB activity. A plausible model posits that, in analogy to other GEFs, the SH3 domain exerts an auto-inhibitory effect on the DH domain (and hence on activation of Cdc-42) [35]. CB binds gephyrin in the C-domain, whereas the binding site of gephyrin on CB partially overlaps with the binding of Cdc42, which led to speculation that they might be mutually exclusive [45] (but see [46]). A recent study suggested, however, that CB-mediated gephyrin postsynaptic clustering is not dependent on a functional DH domain, but requires the PH domain [47]. Because gephyrin postsynaptic clusters were observed in the brain of conditional Cdc42-deficient mice, this study even concluded that Cdc42 is dispensable for gephyrin aggregation.

In addition to gephyrin, CB interacts directly with NL2 via its SH3 domain. Based on data from recombinant systems, this interaction was suggested to cause a conformational change relieving the auto-inhibition of the DH domain, thereby facilitating cell-surface translocation of gephyrin and GABA$_A$R [35]. In neurons, the activation of CB$_{SH3+}$ by NL2 was postulated to facilitate gephyrin clustering at postsynaptic sites upon binding to phosphoinositol-3-phosphate in the cell membrane via its PH domain. This model received further support in a report showing that CB binds to GABA$_A$R α2 (and α3) subunit and that this interaction leads to CB activation and translocation of trimeric CB-gephyrin-α2 subunit complexes to the cell membrane [48]. However, the role of NL2 was not examined in this study. Furthermore, in view of the multiplicity of CB isoforms and of the heterogeneity of

GABA$_A$Rs, which form multiple subtypes differing in subunit composition and cellular/subcellular localization [49], such models are necessarily fragmentary and do not take into account the diversity and exquisite functional specialization of GABAergic synapses across the CNS.

Insight into the roles of CB isoforms for gephyrin clustering was derived from over-expression studies in transfected neurons, which aimed to distinguish between CB2$_{SH3+}$ and CB2$_{SH3−}$ [46, 50], as well as to elucidate the contribution of Cdc42, the only known substrate of CB. In both studies, CB over-expression contributed to stabilize gephyrin, as evidenced by a marked increase in size and density of postsynaptic gephyrin clusters. Interestingly, CB2$_{SH3−}$ facilitated the formation of supernumerary postsynaptic clusters, whereas CB2$_{SH3+}$ favored formation of intracellular gephyrin aggregates, as illustrated in Fig. 2. Further, deletion of the PH domain of CB2 led to gephyrin retention along the dendritic cytoskeleton, whereas deletion of the DH domain strongly interfered with gephyrin postsynaptic clustering. Finally, over-expression of Cdc42, using dominant negative and constitutively active mutants for comparison, revealed that Cdc42 cooperates with CB2$_{SH3−}$ for postsynaptic targeting of gephyrin and that it regulates gephyrin cluster size at postsynaptic sites [46]. These data add to the model proposed by Poulopoulos et al. [35], by unraveling distinct functions of CB2 isoforms for gephyrin transport, postsynaptic targeting, and regulation of postsynaptic clustering in concert with Cdc42. However, they suggest that the postulated conformation-dependent activation of CB$_{SH3+}$ by NL2 might not be crucial for proper CB function at GABAergic PSDs. Further insight into the roles of CB for gephyrin trafficking and clustering will require the analysis of the other splice variants (CB1, CB3), as well as more knowledge of their expression pattern and regulation.

Differences between GABAergic and glycinergic synapses

Because gephyrin is found in both glycinergic and GABAergic synapses, it is generally considered that the assembly of signaling scaffolds by gephyrin is similar in both types of synapses. This concept is reinforced by the homology between GABA$_A$R and GlyR, which are members of the family of Cys-loop ligand-gated ion channels. However, there are a number of important differences between the two types of synapses, which suggest that their formation and regulation might depend on distinct mechanisms (Table 1).

First, despite being closely related, GABA$_A$Rs and GlyRs differ fundamentally in their affinity for gephyrin. Recent reports identified the binding site of the GABA$_A$R $α1–α3$ subunits on the gephyrin E-domain and showed substantial overlap with the GlyR $β$ subunit, albeit with a >30-fold lower affinity [19, 51]. Nevertheless, expression of a mutant GABA$_A$R lacking the interaction site in the M3–M4 loop of the $α3$ subunit disrupted its postsynaptic clustering in cultured hippocampal neurons. These findings extend a previous report, which identified a gephyrin-interaction motif in the M3–M4 intracellular loop of the $α2$ subunit, sufficient to direct chimeric GABA$_A$R to gephyrin-rich postsynaptic sites [20].

Second, as discussed in the next section, postsynaptic clustering of some GABA$_A$R subtypes is possible in the absence of gephyrin. In contrast, GlyR postsynaptic clustering appears to be dependent on the presence of gephyrin clusters [52], mirroring the high affinity of their interaction and confirming observations that GlyR trafficking in neurons is facilitated by association with gephyrin [53].

