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Current Opinion in Pharmacology

Expanding $GABA_AR$ pharmacology via receptor-associated proteins

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Abstract

Drugs directly targeting γ-aminobutyric acid type A receptors ($GABA_ARs$), the major mediators of fast synaptic inhibition, contribute significantly to today's neuropharmacology. Emerging evidence establishes intracellularly $GABA_AR$-associated proteins as the central players in determining cellular and subcellular GABAergic input sites, thereby providing pharmacological opportunities to affect distinct receptor populations and address discrete neuronal dysfunctions. Here, we report on recently studied $GABA_AR$-associated proteins and highlight challenges and newly available methods for their functional and physical mapping. We anticipate these efforts to contribute to decipher the complexity of GABAergic signalling in the brain and eventually enable therapeutic avenues for, so far, untreatable neuronal disorders and diseases.

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Current Opinion in Pharmacology 2021, **57**:98–106

This review comes from a themed issue on **Neurosciences: Ion channel accessory proteins**

Edited by **Daniel Bertrand** and **Guss Smith**

For complete overview about the section, refer Neurosciences: Ion channel accessory proteins

Available online 5 March 2021

https://doi.org/10.1016/j.coph.2021.01.004

Introduction

γ-Aminobutyric acid type A receptors ($GABA_ARs$) are ion channels, gated by GABA, the major inhibitory neurotransmitter in the human brain. $GABA_AR$ subtypes are heteropentamers assembled from 20 subunits. $GABA_AR$ subtype populations exhibit specific cellular and subcellular distributions, are activity-dependently regulated during development [1] and altered in response to substances [2] and pathological conditions such as neurodegeneration [3]. The impressive functional heterogeneity of GABAergic synapses is primarily determined by presynaptic input, local translation [4], alternate splicing of transcripts, and intracellular receptor interactions [5]. Receptor surface numbers, subcellular distribution, nanoscale arrangement [6–10], and turnover are determined by distinct and subunit-specific intracellular interactions with associated proteins, many of which are tightly regulated by post-translational modifications [11,12]. Defects in these intracellular $GABA_AR$ signalling pathways result in GABAergic synapse dysfunction and are at the core of a number of neuropathological conditions including acute brain disorders, neuropsychiatric and neurodegenerative diseases [13] as well as neurodevelopmental disorders [14] and epileptogenesis [15]. Resolving the underlying, largely unexplored, interfaces between $GABA_ARs$ and their associated proteins addresses a key challenge in neuroscience, namely linking the activity of $GABA_AR$ populations at specific input sites and within defined circuits to behavior and has great potential to expand $GABA_AR$ pharmacology [12] (Figure 1).

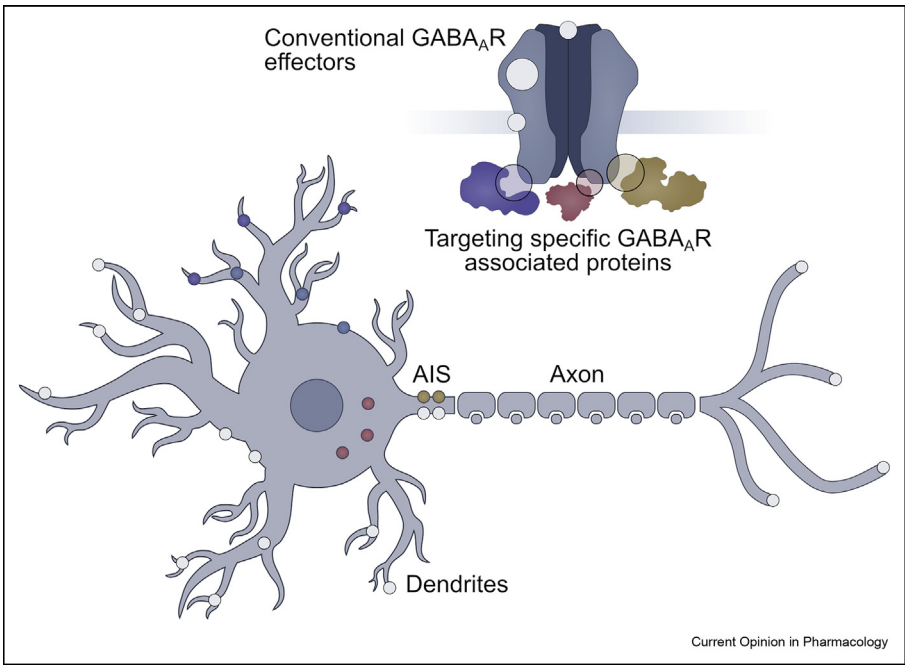
Here, we report on new insights into $GABA_AR$ accessory proteins and their implications for $GABA_AR$ pharmacology. Furthermore, we highlight newly available methodological approaches to identify and study the relevant interaction interfaces of the rapidly increasing number of putative $GABA_AR$-associated proteins, —thereby provide a toolbox to unleash the full neuropharmacological potential of $GABA_AR$-associated proteins and decipher the function of discrete $GABA_AR$ populations and specific GABAergic input sites.

