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Fractional occupancy of synaptic binding sites and the molecular plasticity of inhibitory synapses

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Abstract

The postsynaptic density (PSD) at inhibitory synapses is a complex molecular assembly that serves as a platform for the interaction of neurotransmitter receptors, scaffold and adapter proteins, cytoskeletal elements and signalling molecules. The stability of the PSD depends on a multiplicity of interactions linking individual components. At the same time the PSD retains a substantial degree of flexibility. The continuous exchange of synaptic molecules and the preferential addition or removal of certain components induce plastic changes in the synaptic structure. This property necessarily implies that interactors are in dynamic equilibrium and that not all synaptic binding sites are occupied simultaneously.

This review discusses the molecular plasticity of inhibitory synapses in terms of the connectivity of their components. Whereas stable protein complexes are marked by stoichiometric relationships between subunits, the majority of synaptic interactions have fractional occupancy, which is here defined as the non-saturation of synaptic binding sites. Fractional occupancy can have several causes: reduced kinetic or thermodynamic stability of the interactions, an imbalance in the concentrations or limited spatio-temporal overlap of interacting proteins, negative cooperativity or mutually exclusive binding. The role of fractional occupancy in the regulation of synaptic structure and function is explored based on recent data about the connectivity of inhibitory receptors and scaffold proteins. I propose that the absolute quantification of interactors and their stoichiometry at identified synapses can provide new mechanistic insights into the dynamic properties of inhibitory PSDs at the molecular level.

1. Introduction: the problem with too many interactions

One thing that many synaptic proteins have in common is that they engage in multiple molecular interactions. If each protein could only interact with one binding partner, it would be impossible to build complexes comprising more than two components. If they were to bind to exactly two other molecules each, some clustering may emerge, however, it would be subject to serious constraints as regards the internal organisation and overall stability of the structure, favouring elongated or ring-like arrangements that rely on stable bonds between the components. Anything above an average of two interactions per synaptic protein should be sufficient to create large protein assemblies bordered by steep concentration gradients (Fig. 1, left jigsaw). Most integral components of the PSD have indeed more than two possible binding sites, often substantially more. This is exemplified by the main scaffold protein at inhibitory synapses, gephyrin, that orchestrates the clustering of neurotransmitter receptors in a complex molecular network. The following discussion will be based mostly on several recent reviews that provide exhaustive accounts of the role of gephyrin in the organisation of inhibitory synapses (Tyagarajan and Fritschy, 2014; Choi and Ko, 2015; Alvarez, 2017; Groeneweg et al., 2018; Kasaragod and Schindelin, 2018). I will refer to original articles when needed to support specific arguments or hypotheses, in particular as regards newer publications that have not yet been discussed extensively in the scientific literature.

Gephyrin is a multi-domain protein that is densely clustered at inhibitory synapses. Whereas the N-terminus (G-domain) of gephyrin forms homotrimers, the isolated C-terminal E-domain can form homodimers. With three putative bonds per molecule (two N-terminal and one C-terminal bond) the basic requirement for the clustering of gephyrin at inhibitory PSDs is thus met. Based on its oligomerisation properties, the gephyrin scaffold is widely assumed to take on a planar, hexagonal structure (Kneussel and Betz, 2000). In line with a latticed arrangement, cryo-electron tomographic images confirm that inhibitory PSDs have a thickness of only 10-20 nm (Tao et al., 2018), and quantitative single molecule localisation microscopy (SMLM) has shown that gephyrin is packed at a two-dimensional density of up to $10^4/\mu\text{m}^2$ (Specht et al., 2013). Gephyrin also binds to the intracellular domains (ICDs) of the β -subunit of the glycine receptor (GlyR) and of several GABA_A receptor subunits (reviewed in (Groeneweg et al., 2018)). The primary role of the gephyrin scaffold lies after all in the clustering of inhibitory receptors at synapses (Fig. 2), which directly controls the strength of inhibitory synaptic transmission. Gephyrin may be further tethered to the plasma membrane by palmitoylation of its cysteine residues C212 and C284 (Dejanovic et al., 2014), as well as through a variety of interactions with the adhesion protein neuroligin and the regulatory protein collybistin (Soykan et al., 2014). Additional binding partners of gephyrin include a growing list of cytoskeletal and transport proteins, adaptor and signalling molecules (Uezu et al., 2016).