A third major difference between GABAergic and glycinergic synapses is the dependence of gephyrin on CB for postsynaptic aggregation. Unexpectedly, CB-knockout (KO) mice exhibit no apparent morphological and functional alteration of glycinergic synapses in the brainstem and spinal cord [54]. In particular, they do not show any signs of spasticity typical of hypo-glycinergic function at birth, which are prominent in mice lacking the type 1 glycine transporter (GlyT1) [55]. These results indicated that CB either is dispensable for gephyrin/GlyR clustering at glycinergic PSDs or is functionally replaced by a homologous protein. In contrast, as detailed in the next section, CB deficiency produces marked alterations of gephyrin clustering at GABAergic postsynaptic sites.

A fourth difference is the differential targeting of NLs to GABAergic and glycinergic synapses. As noted above in the section on NL2, GABAergic, but not glycinergic synapses, selectively contain this NL isoform, whereas NL3 and NL4 have been reported in glycinergic synapses, at least in the retina [37]. Since NL2 interacts with CB and gephyrin at GABAergic synapses, this difference in localization might explain why CB deletion does not affect the PSD of glycinergic synapses.

A fifth difference is the existence of multiple, presumably dozens of GABA$_A$R subtypes, differing in subunit composition in adult brain, compared to a few GlyR subtypes, assembled mainly from $α1$ or $α3$ and $β$ subunit variants. The multiplicity of GABA$_A$R underlies molecular and functional heterogeneity of GABAergic synapses, as well as differential targeting to extrasynaptic versus postsynaptic sites. There is evidence that receptors differing in $α$ subunit variant are functionally not interchangeable, and that only GABA$_A$R targeted postsynaptically contribute to formation of gephyrin clusters [56, 57]. Therefore, interactions of GABA$_A$R subtypes with gephyrin and other GABAergic PSD proteins are likely more heterogeneous and synapse-specific than GlyR-gephyrin interactions. These limitations are well evident in single-particle
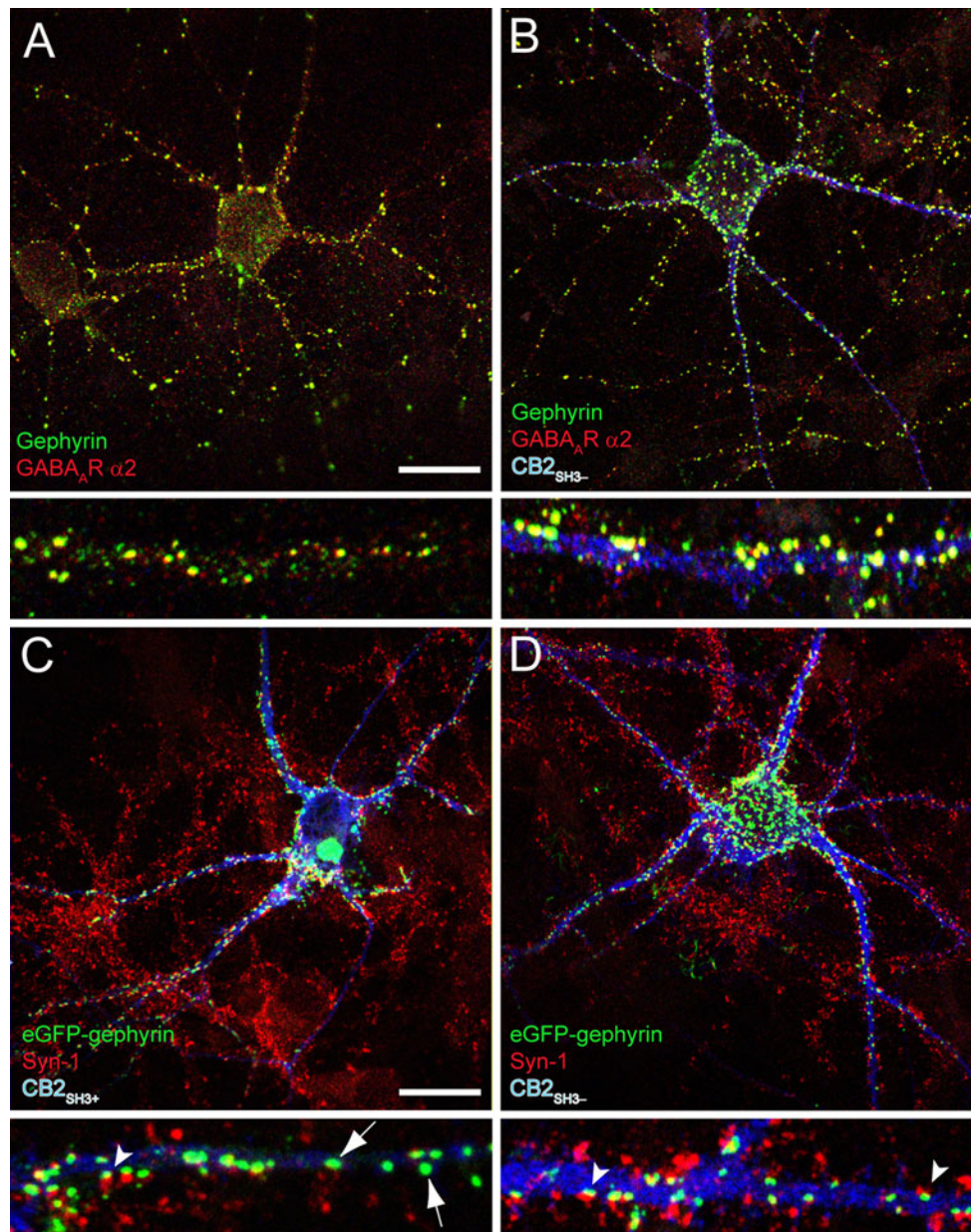
Fig. 2 Differential effects of CB2$_{SH3+}$ and CB2$_{SH3-}$ on the postsynaptic clustering of gephyrin; adapted from [46]. Primary cultures from hippocampal embryonic neurons were transfected with tagged cDNA constructs after 11 days-in vitro (DIV) and processed for immunofluorescence staining with the markers indicated 4–7 days later. **a** Control cell showing extensive co-localization of gephyrin and $\alpha2$ subunit immunofluorescence at postsynaptic sites. **b** Upon transfection with mycCB2$_{SH3-}$, there is a marked increase in the density and size of gephyrin/$\alpha2$ clusters in the soma and on dendrites, suggesting that CB2$_{SH3-}$ stabilizes gephyrin and favors its postsynaptic clustering. **c,d** Similar effects were observed in neurons co-transfected with eGFP-gephyrin and myc-CB2$_{SH3+}$ (**c**) or myc-CB2$_{SH3-}$ (**d**); however, CB2$_{SH3+}$ favored the formation of non-synaptic aggregates (*arrows*), whereas CB2$_{SH3-}$ led to increased density of postsynaptic aggregates, recognized by apposition to the presynaptic marker synapsin 1 (*arrowheads*). The formation of non-synaptic aggregates suggests saturation of molecules, such as NL2, which normally ensure postsynaptic localization of CB2$_{SH3+}$. *Scale bars* 20 μm