Expanding $GABA_AR$ pharmacology via the associated proteins

Intracellularly associated proteins provide bidirectional control over GABAergic activity

Our insight into the molecular mechanisms that determine the diversity of $GABA_ARs$ at inhibitory postsynaptic densities remains largely incomplete, even more so, their contribution to mechanisms of postsynaptic plasticity [16]. $GABA_AR$-associated proteins that act as scaffolds or clustering factors are often membrane-associated via inositol-phosphate binding or palmitoylation [17,18]. By providing receptor-binding sites with varying affinity, density, and subsynaptic

Figure 1



Targeting specific GABAergic input regions via GABA$_A$R-associated proteins. Schematic representation of a pentameric GABA$_A$R bound to associated proteins that determine the clustering, half-life, and nanoscale arrangement of receptor populations. Orthosteric and allosteric effectors adjust function of GABA$_A$R subtypes distributed across brain regions and neuronal substructures (white circles). Conceptually, pharmaceutical interference with intracellular GABA$_A$R interactors could provide the means to modulate GABAergic signalling in specific brain regions, circuits, and even GABAergic inputs (colored circles).

arrangement [6–10], they dynamically recruit GABA$_A$R subtypes to distinct, post-, extra-, or subsynaptic domains or regions (Figure 2) [19,20]. Compounds targeting these binding sites diminish the synaptic clustering of GABA$_A$Rs, resulting in reduced amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) [21,22].

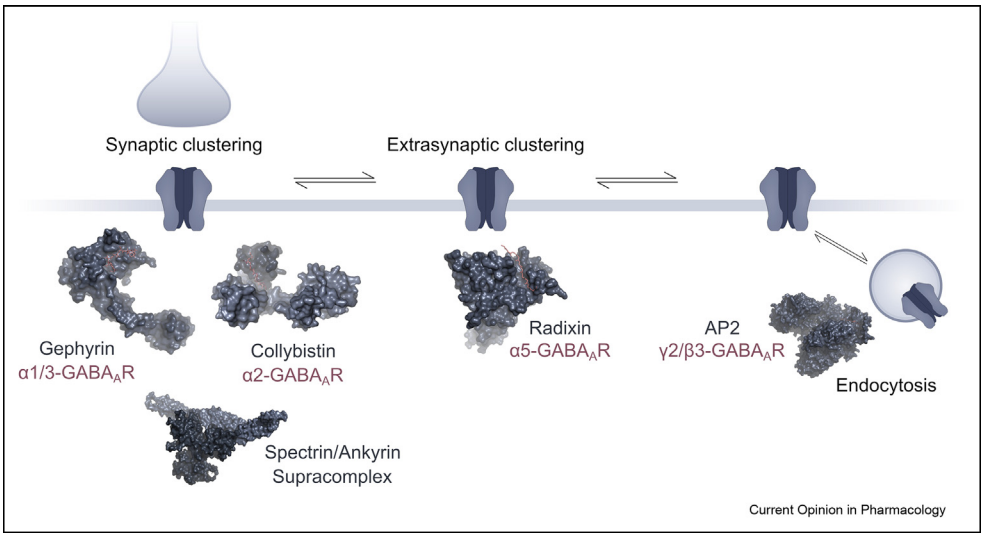
GABA$_A$R-associated proteins mediating receptor trafficking typically control receptor numbers and half-life at the neuronal surface membrane. Direct competition with binding of the clathrin adaptor protein 2 complex (AP2) with GABA$_A$Rs rapidly affects mouse behavior by increasing synaptic GABA$_A$R numbers, inhibitory synaptic strength and neuronal excitability [23] (Figure 2). Recent transgenic proteomic analysis identified Cleft lip and palate transmembrane protein 1 (Clptm1) as a novel GABA$_A$R-associated protein that limits the forward trafficking of a number of GABA$_A$R subtypes via direct interactions [24]. The sorting nexin 27 protein (SNX27) enhances the membrane retrieval of the inhibitory synapse-specific adhesion molecule neuroligin 2 (NL2) by direct binding via its postsynaptic density-95, discs-large, zona occludens 1 (PDZ) domain [25]. The observed enhance in postsynaptic gephyrin and GABA$_A$R clustering and consequently increased inhibitory currents upon knock-down were proposed to result from the stabilization of extrasynaptic GABA$_A$Rs [25]. An additional, recent example is the cytoplasmic FMR1 interacting protein 1 (CYFIP1), its conditional loss alters neuroligin 3 (NL3) and GABA$_A$R β-subunit expression and thereby also increases inhibitory synaptic clusters and hence amplitude of mIPSCs [26].

