



# Targeting GABA<sub>A</sub>R-Associated Proteins: New Modulators, Labels and Concepts

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$\gamma$ -aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) are the major mediators of synaptic inhibition in the brain. Aberrant GABA<sub>A</sub>R activity or regulation is observed in various neurodevelopmental disorders, neurodegenerative diseases and mental illnesses, including epilepsy, Alzheimer's and schizophrenia. Benzodiazepines, anesthetics and other pharmaceuticals targeting these receptors find broad clinical use, but their inherent lack of receptor subtype specificity causes unavoidable side effects, raising a need for new or adjuvant medications. In this review article, we introduce a new strategy to modulate GABAergic signaling: targeting the intracellular protein interactors of GABA<sub>A</sub>Rs. Of special interest are scaffolding, anchoring and supporting proteins that display high GABA<sub>A</sub>R subtype specificity. Recent efforts to target gephyrin, the major intracellular integrator of GABAergic signaling, confirm that GABA<sub>A</sub>R-associated proteins can be successfully targeted through diverse molecules, including recombinant proteins, intrabodies, peptide-based probes and small molecules. Small-molecule artemisinins and peptides derived from endogenous interactors, that specifically target the universal receptor binding site of gephyrin, acutely affect synaptic GABA<sub>A</sub>R numbers and clustering, modifying neuronal transmission. Interference with GABA<sub>A</sub>R trafficking provides another way to modulate inhibitory signaling. Peptides blocking the binding site of GABA<sub>A</sub>R to AP2 increase the surface concentration of GABA<sub>A</sub>R clusters and enhance GABAergic signaling. Engineering of gephyrin binding peptides delivered superior means to interrogate neuronal structure and function. Fluorescent peptides, designed from gephyrin binders, enable live neuronal staining and visualization of gephyrin in the post synaptic sites with submicron resolution. We anticipate that in the future, novel fluorescent probes, with improved size and binding efficiency, may find wide application in super resolution microscopy studies, enlightening the nanoscale architecture of the inhibitory synapse. Broader studies on GABA<sub>A</sub>R accessory proteins and the identification of the exact molecular binding interfaces and affinities will advance the development of novel GABA<sub>A</sub>R modulators and following *in vivo* studies will reveal their clinical potential as adjuvant or stand-alone drugs.

**Keywords:** GABA<sub>A</sub> receptors, gephyrin, collybistin, protein-protein interaction (PPI), super resolution microscopy, fluorescent probes, dimeric peptide, peptide inhibitor design

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**Received:** 31 March 2019

**Accepted:** 12 June 2019

**Published:** 26 June 2019

### Citation:

Khayenko V and Maric HM  
(2019) Targeting  
GABA<sub>A</sub>R-Associated Proteins: New  
Modulators, Labels and Concepts.  
*Front. Mol. Neurosci.* 12:162.  
doi: 10.3389/fnmol.2019.00162

## INTRODUCTION

$\gamma$ -aminobutyric acid type A receptors (GABA<sub>A</sub>Rs) are the principal mediators of phasic and tonic inhibition in the human brain, being a vital part of the molecular machinery that creates cognition, behavior, and consciousness (Fritschy and Panzanelli, 2014). Dysfunctional GABA<sub>A</sub>Rs are involved in the pathogenesis of neurodevelopmental disorders (Ali Rodriguez et al., 2018), schizophrenia (de Jonge et al., 2017), postpartum depression (Mody, 2019), epilepsy (Palma et al., 2017; Hines et al., 2018), Alzheimer's disease (Govindpani et al., 2017), autism (Vien et al., 2015) and stroke (Darmani et al., 2016; Wang et al., 2018). Structurally, these receptors belong to the pentameric ligand-gated ion channels harboring an extracellular domain (ECD), followed by four helical transmembrane domains (TMDs) and loops connecting these helices. GABA<sub>A</sub>Rs display a highly subtype-specific cellular and sub-cellular distribution and exhibit distinct physiological properties, making them very attractive pharmaceutical targets.

First GABA<sub>A</sub>R targeting compounds have been discovered more than a century ago. In 1904, Bayer marketed barbitol, the first barbiturate and positive allosteric modulator of GABA<sub>A</sub>Rs (Löscher and Rogawski, 2012). In the 1960s, benzodiazepines, a new class of GABA<sub>A</sub>R allosteric modulators (Sancar and Czajkowski, 2011), became commercially available. Today, modulators of GABA<sub>A</sub>R activity find broad clinical use as anesthetics (Propofol; Olsen, 2018), anticonvulsants (Gabapentin) or as hypnotics, muscle-relaxants and anxiolytics (Clonazepam, Diazepam), and new experimental medicines are developed. Nonetheless, wider application of these classical GABA<sub>A</sub>R modulators is limited by their lack of receptor subtype specificity, due to the fundamental structural and functional constraints: pharmacologically exploited sites are small hydrophobic pockets with high subunit sequence homology located at the folded ECDs and TMDs of the ion channels (Figure 1; Miller et al., 2017; Kasaragod and Schindelin, 2018; Masiulis et al., 2019). Additionally, binding sites on the interface between two subunits, such as the benzodiazepine binding site, are shared among different synaptic receptor subtypes. Consequently, the action of classical clinically relevant GABA<sub>A</sub>R ligands can be unspecific and provoke unavoidable side effects.