tracking studies of GABA$_A$R cell surface mobility, which cannot be performed on a uniquely identified receptor subtype. Nevertheless, evidence available indicates that diffusion properties and synaptic confinement of GABA$_A$Rs containing the $\gamma2$ subunit are modulated by

enhanced excitatory synaptic activity, in a Ca^{2+}-dependent manner [58].

Several reports suggested that, despite the tight biochemical association between GlyRs and gephyrin, there is no direct correlation between gephyrin availability at

Table 1 Differences between GABAergic and glycinergic synapses related to gephyrin

	Feature	GABAergic	Glycinergic
1.	Gephyrin–receptor interaction	Low affinity (α1, α2, α3 subunits)	High affinity (β subunit); insertion of the binding motif (residues 398–410 of the β subunit) into a recombinant protein is sufficient for interaction with gephyrin [106]
2.	Receptor dependence on gephyrin for postsynaptic clustering	Receptor subtype-dependent (α2 $\gg$ α1)	Very high (clustering of GlyRs depends on gephyrin [107])
3.	Gephyrin dependence on collybistin for postsynaptic clustering	Variable (neuron and/or synapse dependent)	None
4.	Neuroligin isoform present	NL2 (NL3, NL4)	NL4
5.	Receptor heterogeneity	Numerous receptor subtypes (19 subunits)	Limited repertoire

See main text for further explanations and references

postsynaptic sites and synaptic retention time or cell-surface diffusion parameters of GlyRs in neurons. Therefore, the high affinity of gephyrin for the GlyR β loop trafficking does not imply a one-to-one relationship. It is conceivable that gephyrin scaffolds contain a limited number of binding sites for GlyR [25, 59, 60]. Further evidence for a dissociation between GlyR and gephyrin clustering dynamics was provided by analyzing the interaction between gephyrin and heat-shock cognate protein 70 (HSc70), a chaperone modulating ubiquitination of its substrates [61]. These authors showed that Hsc70 selectively regulates gephyrin clustering in cultured spinal cord neurons, without affecting GlyR clustering and cell-surface diffusion kinetics.

Heterogeneity of GABAergic synapses revealed in mice with targeted gene deletions

By immunofluorescence staining and immunoelectron microscopy in brain sections, gephyrin is selectively detected at postsynaptic sites of glycinergic and most GABAergic synapses, forming puncta (clusters) that are co-localized with GlyRs and GABA$_A$Rs, respectively [62–65]. Initial observations of a functional interaction between gephyrin and GABA$_A$R were made in γ2-KO mice, in which postsynaptic clustering of GABA$_A$R and gephyrin was strongly impaired compared to wild-type and heterozygous littermates [66–68]. Conversely, GABA$_A$R clustering was widely disrupted in neurons from gephyrin-KO mice [69]. This interdependence was later shown not to be absolute, as GABA$_A$R containing the α1 subunit, for instance, can form postsynaptic clusters mediating synaptic transmission in the absence of gephyrin [70, 71]. However, ablation of GABA$_A$Rs by targeted deletion of an α subunit variant often results in the disruption of postsynaptic gephyrin clusters and formation of large intra-cellular gephyrin aggregates [56, 72–76], confirming the dependence of gephyrin on

GABA$_A$Rs for postsynaptic localization. Importantly, in thalamic neurons expressing both α1-GABA$_A$R postsynaptically and α4-GABA$_A$R extrasynaptically, gephyrin clustering was not rescued following α1 subunit deletion, indicating that α4-GABA$_A$R cannot interact with gephyrin [56, 75].