Their pharmacological targeting can be expected to provide antagonising and bidirectional control over GABAergic signalling, potentially enabling precise enhancement or reduction of GABAergic inhibition to address individual synaptic dysfunctions (Figures 1 and 2).

The full potential of GABA$_A$R subtype-specific modulation remains largely untapped

Benzodiazepines [27–29] and increasingly neurosteroids [30] contribute significantly to today's neuropharmacology. Side-effects and recognition of functional GABA$_A$R subtype heterogeneity encouraged the development of modulators with improved subtype selectivity. Robust preclinical data recently validated the long-standing α2/α3-GABA$_A$R positive allosteric modulator nonsedating anxiolytic and the α5-GABA$_A$R negative allosteric modulator cognition enhancer hypotheses

Figure 2



GABA$_A$R populations are in dynamic equilibrium [6]. GABA$_A$R-associated proteins enrich GABA$_A$Rs at extra- and postsynaptic sites and mediate endocytosis by interaction with specific subunits. Surface representation of GABA$_A$R-associated proteins in blue together with known or putative binding motifs shown as red stick models. Gephyrin mediates synaptic clustering of α3-GABA$_A$Rs via direct interaction and inhibition affects GABA$_A$R clustering and fast synaptic inhibition [21,22]. Conversely, radixin clusters extrasynaptic pools of α5-GABA$_A$Rs [53]. AP2 controls GABA$_A$R surface numbers of γ2-GABA$_A$Rs [23] and β3-GABA$_A$Rs [72]. Collybistin maintains α2-GABA$_A$Rs at the AIS of cortical pyramidal neurons [45]. Enhanced targeting α1-GABA$_A$Rs provide seizure resilience [46]. Ankyrin/spectrin complexes are a major component of the AIS scaffold and contribute to GABA$_A$R maintenance.

[27], thus providing the long anticipated starting points for the development of innovative treatments of cognitive disorders and anxiety and possibly even stroke, schizophrenia, and depression [27–29]. So far, the development of disease-specific allosteric GABA$_A$R-directed drugs has suffered from innate structural limitations of the druggable orthosteric and allosteric GABA$_A$R sites, especially in the case of compounds designed to differentiate between α1-, α2-, and α3-GABA$_A$Rs [27,28]. All extracellularly binding compounds, including benzodiazepines [27–29], z-drugs [31], and dihydromyricetin [32], some of them claimed to be selective for a specific receptor subtype, exhibit only limited selectivity, which is even further aggravated by their high potency [29].