Molecules modulating receptor signaling through accessory proteins in the central nervous system (CNS; Figure 1) emerged as a new class of pharmaceuticals with superior receptor specificity and potential to treat epilepsy, neuropathic pain, fibromyalgia, migraines, and other diseases (Maher et al., 2017). Therefore, targeting GABA<sub>A</sub>R-associated proteins might be a superior pharmacological strategy compared to the classical approaches. This rational approach, however, requires detailed knowledge and advanced understanding of the intracellular signaling of distinct GABA<sub>A</sub>R subtypes. The large number of post-synaptic candidate proteins that directly or indirectly associate with GABA<sub>A</sub>Rs is still increasing (Krueger-Burg et al., 2017), with functional studies exploring some of their

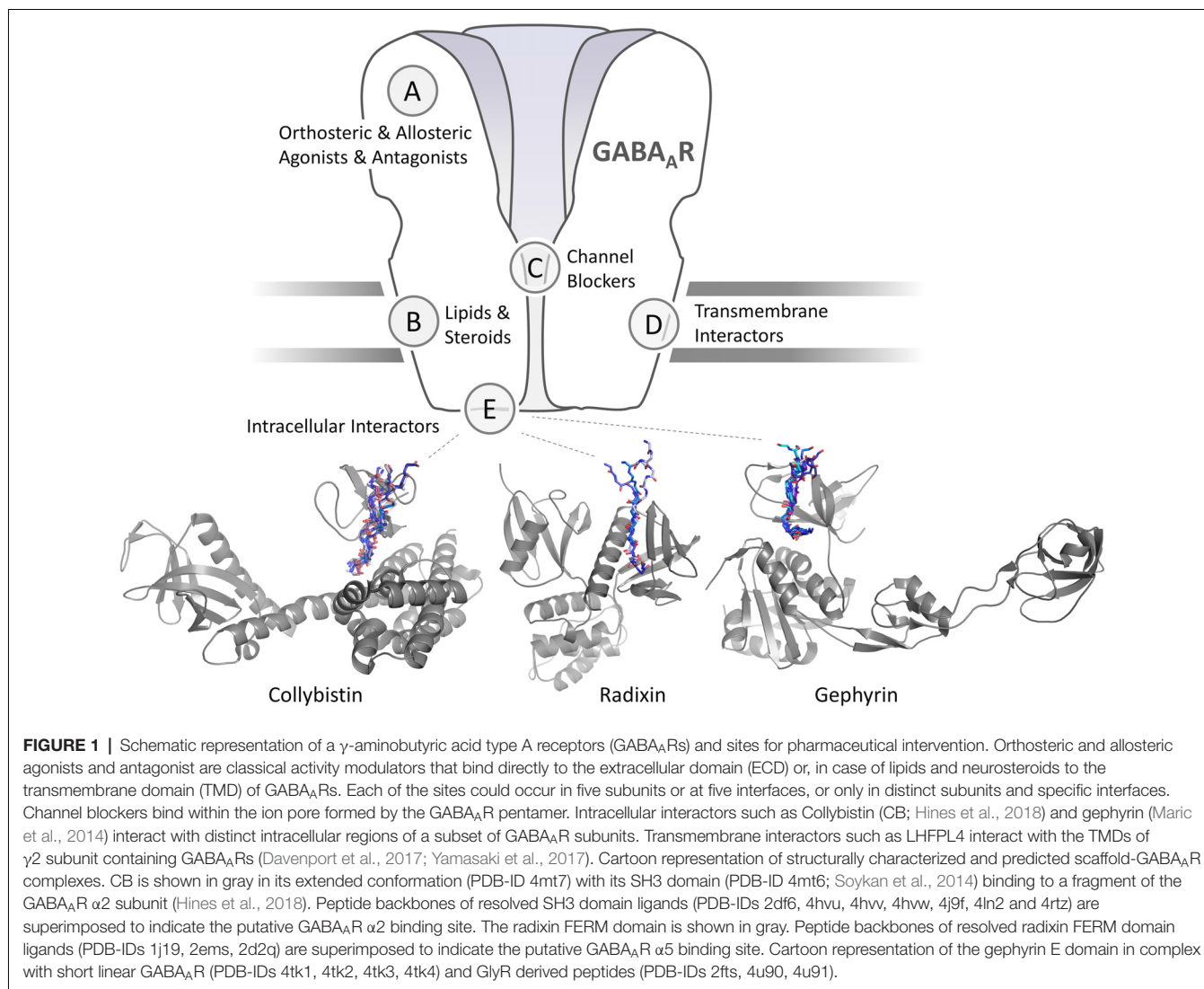
physiological roles and organization (Uezu et al., 2016; Lu et al., 2017), yet, the specific molecular details of these interactions remain largely unknown. We hypothesize that the identification of the exact molecular binding interfaces and binding affinities of known and newly identified GABA<sub>A</sub>R associated proteins will not only greatly expand our basic understanding of CNS function, but also provide new pharmaceutical opportunities.