Beyond GABA$_A$Rs, evidence for multiple mechanisms of gephyrin postsynaptic clustering at GABAergic synapses was provided by the analysis of CB- and NL2-KO mice. In particular, in GABAergic synapses of the forebrain and cerebellum, multiple phenotypes were observed in CB-KO mice, ranging from disruption of both gephyrin and GABA$_A$R clusters (e.g., CA1 pyramidal cells) to complete preservation of these clusters (e.g., PV$^+$ interneurons in CA1), passing by partial alteration (loss of gephyrin, but not GABA$_A$R, clusters in Purkinje cells of the cerebellum) [54, 77]. Although preservation of gephyrin clusters might point to a functional homologue of CB, the phenotype of Purkinje cells rather indicates that clustering of gephyrin and of GABA$_A$R depends on different mechanisms, with only the former requiring the presence of CB. In NL2-KO mice, differential loss of postsynaptic clusters was observed in different subcellular compartments of CA1 pyramidal cells and dentate gyrus granule cells, with the main disruption of gephyrin and GABA$_A$R clusters occurring on the soma, at synapses formed by basket cells [35, 78, 79]. Thus, these reports pointed to distinct clustering mechanisms for postsynaptic proteins of perisomatic versus dendritic GABAergic synapses.

Further insight into GABAergic synapse heterogeneity was provided by the analysis of postsynaptic proteins in the hippocampal formation of α2-KO mice [74]. Focusing on CA1 pyramidal cells, where α2-GABA$_A$R predominate in the perisomatic area and the axon-initial segment [80, 81], a pronounced reduction of gephyrin clusters was observed, notably on cell somata, on the axon-initial segment, as well as in stratum radiatum and stratum oriens (Fig. 3a). While this loss of gephyrin clusters was suggestive of a disruption

of the PSD, only moderate changes in GABAergic miniature inhibitory postsynaptic currents (mIPSC) were observed in mice lacking α2-GABA$_A$R, with unchanged amplitude and 40% reduction in frequency. Therefore, these results indicated preservation of GABAergic function despite strongly altered gephyrin clustering at postsynaptic sites. Analysis of α1-GABA$_A$R and NL2 revealed that their presence at perisomatic postsynaptic sites was preserved in CA1 pyramidal cells of α2-KO mice (Fig. 3b), whereas they were absent from the axon-initial segment. This striking subcellular difference was correlated with the presence of the dystrophin–glycoprotein complex (DGC) in perisomatic synapses (Fig. 3c), suggesting that this protein complex represents an alternate mechanism contributing to formation and maintenance of PSD in a subset of GABAergic synapses [74], and confirming that perisomatic and dendritic GABAergic PSDs are molecularly distinct.

The dystrophin–glycoprotein complex

The DGC is a transmembrane signaling complex present in striate and cardiac muscle cells, kidney tubular epithelial cells, neurons, and astrocytes, linking the extracellular matrix to the actin cytoskeleton (reviewed in [82–84]). The DGC is essential for normal brain development and synaptic function and for maintaining the structural integrity of the sarcolemma. Mutations affecting the formation, post-translational modification, or function of the DGC cause multiple forms of hereditary muscle dystrophies. While the contribution of the DGC to muscle disease has been thoroughly investigated, patients with Duchenne and Becker muscular dystrophies also show signs of cognitive impairments and learning disabilities that have been related to its functions in the CNS [83, 85]. In neurons, dystrophin was shown initially to be present in PSD fraction of purified brain membranes [86]. Subsequent studies demonstrated its association with a subset of GABAergic synapses [87, 88], where it regulates GABA$_A$R clustering and GABAergic synaptic function, as well as glutamatergic synaptic plasticity (reviewed in [82, 83]). In mdx mice, direct involvement of dystrophin in synaptic alterations has been demonstrated by dystrophin rescue experiments, which normalize mIPSC amplitude and frequency, as well as LTP in CA1 pyramidal cells [89, 90].