Inhibitory neurotransmitter receptors likewise have a high level of connectivity. GlyRs and GABA_ARs, both members of the pentameric ligand-gated ion channel (pLGIC) family, are assembled of different combinations of receptor subunits (Kasaragod and Schindelin, 2018). Recent data suggest that heteromeric GlyR complexes most likely contain three α and two β -subunits (discussed in Patrizio et al., 2017; Low et al., 2018). Since only the β -subunits can bind to gephyrin, each pentameric receptor can be attached via two binding sites to the synaptic scaffold. In the case of the GABA_AR, the expression of numerous subunits gives rise to dozens of different receptor complexes displaying a variety of functional and biophysical properties (Mortensen et al., 2012; Chua and Chebib, 2017). Several members of the α and β -subunits were shown to bind directly to gephyrin ($\alpha 1/2/3/5$, $\beta 2/3$; Maric et al., 2011; Mukherjee et al., 2011; Tretter et al., 2011; Kowalczyk et al., 2013; Brady and Jacob, 2015), suggesting that pentameric GABA_ARs may contain up to five synaptic binding sites. The $\alpha 5$ -subunit can also interact in an activity-dependent manner with radixin, an extrasynaptic scaffold protein (Hausrat et al., 2015). Furthermore, the auxiliary subunit Lhfpl4 tethers $\gamma 2$ -containing GABA_AR complexes to neuroligin-2 (Heller et al., 2012; Davenport et al., 2017; Yamasaki et al., 2017). In short, both receptors and gephyrin can potentially interact with several other synaptic molecules simultaneously, suggesting a stable and densely packed network (Fig. 1, left). Such a model, however, is at odds with experimental data showing that synaptic components have a high degree of mobility.

2. Dynamics and disorder at inhibitory synapses

Clear evidence that inhibitory synapses are not static compartments comes from time-lapse imaging. Whereas the synapse as such may persist over many days, its structure and composition undergoes

continuous changes as a result of the addition and removal of molecules on a time scale of seconds to hours (Rubinski and Ziv, 2015). How can the relentless remodelling be reconciled with the apparent tenacity of synapses? The answer may lie in the high local concentration of free binding sites, which means that new arrivals and newly detached molecules are quickly recaptured. Successive episodes of receptor immobilisation separated by small displacements within the same PSD have been spotted by single particle tracking (SPT) using quantum dots (Specht et al., 2013; Renner et al., 2017). Even though individual interactions may be labile, the high availability of binding sites makes it less likely that the detached molecules escape from the PSD. In other words, the sheer size of the synapse and the multiplicity of binding sites stabilises the overall structure. This view is ultimately a generalisation of the so-called ‘slot model’, according to which a constant number of binding sites maintains a steady state of mobile neurotransmitter receptors (Shi et al., 2001).

In consequence, the effective exchange rates of receptors and scaffold proteins at synapses are relatively slow. Fluorescence recovery after photo-bleaching (FRAP) shows that only 40% of the synaptic gephyrin pool is exchanged within 30 minutes in cultured spinal cord neurons, corresponding to a recovery time constant of about two hours (Calamai et al., 2009). Similar gephyrin kinetics were recorded at GABAergic synapses in mature organotypic hippocampal slices (Vlachos et al., 2013). Photo-conversion experiments with GlyRs at motor neuron synapses in zebrafish larvae also returned time constants on the order of hours (Chow et al., 2017). Importantly, the recovery time of GlyRs increased with the size of the synapse, lending support to the idea that higher copy numbers, and by extension a greater availability of binding sites, prolong the effective dwell time of molecules in the PSD. According to this interpretation, cell type specific differences in synaptic GlyR and gephyrin levels are indicative of the relative stability of the PSD (Specht et al., 2013; Chow et al., 2017).

Independent of the size of the synapse, the exchange rate of gephyrin in young hippocampal cultures was much faster than at mature synapses of matched size (Vlachos et al., 2013). The stability of the PSD is therefore subject to additional variations that are likely reflected in the connectedness between synaptic proteins. This suggests that inhibitory PSDs may have different levels of organisation – highly ordered domains with densely packed molecules at one end of the spectrum, loose assemblies of low molecule densities at the other end (Alvarez, 2017). An irregular and disjointed network of filaments was detected using electron tomography of inhibitory PSDs in cultured hippocampal neurons (Linsalata et al., 2014). Super-resolution images confirmed that large synapses can be composed of sub-domains of varying molecule densities that undergo dynamic rearrangements on a time scale of minutes (Specht et al., 2013; Dzyubenko et al., 2016; Orlando et al., 2017; Pennacchietti et al., 2017). In agreement with the interpretation by Alexander Linsalata and colleagues (Linsalata et al., 2014), these data point to a dynamic model of the PSD, whereby sub-synaptic complexes are held together by a network of weak interactions that fail to create long-range ordered structures (Fig. 1, right). Despite the transience of the interactions, the high availability of free binding sites slows the effective exchange rates and maintains the steady state of clustered receptors and scaffold proteins at inhibitory synapses.