## ADJUSTING GABAERGIC SIGNALING THROUGH INTRACELLULAR MODULATION

The majority of GABA<sub>A</sub>Rs assemble as heteropentamers to form GABA-gated chloride channels. Different subunit combinations possess unique pharmacology (Olsen and Sieghart, 2009), divergent brain region distribution (Wisden and Seeburg, 1992), cell-type specific expression (Lee and Maguire, 2014), and varying subcellular localization between synaptic and extra-synaptic sites (Mody and Pearce, 2004). Thus, subtype-specific modulators of GABA<sub>A</sub>R signaling should affect distinct circuits, brain regions or subcellular populations with improved accuracy and more selective pharmacology. Combined structural and functional studies have revealed the molecular details of the interplay of the ECD and TMDs in channel gating (Miller and Aricescu, 2014; Lu et al., 2017; Kasaragod and Schindelin, 2018; Zhu et al., 2018; Laverty et al., 2019). Structural studies of the receptors could, so far, not resolve most of the presumably intrinsically disordered intracellular regions of GABA<sub>A</sub>Rs. Short intracellular receptor regions, however, do adopt defined conformations when engaged with structured intracellular interactors, such as gephyrin (Maric et al., 2014) and the AP2 complex (Kittler et al., 2008; Table 1). Functional studies validated that distinct motifs within these unstructured regions exert tight control over channel biosynthesis, recycling, diffusion and synaptic recruitment (Tretter et al., 2012; Nakamura et al., 2015; Groeneweg et al., 2018; Lorenz-Guertin and Jacob, 2018). Remarkably, these intracellular regions display the highest level of sequence heterogeneity among receptor subunits, thereby enabling subtype-specific modulation of GABAergic signaling. Agents targeting these discrete regions will probably be highly selective and could affect GABA<sub>A</sub>R subtypes with distinct functional and pharmacological properties. It is noteworthy that, so far, all intracellular GABA<sub>A</sub>R interactions that displayed sufficient affinity and specificity ended up being exploited to modulate neuronal communication (Table 1).

## AFFECTING POSTSYNAPTIC GABA<sub>A</sub>R ACCUMULATION BY TARGETING INTRACELLULAR SCAFFOLDS

The concept of neurotransmission modulation through targeting receptor-scaffolding protein interactions originated from studies investigating PSD-95/Discs-large/ZO-1 (PDZ) domain carrying proteins. These showed that through modulation of receptor-scaffolding protein interactions a variety of responses could be achieved, ranging from disruption of glutamate signaling to



neuroprotective effects in ischemic brain damage (Hammond et al., 2006; Sainlos et al., 2011; Bach et al., 2012; **Figure 2A**). These results suggested that modulation of the inhibitory neurotransmission could be accomplished in a similar way, a concept recently proved with the inhibitory scaffold protein gephyrin (Maric et al., 2017).

Scaffolding proteins, such as gephyrin (Specht et al., 2013), radixin (Loebrich et al., 2006; Hausrat et al., 2015) and (collybistin, CB; Mayer et al., 2013; Hines et al., 2018), dynamically regulate the cell membrane distribution of postsynaptic and extrasynaptic GABA<sub>A</sub>Rs. Interestingly, their scaffolding functions are highly receptor specific, potentially allowing a fine tuning of neurotransmission.