The molecular composition of the DGC differs between muscle sarcolemma, neuromuscular junction, brain and retina, and astrocytes [91, 92], where, for instance, the DGC is involved in proper membrane targeting of aquaporin four at end-feet in contact with brain blood vessels [93, 94]. Common components of the DGC include α- and β-dystroglycan, full-length dystrophin (Dp427), sarcoglycans, a dystrobrevin isoform, and syntrophins. The
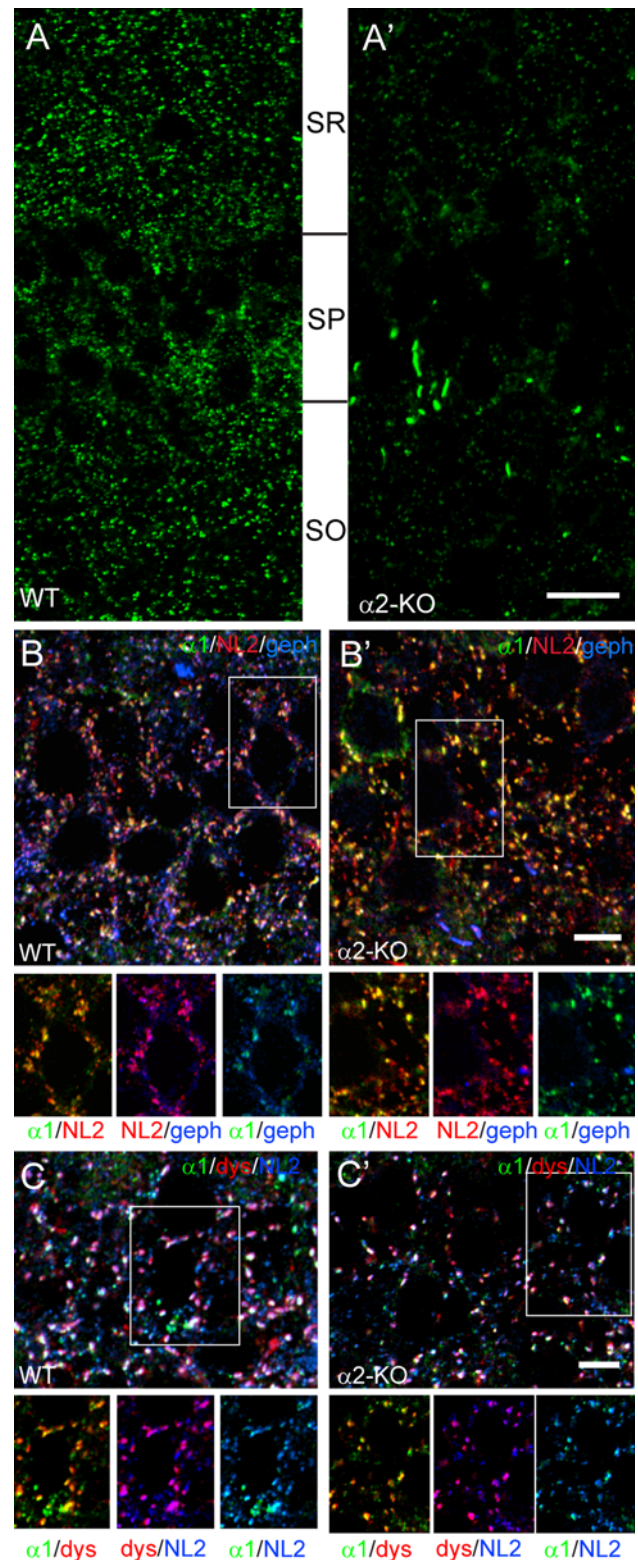
predominant dystrophin isoform found in astrocytes is the short N-terminal variant Dp71.

Association of the DGC with cytoplasmic signaling molecules is ensured by direct protein–protein interactions

◀ **Fig. 3** Loss of gephyrin clusters, but preservation of α1-subunit, NL2, and dystrophin clusters in perisomatic synapses of α2-KO mice; adapted from [74]. **a, a′** Distribution of gephyrin clusters in the CA1 region of the hippocampus, illustrating the strong reduction in α2-KO mice and the appearance of large intracellular gephyrin aggregates. **b, b′** Despite this effect, clustering of α1 subunit and NL2 was not affected around the pyramidal cell body in mutant mice, as seen by triple immunofluorescence staining. The framed area is shown below in color-separated images. **c, c′** Similarly, the postsynaptic clustering of dystrophin and its colocalization with the α1 subunit and NL2 is not affected in α2-KO mice. *Scale bars* **a** 50 μm, **b, c** 10 μm

with β-dystroglycan or dystrophin or by means of PDZ domains present in syntrophin isoforms. Identified partners of syntrophins include nNOS, acquaporin 4, voltage-gated Na^+ and K^+ channels [83], and NLs [95]. Interactions with extracellular signaling molecules critically depends on α-dystroglycan posttranslational modification by glycosylation. Consequently, mutations in glycosylating enzymes contribute to the pathogenesis of a subset of congenital muscle dystrophies associated with lissencephaly and mental retardation [84, 96]. α-dystroglycan function on glial end-feet appears to be especially crucial for normal brain development, whereas neuronal α-dystroglycan glycosylation is necessary for proper synaptic plasticity [97].

Localization of the DGC by immunofluorescence has been documented in neurons, using antibodies to full-length dystrophin, Dp71, utrophin, α-/β-dystroglycan, dystrobrevin, and syntrophins [82, 98]. These studies reveal a systematic colocalization with proteins of the GABAergic PSD in a subset of neurons of cortical areas, including the entire cerebral cortex, hippocampal formation (where Dp71 is selectively present in dentate gyrus granule cells), tectum, and cerebellum. In the hippocampus, the DGC appears to be restricted to principal cells, and is present selectively on the soma and proximal dendrites, suggesting an association with perisomatic synapses (formed by basket cells) [87, 99].

It is not known, however, what determines the presence of the DGC in specific GABAergic synapses of cortical pyramidal cells. In primary neuronal cultures containing only few interneurons, we have shown that presence of a GABAergic terminal is required for the formation of the DGC [100], whereas both gephyrin and GABA_ARs can form ectopic clusters facing glutamatergic terminals. In vivo, it is therefore conceivable that a presynaptic factor present selectively in a subset of GABAergic neurons (such as basket cells, but not chandelier cells or dendritic-targeting interneurons in the hippocampus) induces the formation of the DGC and therefore entails these GABAergic synapses with distinct properties for clustering postsynaptic proteins.