3. Affinity and fractional occupancy of synaptic binding sites

Generally, there is a good correspondence between the stoichiometry of an interaction and its stability (Hein et al., 2015). In other words, the occupancy of binding sites correlates well with the binding affinity. Only few of the possible interactions at synapses can be considered as truly stable, at least on the timescale of their residence within the PSD. Examples of stable, stoichiometric interactions are found in the pentameric complexes of GlyRs and GABA_ARs and trimeric complexes of gephyrin that can be regarded as the smallest structural and functional units. The trimer of gephyrin is held together by strong interactions between the G-domains (Schwarz et al., 2001; Sola et al., 2001). SMLM-based molecule counting indicated that cytosolic gephyrin is mostly trimeric (Patrizio et al., 2017), in agreement with AFM data of purified gephyrin complexes showing primarily trimers and less than 2% of hexamers (Sander et al., 2013). So far, there are no indications that the G-domain trimers dissociate under physiological conditions at synapses.

The overwhelming majority of interactions between synaptic proteins, however, have limited kinetic or thermodynamic stability, meaning that they exist in an equilibrium of bound and unbound forms. The lower the affinity of the interaction, the more the equilibrium is shifted towards sub-stoichiometric binding. It has

been proposed that sub-stoichiometric interactions are essential to create interconnected protein networks. Without them, "stable complexes would remain in isolation, [...] they would not be able to connect to each other or to transient, dynamic regulators" (Hein et al., 2015). This concept is reminiscent of the situation at inhibitory synapses, where the formation of the PSD requires interactions between stable trimers of gephyrin as well as between pentameric receptor complexes and the gephyrin scaffold.

Isolated E-domains of gephyrin can form stable homodimers with a K_D in the nanomolar range, as inferred from measurements with the bacterial homolog MoeA (31 nM; Xiang et al., 2001), however, the strength of this interaction at synapses is not known. It has long been thought that the dimerisation of gephyrin at synapses requires some kind of switch, given that outside of synapses dimerisation of the full-length protein is blocked through an unknown mechanism (Schrader et al., 2004; Sola et al., 2004). A possible trigger could be the binding of inhibitory pLGICs to the receptor-binding site of gephyrin. However, this does not appear to be the case, since soluble gephyrin as well as gephyrin attached to extrasynaptic GlyRs takes on the form of a trimer (Patrizio et al., 2017). An alternative explanation is that E-dimer formation is controlled by the central domain of gephyrin that can assume either elongated or compact conformations (Sander et al., 2013). This protein sequence contains a large number of regulatory phosphorylation sites that can affect gephyrin clustering or receptor binding (see §4).

Conversely, the accumulation of GlyRs at inhibitory synapses depends on gephyrin binding via the β -subunits of the receptor. Whereas GlyR $\alpha\beta$ heteropentamers are trapped at synapses, GlyRs composed only of α -subunits do not co-localise with synaptic gephyrin clusters (Patrizio et al., 2017). Insertion of the gephyrin binding sequence of GlyR β into the α -subunit rescues receptor clustering (Meier et al., 2001). The affinity of the GlyR β -gephyrin interaction is relatively strong. Isothermal titration calorimetry (ITC) yields K_D values from 2 μ M (Maric et al., 2014) to as low as 14 nM (Grunewald et al., 2018). It can therefore be expected that the occupancy of binding sites is quite high. Quantitative SMLM measurements revealed that an average inhibitory synapse in cultured spinal cord neurons contains about four times as many gephyrin molecules (304) as GlyR complexes (83). Since the receptor is thought to have an $\alpha_3\beta_2$ stoichiometry, the nominal occupancy of gephyrin binding sites at these synapses is about 50% (Patrizio et al., 2017). At present, it is not clear how many of the remaining binding sites are occupied by GABA $_A$ Rs or other interacting proteins (§5). If the contributions of all interactors are taken into account, the total occupancy of receptor-binding sites at inhibitory synapses most likely exceeds 50%. The affinity of GABA $_A$ R subunits for gephyrin, however, is much lower than that of GlyR β ; their dissociation constants range from 5-17 μ M for the subunits α_1 and α_3 (Maric et al., 2011; Mukherjee et al., 2011; Tretter et al., 2011), and 17-55 μ M for β_2 (Kowalczyk et al., 2013). As a result, GABA $_A$ Rs are bound less tightly to the synaptic scaffold and retain a higher mobility than GlyRs (Levi et al., 2008). Another consequence of the higher affinity of the GlyR β -gephyrin interaction is that about half of the receptors outside of synapses are also bound to gephyrin (Ehrensperger et al., 2007; Patrizio et al., 2017), while GABA $_A$ Rs are not.