GABA$_A$R subunit-specific intracellular phosphorylation was recently associated with the impressive long-term therapeutic action of the transmembrane-binding neurosteroid Zulresso® [33]. This compound, just like the newly developed, orally bioavailable Zuranolone [34], however, acts across receptor subtypes, thus motivating alternative approaches to achieve selective GABA$_A$R modulation.

Targeting specific neuronal types and circuits via GABA$_A$R-associated proteins

Intracellular interactions of GABA$_A$Rs are highly subtype selective [12] and provide a steadily increasing (Table 1) number of binding pockets for affecting distinct receptor subtype populations. Targeting GABA$_A$R protein complexes, instead of directly adjusting gating or conductance of a single receptor subtype across all brain regions and substructures, may provide the means to affect disease relevant protein complexes in distinct brain regions and circuits and possibly even specific neuronal types and substructures (Figure 1). This invaluable specificity advantage enables the development of sophisticated tools to explore the underlying mechanisms of GABA$_A$R subtype distribution and maintenance. In this framework, specific micro- and macrocircuits and their corresponding behavioral outputs may be selectively modulated. This pharmacological approach can be alternative or additive to future genetic interventions for the cure of currently untreatable neurological diseases and disorders. Acute modulators of intracellular GABA$_A$R interactions are an attractive alternative to genetic approaches, that suffer from poor correlation of functional effects to individual receptor subtype populations, as exemplified by the complex alterations in the GABA$_A$R α1 knockout (KO) mouse brains [35]. Besides overcoming the adaptive responses in GABAergic signalling *in vivo*, acute modulators offer a largely improved temporal resolution and the possibility to tune effects by adjusting the dose. Recently developed chemicogenetic approaches combine acute pharmacology with genetics to achieve

Table 1

Recently studied directly and indirectly $GABA_AR$-associated proteins.

Associated protein	Mode of action	Experimental model	Binding motif
SNX27	Endocytotic recycling of NL2 at GABAergic synapses	Hippocampal neurons [25]	PDZ-mediated binding
CYFIP1	Bidirectional control over NL3 and β2/3-$GABA_AR$ subunit expression	Hippocampal neurons [26]	not defined
Radixin	Clustering of extrasynaptic α5-$GABA_ARs$	Hippocampal neurons and KO mouse model [53], phage display and computational modeling [68]	^{342}NYFTKRGWAWDGKKAL^{357}
Collybistin	Clustering of postsynaptic α2-$GABA_ARs$	$GABA_AR$ α1/2 chimera mouse model [45,46]	^{360}VMIQNNAYAVAVANYA^{375}
Gephyrin	Clustering of postsynaptic α1/3-$GABA_ARs$	$GABA_AR$ α1/2 chimera mouse model [45,46], anxiety mouse model [32]	^{360}LIKKNNTYAPTATSYT^{375} ^{395}FNIVGTTYPIN^{405}
NrCAM	Complex formation with synaptic scaffolding protein gephyrin via astrocytic input	*In vivo* Split-TurboID [48]	Gephyrin-mediated binding
Clptm1	Limiting the forward trafficking of $GABA_ARs$ with differing subunit composition	Transgenic proteomic analysis [24]	not defined

cell-specific control over neuronal signalling proteins, but their complexity hampers broader application [36].

Targeting the associated proteins also provides a new perspective to counter the loss of $GABA_ARs$ and their postsynaptic scaffold in benzodiazepine pharmacoresistance [37]. Disrupting or stabilizing scaffolding/trafficking interactions could help to overcome tolerance and reduce dose escalation, which is urgently required for benzodiazepines that are broadly prescribed for the treatment of anxiety and insomnia [38].

Reconstitution of neuronal dysfunction via $GABA_AR$-associated gephyrin and collybistin

Gephyrin and collybistin are critical for the specification and maintenance of soma and axon targeting GABAergic contacts in the cortex and hippocampus that are implicated in epileptic disorders [39,40]. Gephyrin mediates the alternative recruitment of distinct $GABA_AR$ and glycine receptor subunits via a universal receptor binding pocket [41]. Upon receptor binding and regulated by post-translational modification, gephyrin forms a highly condensed sheet-like assembly via phase separation [42]. Competition with receptor binding modulates fast synaptic inhibition by reducing clustering and thus conductivity of a distinct subset of $GABA_AR$ subtypes. This was initially achieved using native fragments of $GABA_AR$ and glycine receptors [41], later with affinity enhanced variants [21,43] and recently even small molecules [22].