In the context of the present review, interaction of DGC members with GABAergic PSD proteins deserves

particular attention. Interaction between α-dystroglycan and α-/β-neurexins by means of laminin–neurexin–sex hormone-binding globulin (LNS)/laminin G domains has been reported several years ago [101], and was proposed to compete with α-latrotoxin binding on neurexins. In addition, binding of synaptic scaffolding molecule (S-SCAM) to β-dystroglycan and NL2 has been characterized in studies showing the selective presence of S-SCAM at inhibitory synaptic sites [102, 103]. S-SCAM has been identified for its synaptogenic role and interactions with NMDA-receptor subunits and NLs. Its binding to β-dystroglycan involves one of two WW domains, whereas binding to NL2 occurs through the second of its three PDZ domains. Recently S-SCAM was shown to bind also SynArfGEF, which selectively activates Arf6, a GTP-binding protein member of the ADP ribosylation factor family. S-SCAM is able to interact also with several MAGUKs (including PSD-95, SAP97, and Homer) [103]. Further, these authors showed that SynArfGEF also interacts with dystrophin and utrophin via its PDZ domain and that it co-localizes with dystrophin and gephyrin at GABAergic synapses. Besides CB, SynArfGEF is only the second GEF identified so far in GABAergic synapses, and may represent an effector signaling molecule related to the DGC. Owing to the multiple effects of Arf6, the role of SynArfGEF is not yet determined. However, it is clear that it cannot substitute CB for gephyrin or GABA_AR clustering at synapses containing the DGC, as shown in CB-KO mice.

While interactions with S-SCAM appear important for the association of the DGC with NL2 at GABAergic postsynaptic sites, they also provide for the possibility of cross-talk with glutamatergic synapses, in particular via activation of Arf6. Further, the selective association of S-SCAM with NL2 might be of relevance to explain the selective loss of inhibitory PSD proteins in perisomatic synapses of NL2-KO mice (see next section).

Model of PSD protein clustering at GABAergic synapses

Differences in PSD protein clustering properties in GABAergic synapses in relation to the presence or absence of the DGC indicate that models of GABAergic synapse formation and maintenance need to incorporate the role DGC proteins (and possibly SynArfGEF and Arf6) to account for published results. However, since the DGC is present in a subset of GABAergic synapses only, and is dispensable for GABAergic synapse formation in cultured hippocampal neurons [104], models of clustering that do not involve the DGC are also required.

The models proposed here (Fig. 4) are elaborated on the basis of observations made in α1-KO, α2-KO, NL2-KO,

CB-KO, and mdx mice and take the following considerations into account:

1. In wild-type mice, while gephyrin, NL2, and CB likely are present in the majority of GABAergic synapses [14, 32, 41, 65], a major distinction is brought about by the presence or absence of the DGC, as exemplified by thalamic relay neurons and cerebellar neurons (Fig. 4a, b). Some cells, like hippocampal pyramidal cells, carry a mixture of both types of synapses (Fig. 4e, f).

2. Interactions between CB, NL2, gephyrin and possibly GABA$_A$R contribute to the formation of the postsynaptic scaffold (Fig. 4a, b) [14, 15, 50].

3. In synapses containing multiple α subunit variants, such as α1- and α2-subunits in perisomatic synapses of CA1 pyramidal cells (Fig. 4e) [74, 81], it is not known whether they correspond to different receptor subtypes, or whether they are intermingled within single receptors [74].

4. Gephyrin clustering, but not NL2 clustering, requires the presence of postsynaptic GABA$_A$R (Fig. 4c, d) [105], in particular those containing the α2 subunit (Fig. 4g) [74].

5. In contrast, the DGC is not altered in neurons lacking functional GABAergic transmission or gephyrin clusters (Fig. 4d, g) [74, 105], indicating that its formation and postsynaptic localization is not dependent on GABA$_A$R or gephyrin.

6. Preservation of NL2 clustering in the absence of GABA$_A$R and/or gephyrin suggests a mandatory association between NL2 and S-SCAM/β-dystroglycan complex (Fig. 4d, g) [102]. Further, this association raises the unresolved issue whether the DGC requires NL2 for localizing at GABAergic synapses.

7. Disruption of gephyrin and GABA$_A$R clusters in perisomatic, but not dendritic synapses of hippocampal neurons of NL2-KO mice [35, 79] suggests that NL2 is the only NL isoform that interacts with S-SCAM/β-dystroglycan [102] and indicates that postsynaptic clustering of GABA$_A$R requires the presence of a NL isoform. As a corollary, gephyrin cluster preservation in dendritic synapses of NL2 mice suggests compensation by another NL isoform.

8. However, these observations cannot be generalized throughout the brain, as, for example, there is no loss of α1-GABA$_A$R and gephyrin clusters in Purkinje cells of NL2-KO mice (personal observation); the nature of the mechanisms compensating for the absence of NL2 in these cells in not known, but a compensation by NL3 or NL4 is not unlikely.