## Radixin

Radixin is involved in the anchoring of numerous membrane proteins to the actin cytoskeleton (Kawaguchi et al., 2017). Its C-terminal domain mediates actin binding, while the N-terminal FERM domain functions as a universal protein-binding module

that directly interacts with receptors, extracellular matrix components, transmembrane and adhesion proteins (Kitano et al., 2006; Takai et al., 2007; Terawaki et al., 2007, 2008; Yogesha et al., 2011; **Figure 1**). Radixin also harbors a central  $\alpha$ -helical domain, which either adopts a closed or elongated conformation to allow its auto-inhibitory module to mask the FERM domain. In neurons, radixin is activated through phosphorylation, which enables its simultaneous binding to cytoskeletal elements and transmembrane proteins, including  $\alpha$ 5 subunit containing GABA<sub>A</sub>Rs (Loebrich et al., 2006; Hausrat et al., 2015). In primary hippocampal neuronal cultures, the association of radixin with  $\alpha$ 5-containing GABA<sub>A</sub>Rs at extrasynaptic sites decreases upon maturation, in contrast, the number of  $\alpha$ 5-containing GABA<sub>A</sub>Rs, that associate with gephyrin at post-synaptic sites remains constant (Brady and Jacob, 2015). Structural and thermodynamic details of the radixin-GABA<sub>A</sub>R  $\alpha$ 5 complex will reveal whether modulation can be achieved without simultaneously affecting the binding of other ligands.

**TABLE 1** | List of all known intracellular GABA<sub>A</sub>R-associated proteins that display micromolar or better binding affinity.

GABA <sub>A</sub> R Interactor	GABA <sub>A</sub> R Subunit	Mapped Binding Site	Affinity [nM]	PDB ID	Physiological function	Inhibition or Interference
Gephyrin	α1	LIKKNITYAPTATSYT <sup>1</sup>	17 <sup>7</sup>	-	Clustering of distinct subsets of GABA <sub>A</sub> Rs at post-synaptic sites. <sup>1,2,3,7</sup>	Redistribution of post-synaptic GABAARs towards extra-synaptic sites. Decreased amplitude and frequency of phasic inhibitory currents.
AP-2	α3	FNIVGTTYPIN <sup>2</sup>	5 <sup>3,7</sup>	4TK1 <sup>8</sup> 4U90 <sup>9</sup>	Surface stabilization; increased receptor numbers, enhanced inhibitory post-synaptic currents <sup>5</sup>	Surface stabilization; increased receptor numbers, enhanced inhibitory post-synaptic currents <sup>5</sup>
	β2 β3	AGLPRHSFGRNALERHVAQKKSRL <sup>3</sup> KTHLPRRRS <sup>4</sup>	17 <sup>3</sup> 1 <sup>4</sup>	- -		
Collybistin	γ2	YECL <sup>5</sup>	0.4 <sup>5</sup>	2PRG <sup>5</sup>	Clustering of α2 subunit containing GABA <sub>A</sub> Rs at post-synaptic sites. Pronounced importance for receptors at the axon initial segment. <sup>6</sup>	Reduced GABA <sub>A</sub> R α2 cluster size and loss of GABA <sub>A</sub> R α2 subunit containing receptors. Reduced inhibitory post synaptic current amplitudes and decay times. Anxiety and seizure susceptibility. <sup>6</sup>
	α2	VMIGNNAYAVANVAPNL <sup>6</sup>	1 <sup>6</sup>	-		

Interacting GABA<sub>A</sub>R subunits, mapped binding sites, determined affinities and PDB-IDs of structures are listed as well as the reported function and the consequences of an acute inhibition or genetic interference. <sup>1</sup>(Mukherjee et al., 2011), <sup>2</sup>(Treiter et al., 2011), <sup>3</sup>(Kowalczyk et al., 2013), <sup>4</sup>(Kittler et al., 2005), <sup>5</sup>(Kittler et al., 2008), <sup>6</sup>(Hines et al., 2018), <sup>7</sup>(Maric et al., 2011), <sup>8</sup>(Maric et al., 2014), <sup>9</sup>(Maric et al., 2015).

### Collybistin

Collybistin (CB) is a guanine nucleotide exchange factor for Cdc42, a gephyrin binding partner (Kins et al., 2000) and an important determinant of inhibitory postsynaptic membrane formation and plasticity (Harvey et al., 2004; Tyagarajan et al., 2011a). Robust evidence supports the role of CB in GABA<sub>A</sub>R scaffolding with yeast three-hybrid studies (Saiepour et al., 2010) and proteomic studies (Nakamura et al., 2016) suggesting the presence of a tripartite complex between gephyrin, CB and α2 subunit containing GABA<sub>A</sub>Rs. Recently, a thermodynamic analysis revealed that CB binds GABA<sub>A</sub>R α2-subunits with high selectivity and affinity (Hines et al., 2018; **Figure 1** and **Table 1**). CB is targeted to the neuronal surface membrane through phosphoinositides (Reddy-Alla et al., 2010; Ludolphs et al., 2016) and interfering human mutations result in cognitive deficits (Long et al., 2016; Chiou et al., 2019). Deficiency in CB reduces gephyrin and GABA<sub>A</sub>R clustering and impairs spatial learning (Papadopoulos et al., 2007, 2008). Moreover, mice with a mutation in the α2-subunit binding region of CB display a loss of a distinct subset of inhibitory synapses and a decreased amplitude of inhibitory synaptic currents, which results in a phenotype with increased susceptibility to seizures and early mortality (Hines et al., 2018). Notably, treatment with the α2/α3-selective positive modulator AZD7325 improves the conditions of affected mice, suggesting that compounds targeting the CB-GABA<sub>A</sub>R α2 complex could provide an alternative route to specifically affect GABA<sub>A</sub>Rs containing the α2 subunit.