The connectivity of the inhibitory PSD is evidently shaped by the co-existence of high- and low-affinity interactions that result in different fractional occupancies of binding sites (Fig. 1, right jigsaw). Whereas stable interactions form stoichiometric complexes and ordered sub-domains with a high level of interconnectedness, the role of transient interactions is to prevent the fragmentation of the overall network into small modules (Hein et al., 2015). This model reconciles the tenacity of the overall structure with the diffusion dynamics of individual molecules, and is consistent with the observation that the multivalent receptors and scaffold proteins show reciprocal stabilisation at inhibitory synapses (§4).

4. Plasticity of synaptic interactions

The PSD is subject to signalling processes that modulate its composition and/or the function of its components. A common mechanism to induce changes in the organisation of the PSD is by changing the connectivity between synaptic proteins. Several post-translational modifications are known to affect the affinities of receptors and scaffold proteins. The central domain of gephyrin contains numerous *in vivo* phosphorylation sites that are prone to alter its conformation and oligomerisation properties. Phosphorylation of amino acid residues S268 by ERK1/2, S270 by GSK3 β or CDK5, and S305 by CaMKII differentially regulates the number and size of gephyrin clusters in hippocampal neurons, with downstream consequences on GABA $_A$ R clustering and by extension inhibitory synaptic strength (Tyagarajan et al., 2011b; Kuhse et al.,

2012; Tyagarajan et al., 2013; Kalbouneh et al., 2014; Flores et al., 2015). As a target of converging signalling pathways, residue S270 appears to hold a special role in gephyrin clustering. A recent study has raised the interesting prospect that phosphorylation of this site may be associated with other post-translational modifications of gephyrin such as SUMOylation, giving rise to a complex regulatory system (Ghosh et al., 2016). This may also explain the diversity of the effects of S270 phosphorylation, which has been variously linked to a reduction in cluster density (Tyagarajan et al., 2011b) or an increase in the number (Kalbouneh et al., 2014) or the size and intensity of gephyrin clusters (Battaglia et al., 2018).

It is noteworthy that changes in gephyrin clustering are often paralleled by similar changes in GABA_AR distribution (e.g. Kalbouneh et al., 2014; Battaglia et al., 2018). This demonstrates that there is a reciprocal stabilisation of receptors and scaffold proteins at inhibitory synapses. It is therefore possible that the diverse effects of S270 phosphorylation on gephyrin clustering result from changes in the receptor-gephyrin affinity as well as from changes in gephyrin-gephyrin binding. Along these lines, phosphorylation of residues S188, S194 and S200 followed by Pin1-dependent proline cis-trans-isomerisation increases the affinity of the gephyrin scaffold for GlyR β (Zita et al., 2007), whereas Pin1 acting on neuroligin-2 reduces both gephyrin binding and GABAergic transmission (Antonelli et al., 2014). PKC-dependent phosphorylation of GlyR β at residue S403 reduces the affinity of the receptor for gephyrin (Specht et al., 2011). Residue T375 of GABA_AR α 1 is also subject to phosphorylation *in vivo* (Trinidad et al., 2012). Introduction of a phosphomimetic mutation at this site reduces gephyrin binding, receptor clustering at synapses as well as mIPSC amplitudes in hippocampal neurons (Mukherjee et al., 2011).

Since the molecular composition of the PSD controls the efficacy of inhibitory neurotransmission, the above mechanisms link the plasticity of molecular interactions with the functional plasticity of inhibitory synapses. What is interesting is that in those cases where the precise mechanism has been identified, post-translational modifications target interactions that have fractional occupancy, such as the GlyR β -gephyrin interaction that is sustained at about half of the extrasynaptic receptors (Ehrensperger et al., 2007; Patrizio et al., 2017). By shifting the equilibrium between bound and unbound molecules, the modulation of such interactions has a gradual effect on the clustering of synaptic proteins. The phosphorylation of residues in the gephyrin-binding motifs of GlyR β and GABA_AR α 1, for instance, reduces their affinity for gephyrin from 0.02 to 0.9 μ M and from 17 to 183 μ M, respectively (Mukherjee et al., 2011; Specht et al., 2011). Since these residues are masked when bound to gephyrin, it can also be inferred that their phosphorylation most likely occurs outside of synapses. As such, this mechanism may serve to restrict the recruitment of new receptors to the synapse.