Fig. 4 Models of GABAergic PSD scaffold structure in distinct cell► types of α1-KO, α2-KO, mdx (lacking full-length dystrophin), and wild-type mice. **a, b** In wild-type mice, GABAergic PSDs contain GABA$_A$Rs, gephyrin, and, in most cases, NL2/3, and CB2$_{SH3+}$ and CB2$_{SH3-}$, although these have not been demonstrated, for example in the thalamus (?). In a subset of synapses, for example in Purkinje cells, the DGC is also present, along with its interacting proteins S-SCAM and SynArfGEF. The PSD is anchored to the actin cytoskeleton. Phosphorylation sites on gephyrin and GABA$_A$Rs (-P) contribute to dynamic regulation of GABAergic synapses. **c, d** In α1-KO mice, gephyrin clusters are disrupted in neurons expressing α1-GABA$_A$R, but NL2 clustering is preserved in the presence of the DGC, presumably via binding to S-SCAM. **e, f** In CA1 pyramidal cells, distinct GABAergic PSDs are present on the soma and dendrites, characterized by the presence or absence of the DGC, respectively. These synapses contain both α1- and α2-GABA$_A$Rs, or possibly receptors containing both subunit variants. In these cells, CB2$_{SH3-}$ and Cdc42 contribute to postsynaptic targeting of gephyrin, as well as regulation of the gephyrin scaffold shape and size. CB2$_{SH3-}$ interacts with NL2/3 and GABA$_A$Rs (see [46] for details). **g** In α2-KO mice, gephyrin clustering is disrupted in synapses containing α2-GABA$_A$R. In perisomatic synapses of CA1 pyramidal cells, the DGC anchors NL2 via S-SCAM, thereby preserving NL2 and α1 subunit clustering. **h** Conversely, in mdx mice, a partial reduction of GABA$_A$R at perisomatic synapses might be due to absence of NL2 linked to the DGC. Preservation of gephyrin clustering is best explained by the interaction between CB2/NL2/α2 subunit. Our model postulates a tight interaction between NL2 (*black*) and the DGC, whereas NL isoforms interacting with CB2$_{SH3+}$ might be either NL2 or NL3 (*dark grey*)

9. In α2-KO mice, a possible direct interaction between α1-GABA$_A$R and NL2 might explain preservation of α1-GABA$_A$R clusters in perisomatic synapses (Fig. 4g); whereas the loss of gephyrin clusters in both perisomatic and dendritic synapses (not shown) reveals their dependence on α2-GABA$_A$Rs, which interact directly with both gephyrin and CB2$_{SH3+}$ [48].

10. Gephyrin-independent α1-GABA$_A$R clustering has been observed in CB-KO mice [54], indicating that these GABA$_A$R do not require CB for their proper localization.

11. Absence of dystrophin, such as occurs in mdx mice, causes a partial disruption of α1- and α2-GABA$_A$R clustering, but not gephyrin clustering (Fig. 4h) [87]. Therefore, the mdx mutation might cause loss of postsynaptic NL2 interacting via S-SCAM, whereas the CB2$_{SH3+}$/NL2/gephyrin complex is likely preserved [35, 46]. It is conceivable that the function of NL2 associated with the DGC is distinct from that of NL2/3 associated with CB and gephyrin.

These models also incorporate the postulated functions of CB2 isoforms and Cdc42 (Fig. 4e, f) [35, 46]:

(1) The ability of CB2$_{SH3-}$ to form trimeric complexes with gephyrin and Cdc42 likely contributes to gephyrin postsynaptic targeting, (2) the role of membrane anchored
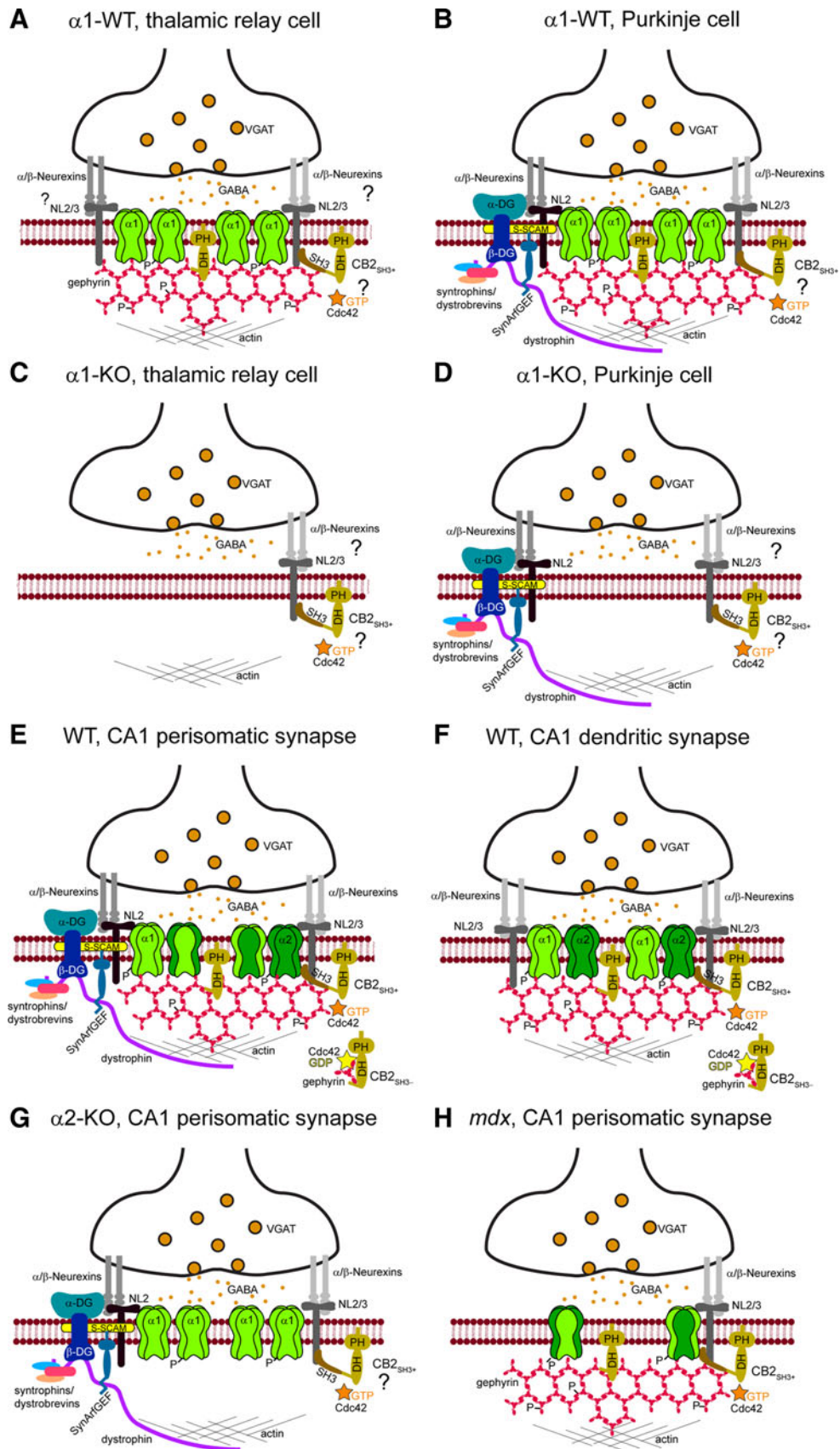
CB2$_{SH3+}$, interacting with NL2, gephyrin, and α2-GABA$_A$R for PSD formation and gephyrin clustering, (3) the stabilization of gephyrin clusters might be mediated by membrane-anchored CB2$_{SH3-}$, (4) the regulation of gephyrin postsynaptic cluster size and shape relies on activated (GTP-bound) Cdc-42.

To explain the phenotype of CB-KO mice, in which a differential loss of gephyrin and GABA$_A$R occurs in various cell types, the presence or absence of the DGC is not sufficient. For instance, α1-GABA$_A$Rs are retained in Purkinje cells of CB-KO mice in the absence of gephyrin [54], suggesting their synaptic anchoring via the DGC and NL2. In contrast, in CA1 pyramidal cells, gephyrin, α1- and α2-GABA$_A$R disappear in CB-KO mice, whereas in PV$^+$ interneurons (and in thalamic VB neurons), gephyrin and α1-GABA$_A$R are retained [77]. However, since the α2-subunit interacts directly with CB2 [48], one might hypothesize that GABAergic synapses containing α2-GABA$_A$R are selectively affected in CB-KO mice. Consequently, in CA1 pyramidal cells, perisomatic synapses might be made of heteromeric α1/α2/β/γ2 receptors, which would fail to cluster in the absence of CB.

Conclusions

Taken together, the results discussed in this review indicate that GABAergic synapses are molecularly heterogeneous, and that this heterogeneity on the postsynaptic side largely arises from the multiple proteins regulating gephyrin clustering and its function as signaling scaffold at the PSD. Moreover, the view that gephyrin forms an inert structure anchoring GABA$_A$Rs needs to be replaced by the concept that these receptors are required at postsynaptic sites to enable gephyrin clustering and to establish a signaling hub in the GABAergic PSD. Therefore, the diversity of GABA$_A$R subunits likely reflects a diversity of signaling pathways operating at GABAergic synapses. While the precise mechanisms underlying GABA$_A$R postsynaptic clustering remain to be elucidated, the models of PSD formation proposed here impart a major role to the neurexin/NL2 complex for specifying the location of GABAergic PSDs in relation to presynaptic terminals. The model is compatible with the proposed seeding role of CB/NL2 interaction for initiating GABA$_A$R and gephyrin clustering and for CB2/gephyrin/Cdc-42 interactions for regulating shape and size of the PSD. Further, extending previous concepts, our model introduces the DGC and its interaction with NL2 via S-SCAM as a second molecular complex present in a subset of GABAergic synapses, possibly under the influence of presynaptic factors. This molecular complex might enable gephyrin-independent α1-GABA$_A$R clustering, possibly via interactions between NL2 and the α1 subunit. Moreover, the differential clustering of α1- and α2-GABA$_A$R observed in mutant mice raises the possibility that these two receptor subtypes belong to functionally distinct signaling scaffolds in GABAergic synapses. Although the DGC is present at a subset of GABAergic synapses only, it might fulfill at least three major functions: stabilization and maintenance of GABAergic synapses, anchoring signaling molecules, notably those containing a PDZ domain, such as Syn-ArfGEF or nNOS, in close vicinity to GABAergic synapses, transducing extracellular signals acting on dystroglycan to regulate the function and plasticity of GABAergic transmission.

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