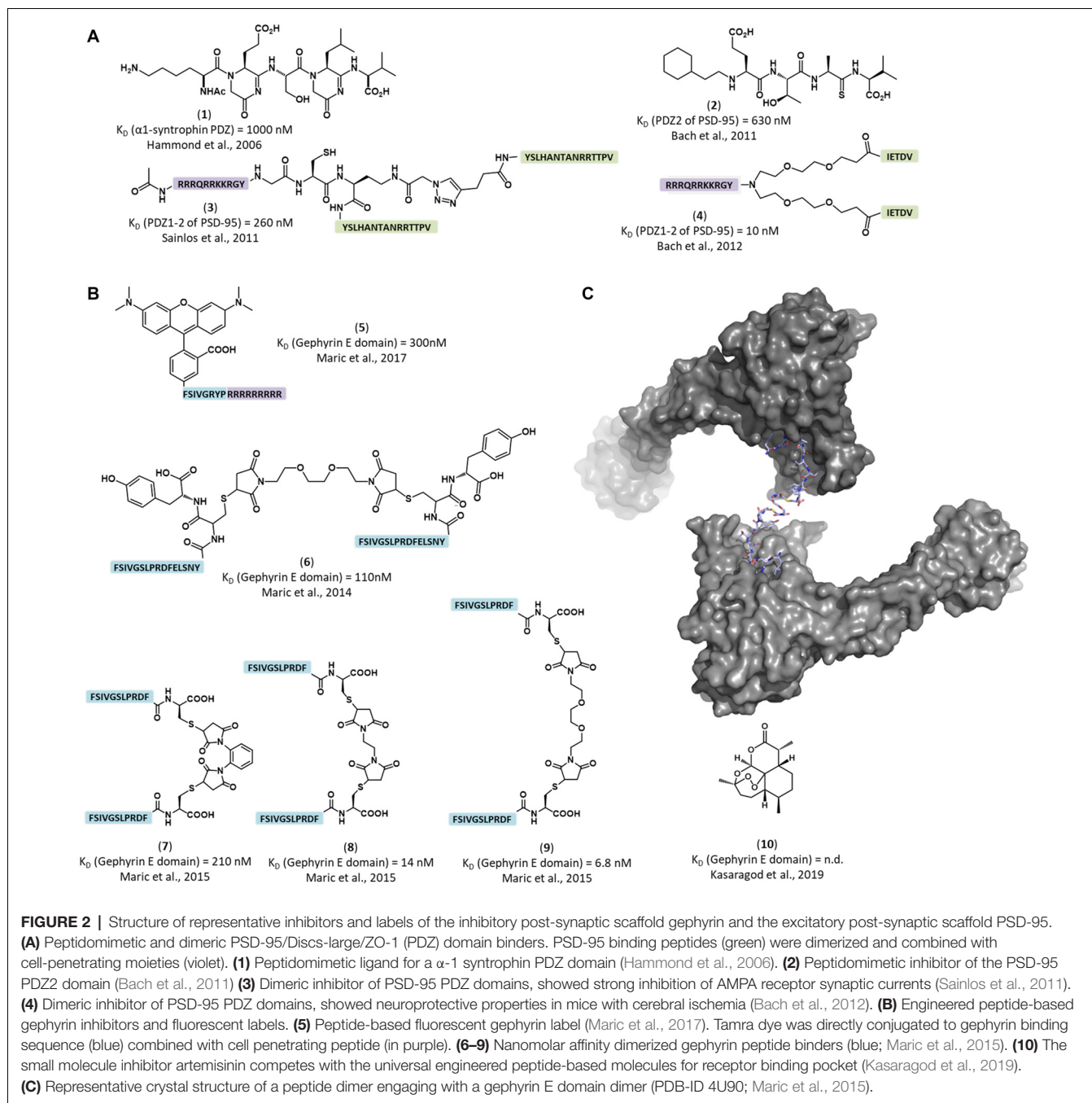
### Neurologin 2

Proteomic studies (Kang et al., 2014; Nakamura et al., 2016) revealed that the synapse-specific adhesion molecule neurologin 2 (NL2; Varoqueaux et al., 2004) strongly associates with a subset of GABA<sub>A</sub>R subtypes and GABA<sub>A</sub>R scaffolds. Neurologin dysfunction has been implicated in autism (Pettem et al., 2013) and specific intracellular residues in NL1 (Nguyen et al., 2016; Letellier et al., 2018) and NL2 (Poulopoulos et al., 2009; Kang et al., 2014) are critical for proper GABAergic signaling. Yet, the exact molecular interfaces, that mediate the direct or indirect gephyrin or CB dependent (Soykan et al., 2014) interactions of neurologin with GABA<sub>A</sub>R, remain uncharacterized. These molecular insights could greatly contribute to our understanding of the development of the inhibitory synapse, as well as the underlying molecular causes of developmental diseases. Neurologin family members exert distinct roles in the formation and stabilization of inhibitory and excitatory synapses and display distinct cellular and subcellular distributions. Accordingly, molecules that interfere with their isoform-specific interactions could act as highly cell-type selective modulators of neurotransmission.

### Gephyrin

Gephyrin is a prime candidate for the role of master regulator of neuronal function at inhibitory sites (Tyagarajan and Fritschy, 2014) and specifically the GABAergic synapses (Choi and Ko, 2015). Initially identified as a glycine receptor binding (Pfeiffer et al., 1982) and scaffolding protein (Feng et al., 1998), gephyrin was later found to be responsible for the





post-synaptic accumulation of GABA<sub>A</sub>Rs. The loss of gephyrin clusters following the loss of the GABA<sub>A</sub>R  $\gamma$ 2 subunit (Essrich et al., 1998) and the loss of GABA<sub>A</sub>R clusters upon gephyrin deficiency (Kneussel et al., 1999) substantiates their critical mutual dependency. Direct binding of gephyrin to  $\alpha$  and  $\beta$  GABA<sub>A</sub>R subunits could be confirmed (Tretter et al., 2008, 2011; Maric et al., 2011; Mukherjee et al., 2011; Kowalczyk et al., 2013; Brady and Jacob, 2015), but the exact binding interfaces and affinities are still largely undefined. Structural, thermodynamic and high-end microscopic approaches elucidated the complex structure-function relationships between gephyrin and a distinct