Collybistin plays a significant role in the regulation [44] of the GABAergic postsynapse and enriches α2-$GABA_ARs$ at the axon-initial-segment (AIS) of cortical pyramidal cells [45]. Its dysfunction has been linked to hyperekplexia, epilepsy, intellectual disability, and developmental disorders. Mutation of the α2-$GABA_AR$ binding region of collybistin to the α1-$GABA_AR$ sequence results in loss of a distinct subset of inhibitory synapses and increased susceptibility to seizures, thus reproducing the effects of human epileptogenic collybistin mutations [45]. The observation that the seizure susceptibility could be rescued by treatment with AZD7325, an α2/3-selective positive allosteric $GABA_AR$ modulator, suggests a value of this compound for treating a subgroup of epilepsy patients. In a follow-up study, where α1-$GABA_AR$ was mutated to contain the α2 subunit binding region of collybistin, Nathanson et al. generated mice that showed a robust resilience to kainate induced seizures. Notably, these mutations were sufficient to fully rescue the lethal seizure phenotype of the existing α2/α1 chimera mice [46]. Thus, substantiating that specific dysfunctional neuronal pathways can indeed be reconstituted via the intracellularly associated proteins.

Targeting specific GABAergic input regions via $GABA_AR$-associated proteins

Most GABAergic contacts are formed onto postsynaptic dendrites, including spines. Compared with soma and AIS, dendritic inhibition is overwhelmingly represented and targeting this key element of GABAergic inhibition blocks important forms of synaptic plasticity [4,16]. The complexity of GABAergic signalling is further increased by the recent observation that dominant GlyR α1 mutants assemble into pre- and extrasynaptic $GABA_ARs$ [47] and that GABAergic inputs also encompass

astrocyte—neuron junctions, stabilized via transcellular coupling of neuronal cell adhesion molecules to gephyrin [48].

The AIS is a crucial subcellular domain in neurons where action potentials are initiated and plays a pivotal role in neuronal physiology. The enrichment of distinct $GABA_AR$ subtypes to the AIS appears to vary across brain regions [49] and depends on gephyrin and ankyrin/spectrin supracomplexes [50], major genetic risk factors in a number of neuronal disorders including epilepsy [51]. Notably, the enrichment of α1-, α2-, and α3-$GABA_ARs$ and their co-localization with gephyrin and/or ankyrin shows strong regional differences [49] and appears to be mediated by independent and distinct receptor protein complexes, as the enrichment of one subunit was unaffected by the genomic deletion of the other [49]. Thus, indicating the presence of unique forms of GABAergic inhibitory transmission and hence addressable pharmacological profiles at the AIS. These could be exploited to guide existing treatments to the relevant patient cohorts and eventually result in the development of new personalized treatments [27,51].

Targeting extrasynaptic $GABA_AR$ reserve pools

Extrasynaptic reserve pools represent an exciting key structure to expand $GABA_AR$ pharmacology beyond the postsynapse and the AIS. Postsynaptic localization of α5-$GABA_ARs$ through gephyrin [52] is countered by extrasynaptic targeting through radixin [53]. Blockade of α5-GABARs accelerates reversal learning [54] and mice with a selective knockdown of α5-$GABA_ARs$ in pyramidal neurons show improved spatial and trace fear-conditioning memory [55]. Allosteric modulators with improved α5-$GABA_AR$ selectivity are being tested in neurodevelopmental disorders, depression, schizophrenia, and to improve cognitive function in neurodegenerative disorders and facilitate poststroke recovery [27] and are already being developed as photosensitive epilepsy medication [56]. Despite these impressive advances in targeting α5-$GABA_ARs$ [57], the involved intracellular motifs and binding sites remain largely untapped. Insights into α5-$GABA_AR$ endogenous signalling will not only advance our understanding of memory and learning but could also contribute to the development of cognitive enhancers.