In the case of gephyrin, it is not known by which mechanism the phosphorylation of residues in the central domain alters the clustering of the scaffold protein. Stable interactions such as the trimerisation of the gephyrin G-domain are unlikely to be affected, since the dissociation of the trimer would produce totally different structural units. Instead, gephyrin phosphorylation probably acts on the dimerisation of the E-domain, receptor binding, or on other transient interactions of gephyrin. The fact that the majority of phosphorylation sites of gephyrin are located in the central domain that itself is not implicated in dimerisation and receptor-binding further suggests that these signalling processes may act not only on soluble gephyrin but also on clustered gephyrin at synapses.

5. Competition for synaptic binding sites

Interactions between pairs of proteins in the PSD should not be considered in isolation. This is because many proteins have overlapping binding sites, making their interactions mutually exclusive. An example is the binding of various GABA_AR subunits and GlyR β to the same binding pocket of gephyrin (Maric et al., 2011; Kowalczyk et al., 2013). Both collybistin and neuroligin-2 appear to bind to the very same region (Tretter et al., 2011; Antonelli et al., 2014), which introduces a strong competition for synaptic binding sites. Certain interactions on the other hand may be compatible with each other. Tripartite complexes consisting of gephyrin and collybistin together with neuroligin-2, GABA_AR α 2 or the signalling protein Cdc42 have been proposed (Poulopoulos et al., 2009; Saiepour et al., 2010; Tyagarajan et al., 2011a). The binding of neuroligin-2 or GABA_AR α 2 relieves an auto-inhibition of collybistin and triggers membrane binding and gephyrin recruitment. Since most of the binding affinities between these components are not known, however, it is difficult to estimate the true occupancy of these interactions at synapses. Some of the tripartite binding modes could in fact result from the multivalency of the interactors, such as the simultaneous binding

of heteropentameric GABA_ARs to collybistin (via the $\alpha 2$ subunit, $K_D = 1 \mu\text{M}$) and gephyrin (via $\alpha 1$ or $\alpha 3$; Hines et al., 2018), or the binding of different E-domains of a single gephyrin trimer to both, collybistin and GABA_ARs.

Competition for synaptic binding sites is best exemplified by the observation that dynamic changes in $\alpha 2$ -containing GABA_AR complexes at hippocampal synapses are counterbalanced by inverse changes in GABA_AR $\alpha 5$ (Garrow and Triller, 2014). Similarly, overexpressed membrane constructs containing the gephyrin binding sequence of GlyR β displace endogenous glycine receptors from spinal cord synapses (Specht et al., 2011). More recently, Hans Michael Maric and colleagues have developed dimeric peptides that bind gephyrin with picomolar affinity (Maric et al., 2017). These super-binding peptides efficiently outcompete GlyR β and GABA_AR $\alpha 3$ in competition assays and, when delivered to neurons, reduce glycinergic mIPSCs in a dose-dependent manner, demonstrating their pharmacological potential as negative regulators of inhibitory neurotransmission.

The observation that synaptic gephyrin clusters with the highest GlyR occupancy in spinal cord neurons have the lowest GABA_AR levels and *vice versa* (Specht et al., 2013) may also reflect some kind of competition between inhibitory receptors at mixed synapses. However, since the expression of the two types of receptor is cell-type dependent, the relative occupancy of binding sites stems from differences in availability rather than from the direct displacement of one receptor type by another. At a given ratio, the occupancy of binding sites is dictated by the affinities of the competitors for gephyrin and would be roughly the same at all synapses (Fig. 2). Cell-type specific receptor profiles thus point to a regulation at the level of gene expression (Steiger and Russek, 2004). This, together with post-translational mechanisms that act on receptor assembly, trafficking and clustering (e.g. Hausrat et al., 2015; Martenson et al., 2017; Ge et al., 2018), regulates the relative quantities of inhibitory receptor subtypes at the cell surface.