subgroup of inhibitory neurotransmitter receptors (Kasaragod and Schindelin, 2018) and indicated an important role of the nanoscale arrangement of gephyrin and the associated receptors at post-synaptic sites (Groeneweg et al., 2018; Specht, 2019). Further functional and *in-silico* studies (Pennacchiotti et al., 2017) confirmed that gephyrin organizes the receptors in distinct nanoscale structures, which shape GABAergic synaptic potentiation and reduce current variability. The stability, oligomerization and receptor binding of gephyrin are highly regulated and exert tight control over receptor numbers at post-synaptic sites, affecting synaptic strength (Alvarez, 2017;

Groeneweg et al., 2018). Biophysical (Maric et al., 2011) and structural (Maric et al., 2014, 2015) studies provided first insights into the structure and function of the gephyrin-GABA<sub>A</sub>R complexes and demonstrated that at least the GABA<sub>A</sub>R  $\alpha 1-3$  and GlyR  $\beta$  subunits bind to an overlapping site within gephyrin in a mutually exclusive fashion (Maric et al., 2011, 2014; **Figure 1** and **Table 1**). Microscopy studies substantiated that gephyrin acts as a dynamic post-synaptic platform for both, GABA<sub>A</sub>Rs and GlyRs (Specht et al., 2013), and that receptor residence times at the postsynapse depend on binding affinities and distinct post-translational modifications (Mukherjee et al., 2011; Specht et al., 2011). The concept of ligand competition for gephyrin binding, therefore, lends an elegant explanation for the comparably high diffusion dynamics of high-affinity gephyrin binding receptors. This phenomenon may be the natural solution to the biological requirement to maintain distinct subsets of receptor subtypes in high density at post-synaptic sites, while at the same time allowing for the rapid exchange of these receptors and scalability through diffusion dynamics (Specht, 2019). This model is also consistent with the observation of reciprocal stabilization of receptors, and the underlying protein scaffold, at inhibitory synapses (Essrich et al., 1998).