$GABA_AR$-associated proteins: challenges and opportunities

Challenge: functional and physical mapping of intracellular $GABA_AR$ interactions

Targeting $GABA_AR$-associated proteins requires precise knowledge about their distribution in specific brain areas, neuronal types and subregions, which, however, is still only rudimentary. The necessary functional and physical mapping of the involved intracellular $GABA_AR$ interaction surfaces demands the development and rigorous application of technologies with enhanced throughput and level of detail.

Mass spectrometric (MS) approaches reported numerous putative $GABA_AR$-associated proteins [58]. As intracellular receptor interactions can be resolved and validated using short linear peptide fragments of the intracellular receptor regions [59], high-throughput MS approaches such as Protein Interaction Screen on Peptide Matrices [60] can be applied to resolve and map receptor subunit interactomes and their post-translational regulation. The application of advanced methods such as protein cross-linking coupled with MS (XL-MS) [61], multiplexed proximity biotinylation [62], and their bioinformatic integration has been outlined recently [63]. These developments as well as long read sequencing will aid in grasping the complexity of gene regulation, specifically alternative splicing [64], and disease-relevant post-translational modifications at GABAergic synapses. Integration of experimental results into computational platforms, as recently provided by Lupascu et al., could allow to decipher how subcellular and intracellular pathways precisely affect individual GABAergic signalling events in physiology and pathology [65].

Opportunity: short linear receptor motifs provide specificity

Characterization of a short list of $GABA_AR$-associated proteins enabled the design of modulators of GABAergic signalling and postsynaptic labels [12] and recently studied $GABA_AR$-associated proteins may be exploited in the same way (Table 1). Structural characterization, including X-ray crystallographic studies and recent cryoelectron tomograms [66], defined the intracellular $GABA_AR$ regions as intrinsically disordered, devoid of fixed secondary structural elements. By only adopting a fold on binding, short linear motifs within the intracellular receptor regions allow for specific interactions with more than one target. Such domain—motif interactions are observed across the proteome and are characterized by structural flexibility and tight post-translational control. This enables immediate adaption in response to changing stimuli, which may be a requirement for their role in structural postsynaptic plasticity.

Opportunity: resolving the nanoscale arrangement of the inhibitory postsynapse

The inherently high binding energy densities of domain—motif interactions [12] suggest they could be harnessed not only as highly specific and potent inhibitors but also as compact fluorescent labels. $GABA_AR$-associated protein targeting molecules may be turned into sophisticated tools for simultaneous visualization and manipulation of the neuronal system with molecular precision. When combined with fluorescent dyes and cell penetrating tags, they can be expected to

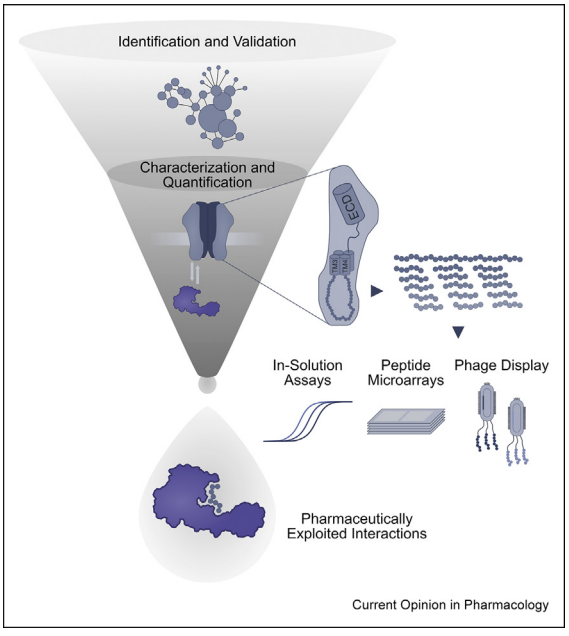
visualize the nanometer-scaled machinery that underlies higher brain functions such as learning and memory, possibly even in living cells and tissue slices. As the fluorophore stays close to the target surface, they will provide the necessary nanometer resolution and localization precision. Moreover, highly affine and selective peptides could achieve stoichiometric labeling, enabling quantification of the target protein, and resolve the specific distribution of $GABA_AR$ subtypes and intracellular interactors [21,75]. When combined with UV-inducible photoinhibitors, they could provide photocontrol of $GABA_AR$ function *in situ* [67].