This raises the interesting question whether the total number of receptor-binding sites in the cell (that is the number of gephyrin molecules) equals the number of interactors (i.e. the sum of GlyR β and GABA_AR subunits, collybistin and neuroligins). The fact that extrasynaptic GlyR-gephyrin complexes are in equilibrium with free receptor complexes and soluble gephyrin trimers (Patrizio et al., 2017) suggests that the concentrations of the reactants are commensurate. The presence of GlyRs and GABA_ARs at mixed synapses also shows that the two types of receptor are in a position to compete for synaptic binding sites despite differences in affinity. If there were a significant shortage of gephyrin, the low-affinity interactors would be outcompeted. An excess of gephyrin, on the other hand, is counterbalanced by the formation of so-called *blobs*, large cytosolic aggregates that reduce gephyrin availability (Lardi-Studler et al., 2007). It is therefore highly likely that the cellular gephyrin levels generally match the total number of molecules vying for its binding sites. Only then has the regulation of receptor-gephyrin binding by post-translational modifications the greatest impact on the composition and the functional properties of the PSD.

6. Putting a lid on the strength of receptor-scaffold interactions

The relatively strong binding between GlyR β and gephyrin could in principle hamper the molecular plasticity of glycinergic synapses. It has been observed in some studies, however, that GlyR β -gephyrin binding is bimodal (discussed in Kasaragod and Schindelin, 2018), which could limit the receptors' stability at synapses. Surface plasmon resonance (SPR) and ITC have shown that peptides containing the intracellular domain (ICD) of GlyR β can bind to trimeric full-length gephyrin with high affinity or with low affinity *in vitro* (Schrader et al., 2004; Sola et al., 2004). A recent study reported that high-affinity binding occurs at roughly one third of the binding sites ($K_D = 14 \text{ nM}$), whereas the remainder of the sites have low affinity ($1.5 \mu\text{M}$; Grunewald et al., 2018). These data imply that only one high-affinity interaction per gephyrin trimer is possible. Since the β -ICD peptides used in ITC experiments can be considered as identical, the bimodality of GlyR β -gephyrin binding must be a function of trimeric gephyrin. In other words, binding of GlyR β to one E-domain would trigger conformational changes that reduce the affinity of the other two E-domains in the trimer, constituting a form of negative cooperativity. The central domain of gephyrin is likely to play an essential role in this mechanism (Sander et al., 2013). When β -ICD peptides were reacted with dimers of the gephyrin E-domain, both the affinity and the binding stoichiometry were reduced (Grunewald et al., 2018). As suggested in an earlier study this could reflect an additional steric hindrance or allostery between the two binding sites in the E-dimer (Sola et al., 2004). It should be noted, however, that ITC data vary considerably

between laboratories, and that bimodal binding has not been observed consistently (e.g. Maric et al., 2014). Despite the lack of consensus, it can be said that long β -ICD peptides encompassing the core motif as well as adjacent regions generally display either bimodal binding or fractional occupancy (Kasaragod and Schindelin, 2018).

If the dual GlyR β -gephyrin binding is accepted several consequences are to be expected. Low-affinity binding would weaken the avidity of divalent GlyR $\alpha_3\beta_2$ complexes for gephyrin and enhance the mobility of the receptors at synapses. Using SMLM-based molecule tracking, both high- and low-affinity binding was shown to contribute to the immobilisation of GlyRs at synapses, even though the high-affinity interaction obviously had a greater effect (Grunewald et al., 2018). Outside of synapses, it is unlikely that a single gephyrin trimer could form a bridge between different GlyR complexes, since only one of its binding sites would have the required stability. Likewise, extrasynaptic GlyR $\alpha_3\beta_2$ complexes do not appear to crosslink different trimers of gephyrin (Patrizio et al., 2017). The underlying reason is not known. One possibility is that for this to happen, the E-domains would have to dimerise in order to come sufficiently close to one another (Maric et al., 2014). Since the dimerisation of the E-domain is suppressed outside of synapses (§3), steric constraints may prevent gephyrin crosslinking by GlyRs. At synapses, where E-domain dimers do occur, the associated loss of receptor occupancy balances receptor binding against the oligomerisation of the gephyrin scaffold (Sola et al., 2004; Grunewald et al., 2018). Hence, the importance of the bimodal GlyR β -gephyrin interaction could be an overall dampening of the receptor-scaffold connectivity.