Gephyrin itself is dynamically regulated, affecting GABA<sub>A</sub>R diffusion and contributing to input-specific adaptations at postsynaptic sites (Chen et al., 2012; Villa et al., 2016; Battaglia et al., 2018). Gephyrin phosphorylation at Ser268 and Ser270, located in the intrinsically disordered central region of the protein, directly affects GABAergic signaling (Tyagarajan et al., 2011b, 2013) and induce gephyrin-mediated remodeling of GABAergic synapses in specific neuronal cell-types (Flores et al., 2015). Despite its major functional relevance only a few of the molecular interfaces that engage with the central region of gephyrin could be identified (Groeneweg et al., 2018). The underlying molecular mechanisms for these gephyrin phosphorylation-induced GABA<sub>A</sub>R synapse dynamics remain to be explored in a comprehensive approach that includes an extensive alternative splicing and complex post-translational modification patterns of this region. Identification of the targeted binding pockets and insights into the binding affinities of the modified and unmodified peptide regions within the central region of gephyrin could shed light on the enigmatic molecular mechanisms of gephyrin multimerization, degradation and the tuning of its ligand binding affinities. Additionally, gephyrin isoforms are tissue-specific (Paarmann et al., 2006), therefore, molecules targeting distinct gephyrin splice variants may display pronounced effects in distinct tissues or brain regions.

## TARGETING THE GEPHYRIN-GABA<sub>A</sub>R COMPLEX

Gephyrin's crucial role in glycinergic and GABAergic transmission made it a major pharmacological target. The modulation of synaptic responses *via* gephyrin was achieved more than a decade ago using intrabodies (Zacchi et al., 2008), and a related approach turned out to be useful for acutely removing inhibitory synapses (Gross et al., 2016). Since then, several studies made an impressive progress in the development

of agents affecting the intracellular interplay of GABA<sub>A</sub>Rs. One such example is artemisinins [**Figure 2B(10)**]. Li et al. (2017) found that artemisinins, lactones derived from the Qinghao plant, affect pancreatic cells by binding gephyrin and modifying GABA<sub>A</sub>R signaling. Kasaragod et al. (2019) identified the artemisinin binding site within gephyrin and showed that application of artemisinins reduces gephyrin and GABA<sub>A</sub>Rs clustering, making artemisinins the first small molecule lead compounds for a new class of inhibitory neurotransmission modulators. Strikingly, the druggable artemisinin-binding pocket overlaps with the universal receptor binding region of gephyrin, which is critical for the interaction with GABA<sub>A</sub> and glycine receptors (Kasaragod et al., 2019). Thermodynamic and structural studies (Maric et al., 2011, 2014) identified the "hotspot" fragments of GABA<sub>A</sub> and glycine receptors that bind to gephyrin. Biomimetic optimization of the "hotspots" amino acid sequence, enhanced the affinity of the resulting peptide ligands 46,000-fold compared to the corresponding native peptides (Maric et al., 2015, 2017; **Figures 2B,C**). Further *in vitro* applications of these new super binder peptide reduced GABA<sub>A</sub>R  $\alpha 2$  conductivity and clustering, providing evidence that GABA<sub>A</sub>R-associated proteins can be successfully targeted with modified peptides to modulate fast synaptic inhibition (Maric et al., 2017).

## TARGETING NON-SCAFFOLD GABA<sub>A</sub>R ASSOCIATED PROTEINS

GABA<sub>A</sub>R trafficking is pivotal for the plasticity (Luscher et al., 2011) and the development (Lorenz-Guertin and Jacob, 2018) of inhibitory synapses, consequently, dysfunction of the GABA<sub>A</sub>R cycling is involved in various neurological disorders (Smith and Kittler, 2010; Mele et al., 2019). Noteworthy, phosphorylation of the intracellular GABA<sub>A</sub>R sites, that are involved in the trafficking of the receptors, has been identified to control receptor numbers and their concentration at synaptic sites (Comenencia-Ortiz et al., 2014; Nakamura et al., 2015), a mechanism that proves to be critical for the physiological function of inhibitory synapses (Vien et al., 2015). Therefore, targeting protein-protein interactions (PPIs) that mediate GABA<sub>A</sub>R trafficking, endocytosis, degradation or recycling, is a promising pharmacological strategy. The proposed direct protein interactors are numerous, among them are muskellin (Heisler et al., 2011), GABARAP (Wang et al., 1999), the brefeldin-A inhibited GDP/GTP exchange factor 2 (Charych et al., 2004), phospholipase C-related catalytically inactive proteins 1 and 2 (Mizokami et al., 2007), N-ethylmaleimide sensitive factor (Goto et al., 2005), neurobeachin (Nair et al., 2013), Huntingtin-associated protein 1, calcium-modulating cyclophilin ligand (Kittler et al., 2004; Yuan et al., 2008) and the clathrin adaptor protein AP2 (Kittler et al., 2005).