A toolbox for exploring intracellular $GABA_AR$ interactions

A range of $GABA_AR$ binding motifs was successfully transformed into bioactive actuators [12]. $GABA_AR$ modulation and visualization in brain slices has been achieved with peptides as well as the natural small molecule drug artemisinin [21–23]. Currently, the major bottleneck (Figure 3) in the development of such peptide-based effectors lies in the mapping and characterization of the involved interfaces that, until recently, necessitated laborious biophysical studies as outlined above [12].

Screening followed by iterative affinity maturation by sequence optimization and chemical modification in microarray format recently empowered the development of intracellularly active peptide-based modulators of GABAergic signalling [21]. Likewise, approaches combining large-scale phage display libraries with *in silico* modeling provided attractive starting points for the detailed characterization of $GABA_AR$-associated protein interactions as recently exemplified by a study on 4.1 protein, erzin, moesin and radixin (FERM) domains [68]. The toolbox (Figure 3) for development of high-affinity peptide binders was furthermore expanded by nano- to picomolar scaled affinity determination of peptide–protein interactions in-solution [69] and size exclusion-based enrichment from pools of synthetic peptides combined with liquid chromatography–tandem MS–based peptide sequencing [70]. Setups that combine miniaturized and automated peptide synthesis with high-throughput binding evaluation could be ideal to map and characterize intracellular $GABA_AR$ interactions and convert identified linear motifs into bioactive competitive binders (Figure 3).

Figure 3



Toolbox for studying and targeting $GABA_AR$-associated proteins. Newly available methods to empower molecular studies in high throughput and the development of pharmaceutical effectors acting on $GABA_AR$-associated proteins. Binding sites and hierarchies that define $GABA_AR$ intracellular signalling are largely unknown. The depicted funnel symbolizes the striking discrepancy between numbers of candidate proteins and validated or characterized $GABA_AR$-associated proteins. A major bottleneck is the elucidation of identified protein–protein interactions at molecular detail and affinity quantification. A conceptual workflow to achieve this task may include the screening of binding interfaces using synthetic biology approaches such as phage display, and microarrays obtained from parallel peptide synthesis. Newly reported methodological approaches could be applied to iteratively maturate peptide binders into bioactive $GABA_AR$ effectors or facilitate structural investigations to resolve the involved binding pocket. ECD: extracellular domain, TM: transmembrane domain.

Conclusions

The pharmacological targeting of neurotransmitter-gated ion channel and G protein–coupled receptor associated proteins and protein complexes in the brain represents an emerging field with several compounds currently in clinical trials and first approved for clinical application [71]. In stark contrast, and despite the increasing number of newly identified transmembrane [73,74] and intracellularly $GABA_AR$-associated proteins, the brain's natural inhibitory signalling pathways remain largely unexplored. Yet, in contrast to compounds that allosterically affect $GABA_ARs$ across the brain, targeting the associated proteins has potential to adjust GABAergic activity with circuit precision, thereby potentially overcoming the severe side-effects of conventional $GABA_AR$ modulators and addressing unmet clinical need. To this end, the systematic application of novel approaches for the large-scale identification of $GABA_AR$-associated proteins will not only determine their unique modes of action but also contribute to expanding $GABA_AR$ pharmacology.

Author contributions

C.S. and H.M.M. wrote the manuscript and prepared the figures.

Conflict of interest statement

C.S. and H.M.M. have no conflict of interest on this topic.

Acknowledgements

The authors thank Joshua Smalley and Stephen J Moss for helpful suggestions, discussions, and editing. This work was supported by the German Research Foundation (DFG, MA 6957/1-1 to H.M.M.).

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