It can also be deduced that different mechanisms govern the accumulation of GlyRs and GABA_ARs at inhibitory synapses. The existence of extrasynaptic GlyR-gephyrin complexes suggests that GlyR recruitment occurs to some extent via the fusion of these building blocks. A partial dimerisation of the E-domains is therefore necessary for gephyrin clustering at glycinergic synapses (Saiyed et al., 2007; Calamai et al., 2009). Unbound GlyRs could also be trapped by the gephyrin scaffold itself. However, since the high-affinity binding sites at synapses are likely to be occupied, the docking of a freely diffusing GlyR would have to rely on low-affinity binding (Grunewald et al., 2018). In contrast, diffusing GABA_ARs must be directly trapped by the available binding sites. This is compatible with the observation that GABA_ARs can cluster in the absence of gephyrin at certain synapses (Kneussel et al., 2001; Panzanelli et al., 2011). Interestingly, the $\gamma 2$ -subunit appears to have an important role in GABA_AR clustering (Essrich et al., 1998), although no gephyrin binding motif has been identified. The discovery that Lhfp14 forms tripartite complexes with GABA_AR $\gamma 2$ and neuroligin-2 (Davenport et al., 2017; Martenson et al., 2017; Yamasaki et al., 2017) points to an alternative clustering mechanism. The recruitment of $\gamma 2$ -containing GABA_ARs promotes the accumulation of gephyrin and *vice versa*, illustrating once more the reciprocal stabilisation of the two components at GABAergic synapses (Essrich et al., 1998). This interdependence enables signalling processes to modify GABAergic synapses by acting on the connectivity of various interactors. In addition to changes in the number of molecules, plasticity paradigms were shown to alter the sub-synaptic distribution of receptors and scaffold proteins (Orlando et al., 2017; Pennacchietti et al., 2017; Battaglia et al., 2018). To what extent these changes are specific for certain types of synapses remains to be seen, however, it can be hypothesised that differences in composition and connectivity generate unique forms of molecular plasticity (§7).

7. Conclusion: spatio-temporal variations of synaptic proteomes

The fractional occupancy of binding sites at synapses can be due to a variety of causes such as low affinity, the redundancy of binding sites for the same molecular target or negative cooperativity. In addition, the co-existence of interaction partners at synapses can be limited, since synaptic proteomes display strong spatio-temporal variations. GlyRs, for instance, are conspicuously absent from (most) inhibitory synapses in the frontal cortex and hippocampus (Uezu et al., 2016). It is therefore not surprising that the gephyrin scaffold at cortical synapses is fundamentally different from the one at spinal cord synapses that express both glycine and GABA_A receptors at different ratios. The absolute number of gephyrin molecules at an average glycinergic synapse in the spinal cord (~600) largely exceeds that of GABAergic synapses in spinal cord and cortical neurons (~190 and 130, respectively; (Specht et al., 2013). Given the lower affinity of GABA_ARs for gephyrin, indirect interactions between the receptors and the gephyrin scaffold (via collybistin, neuroligin and auxiliary proteins) play a greater role in the assembly of GABAergic synapses (Papadopoulos et al., 2007; Soykan et al., 2014; Davenport et al., 2017; Yamasaki et al., 2017). A unified definition of a

stereotypic inhibitory PSD thus becomes meaningless, and the behaviour of each synapse has to be seen in relation to its exact composition.

Quantitative information that directly compares the copy numbers of associated receptors, scaffold and adhesion proteins at identified synapses, however, is largely lacking. This is because synaptic proteomes are generally considered by inclusive reckoning, using biochemical approaches with poor spatial and temporal resolution. SMLM-based super-resolution imaging provides a means to explore the relationship between the most important interactors and competitors in a synapse-specific manner. SMLM not only can visualise the internal organisation of inhibitory synapses with high spatial precision, it also gives access to quantitative information about the stoichiometry of synaptic components. Furthermore, single molecule tracking can be used to evaluate the strength of biochemical interactions in living cells (Salvatico et al., 2015). Experimental approaches that measure interaction lifetimes such as *in vitro* single molecule binding may yield additional information about the kinetics of specific interactions. The combination of binding rates and equilibrium constants, together with absolute numbers of interacting molecules and the occupancy of binding sites can serve as a basis for modelling of the molecular dynamics and connectivity of the PSD and give new mechanistic insights into the plasticity of inhibitory synapses, provided that the synaptic proteome is well defined.