The AP2-GABA<sub>A</sub>R interaction rapidly modulates synaptic GABA<sub>A</sub>R numbers, inhibitory synaptic strength, neuronal excitability, and notably, affects animal behavior (Kittler et al., 2000, 2005, 2008; Tretter et al., 2009). The  $\mu 2$  subunit of the clathrin adaptor protein AP2 binds with high affinity to linear and short peptide motifs within the intracellular

regions of specific GABA<sub>A</sub>R subunits (**Table 1**). Short GABA<sub>A</sub>R derived peptides, that effectively compete with AP2 binding, were successfully used to block the receptor internalization in hippocampal neurons, increasing surface concentration of GABA<sub>A</sub>R clusters by 50% (Smith et al., 2012) and enhancing the strength of inhibitory synapses (Kittler et al., 2008). AP2 antagonists demonstrate that the modulation of GABA<sub>A</sub>R interactions with its intracellular trafficking partners is an alternative way to influence GABAergic signaling.

## PERSPECTIVES

Ongoing research uncovered original, seemingly contrasting, strategies of GABAergic signaling modulation. On the one hand, ligands disrupting gephyrin-GABA<sub>A</sub>R clustering, like artemisinin or “super binding peptides,” could reduce the GABA<sub>A</sub>R synaptic concentration and function. On the other hand, peptides hampering receptor interaction with AP2 trafficking protein increased the synaptic receptor levels. In theory, these approaches could be applied together to achieve bi-directional modulation of inhibitory neurotransmission, promoting a shift in the dynamic equilibrium from phasic to tonic neuronal response.

Those new strategies of GABAergic neurotransmission modulation possess an untapped clinical potential. Agents targeting GABA<sub>A</sub>R associated scaffold or trafficking proteins could be applied wherever abnormal GABAergic activity or regulation is involved in pathogenesis. In status epilepticus patients develop a time-dependent pharmacoresistance to GABAergic agents, probably, due to GABA<sub>A</sub>R internalization (Naylor et al., 2005). In benzodiazepine tolerance linked to prolonged benzodiazepine use, neurons continuously exposed to diazepam lose postsynaptic GABA<sub>A</sub>Rs (Nicholson et al., 2018). Both pathologies are related to the reduction of available postsynaptic GABA<sub>A</sub>Rs and both could potentially be alleviated by targeting GABA<sub>A</sub>R-associated proteins. Stabilization of the gephyrin-receptor scaffolds at inhibitory postsynapses with molecules that mimic the stabilizing action of CB (Saiepour et al., 2010) could help prevent GABA<sub>A</sub>R loss and preserve inhibitory neurotransmission, alternatively, applying AP2 inhibitors could reduce GABA<sub>A</sub>R internalization and reverse the loss of postsynaptic GABA<sub>A</sub>Rs. Those examples illustrate the potential of GABAergic modulators as adjuvants ameliorating the effect of existing potent drugs, whereas in epilepsy or other diseases involving deregulation of inhibitory neurotransmission they could be applied as stand-alone therapeutics.

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We expect that the study of GABA<sub>A</sub>R intracellular interactors, accelerated by *in-silico* predictions and high throughput approaches, will lead to the discovery of novel GABAergic modulators. Affinity, selectivity, bioavailability and immunogenicity of these compounds would have to be optimized for clinical applications, where peptide-based ligands could be further evolved by the introduction of unnatural amino acids, cyclization and other chemical modifications.

Microscopy is an additional intriguing application of these molecules. The enhanced affinity and specificity of the engineered peptide-based compounds allowed to pioneer their use as fluorescent probes [**Figure 2B(5)**], enabling live neuronal staining and visualization of inhibitory post synaptic sites with submicron resolution (Maric et al., 2017). Compact fluorescent peptides, developed from these super binding peptides, bring several advantages over conventional staining agents, namely the antibodies. In contrast to antibodies, peptide probes are live cell compatible and could provide better resolution and localization precision, since the fluorophore, owing to its small size, stays close to the target surface, reducing the linkage error. Moreover, highly affine and selective peptides could achieve stoichiometric labeling, enabling quantification of the target protein.

Here, we discussed how the targeting of GABA<sub>A</sub>R associated proteins could prove to be a versatile pharmacological strategy with clinical potential. Further, we suggested that when combined with state-of-the-art super-resolution microscopy methods, the peptide-based fluorescent probes may resolve the nanoscale architecture of synapses in unprecedented detail. We anticipate that the discovery of additional GABA<sub>A</sub>R interactors could open the way for the development of new imaging tools and alternative pharmacological approaches.

## AUTHOR CONTRIBUTIONS

VK and HM wrote the manuscript and prepared the figures.

## FUNDING

This work was supported by Association of the Chemical Industry (Verband der Chemischen Industrie).

## ACKNOWLEDGMENTS

We thank Drs Vikram Babu Kasaragod and Hermann Schindelin for helpful suggestions, discussions and editing.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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