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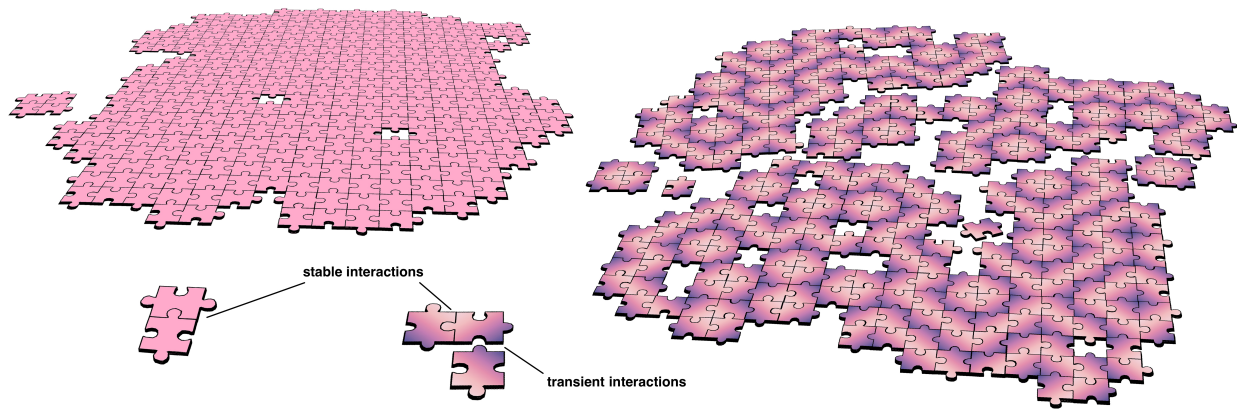


Figure 1. Jigsaw model of the postsynaptic density.

Many integral synaptic proteins have multiple binding partners. The multiplicity of interactions creates dense clusters bordered by steep concentration gradients. If two or more high-affinity bonds were required to stabilise a protein at the synapse, this would limit the dynamic exchange of individual components and impose a strong constraint on the internal organisation and the overall shape of the cluster (left puzzle). Such an arrangement has been proposed for the gephyrin scaffold at inhibitory synapses, where each gephyrin molecule is thought to maintain simultaneous interactions with several other synaptic proteins. As opposed to this model, recent experimental data point to a more flexible arrangement that is characterised by the fractional occupancy of binding sites (right puzzle). The presence of low-affinity interactions implies that many binding sites are not occupied, allowing the addition or the loss of individual molecules or groups of molecules. At the same time, high-affinity interactions create more stable sub-domains with a higher order and occupancy. The co-existence of low- and high-affinity interactions (dark and light patterns, respectively) gives rise to internal structures, where low-affinity sites generally occupy the periphery of the sub-domains. Please note that this scheme is merely intended to illustrate the concept of fractional occupancy. Any resemblance to actual synaptic structures, excitatory or inhibitory, is purely coincidental.

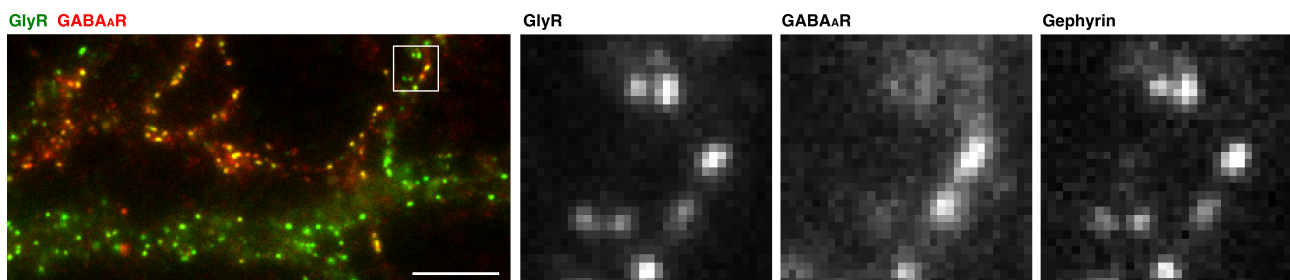


Figure 2. GlyRs and GABA_ARs at mixed synapses in cultured spinal cord neurons.

Rat spinal cord cultures were labelled with antibodies against endogenous GlyR α 1, GABA_AR α 2 and gephyrin (Xiaojuan Yang, unpublished data). Left: Dendritic segments of two neurons expressing different ratios of GlyR (green) and GABA_AR (red). Scale: 10 μ m. Right: High magnification images (5 x 5 μ m, pixel size 160 nm) showing the accumulation of GlyRs and GABA_ARs at synaptic gephyrin clusters (labelled with mAb7a antibody, Synaptic Systems).