Looking for Novelty in an “Old” Receptor: Recent Advances Toward Our Understanding of GABA<sub>A</sub>Rs and Their Implications in Receptor Pharmacology

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Diverse populations of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) throughout the brain mediate fast inhibitory transmission and are modulated by various endogenous ligands and therapeutic drugs. Deficits in GABA<sub>A</sub>R signaling underlie the pathophysiology behind neurological and neuropsychiatric disorders such as epilepsy, anxiety, and depression. Pharmacological intervention for these disorders relies on several drug classes that target GABA<sub>A</sub>Rs, such as benzodiazepines and more recently neurosteroids. It has been widely demonstrated that subunit composition and receptor stoichiometry impact the biophysical and pharmacological properties of GABA<sub>A</sub>Rs. However, current GABA<sub>A</sub>R-targeting drugs have limited subunit selectivity and produce their therapeutic effects concomitantly with undesired side effects. Therefore, there is still a need to develop more selective GABA<sub>A</sub>R pharmaceuticals, as well as evaluate the potential for developing next-generation drugs that can target accessory proteins associated with native GABA<sub>A</sub>Rs. In this review, we briefly discuss the effects of benzodiazepines and neurosteroids on GABA<sub>A</sub>Rs, their use as therapeutics, and some of the pitfalls associated with their adverse side effects. We also discuss recent advances toward understanding the structure, function, and pharmacology of GABA<sub>2</sub>A<sub>2</sub>Rs with a focus on benzodiazepines and neurosteroids, as well as newly identified transmembrane proteins that modulate GABA<sub>A</sub>Rs.

Keywords: GABA, GABA<sub>A</sub>R, benzodiazepines, neurosteroids, pharmacology, LH4, Clptm1, Shisa7

INTRODUCTION

Efforts aimed at uncovering mechanisms driving inhibitory transmission have not only contributed to our understanding of nervous system function, but have also led to the development of several drugs used in the treatment of neurological and psychiatric disorders. In the brain, fast inhibitory transmission is predominantly mediated by GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs), which are pentameric...
ligand-gated ion channels that conduct Cl⁻ upon activation (Olsen and Sieghart, 2009; Sigel and Steinmann, 2012; Gielen and Corringer, 2018). Thus far, nineteen GABAₐR subunits, α(1–6), β(1–3), γ(1–3), δ, ρ, θ, and π, and κ, have been identified in humans (Farrant and Nusser, 2005; Olsen and Sieghart, 2009; Sieghart and Savić, 2018) and the subunit composition, as well as arrangement, of GABAₐRs contribute to receptor properties such as trafficking, localization, kinetics, and pharmacology (Levitan et al., 1988a; Sigel et al., 1990; Lavoie et al., 1997; Belletti and Lambert, 2005; Herd et al., 2007; Sigel and Steinmann, 2012).

Neuronal activity is dynamically regulated by both phasic and tonic inhibition resulting from GABAₐRs localized at synaptic or extrasynaptic regions, respectively (Mody and Pearce, 2004; Farrant and Nusser, 2005; Jacob et al., 2008; Lorenz-Guertin and Jacob, 2018; Chiu et al., 2019; Tomita, 2019). Furthermore, GABAₐRs are ubiquitously expressed across the brain, albeit in a region-, circuit- and cell-specific manner (Sieghart and Sperk, 2002; Engin et al., 2018). Deficits in GABAergic signaling are associated with the pathophysiology behind several neurological and psychiatric conditions (Macdonald et al., 2004; Ramamoorthi et al., 2002; Engin et al., 2018; Amengual-Gual et al., 2019). Mutations of GABAₐR subunit variants, and the discovery of GABAₐR dysfunction has been observed in a myriad of conditions including seizures, sleep disorders, and psychiatric disorders (Greenfield, 2013; Nuss, 2015; Engin et al., 2018; Amengual-Gual et al., 2019). Mutations in discrete GABAₐR subunits have been shown to impair receptor properties, such as trafficking and ligand sensitivity disorders, an update on GABAₐR function and pharmacology with respect to benzodiazepines and neurosteroids will be given. Additionally, we discuss recent studies on GABAₐR structures, GABAₐR subunit variants, and the discovery of GABAₐR-associated transmembrane proteins. Lastly, we highlight potential opportunities in GABAₐR pharmacology development as a result of these advancements.

A BRIEF HISTORY ON GABAₐRs AS PROLIFIC DRUG TARGETS AND THEIR THERAPEUTIC USAGE

Drugs targeting GABAₐRs have been in use since the early 1900s (Figure 1), long before the isolation and cloning of receptor subunits in the 1980s (Sigel et al., 1982, 1983; Barnard et al., 1987; Schofield et al., 1987; Seeburg et al., 1990). Barbiturates were first employed for their anticonvulsant and sedative-hypnotic properties (Smart and Stephenson, 2019). However, a decline in their clinical use resulted from high mortality risk due to accidental overdose and the advent of benzodiazepines (López-Muñoz et al., 2005). The initial discovery of chlorzidiazepoxide in 1955 by Leo Sternbach at Hoffman-La Roche and diazepam (DZ) shortly after in 1959 created excitement for benzodiazepines (Figure 1), allowing them to become one of the most widely marketed and prescribed drugs (Mehdi, 2012). However, it was not accepted until decades later that adverse side effects such as addiction could occur with long-term usage (Lalive et al., 2011; Mehdi, 2012; Votaw et al., 2019). Although their site-of-action had not yet been determined, by the mid-to-late 1970s, it was known that barbiturates and benzodiazepines enhanced inhibition by potentiating the actions of GABA (Smart and Stephenson, 2019). Following the discovery of the various GABAₐR subunits, many studies have been devoted toward characterizing the physiological and pharmacological properties of GABAₐRs with respect to subunit composition (Barnard et al., 1987; Schofield et al., 1987; Levitan et al., 1988b; Pritchett et al., 1989; Seeburg et al., 1990; Farrant and Nusser, 2005; Vithlani et al., 2011; Engin et al., 2018; Sieghart and Savić, 2018). GABAₐRs harbor several binding sites for barbiturates, benzodiazepines, general anesthetics, alcohol, and neurosteroids (Sieghart, 2015). Depending on the GABAₐR subtype, many of these compounds exhibit differences in ligand sensitivity, resulting in different physiological and behavioral responses (Olsen and Sieghart, 2009). Accordingly, substantial work has been devoted toward understanding the binding mode and functional responses of various ligands at different GABAₐR subtypes.

Given their pivotal role in mediating fast inhibitory neurotransmission, it is not surprising that alterations to GABAₐR function are involved in many neurological and psychiatric disorders. GABAₐR dysfunction has been observed in a myriad of conditions including seizures, sleep disorders, and anxiety-like disorders (Greenfield, 2013; Nuss, 2015; Engin et al., 2018; Amengual-Gual et al., 2019).
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(Hernandez and Macdonald, 2019; Maljevic et al., 2019). For instance, the first GABA<sub>a</sub>R subunit mutations associated with epilepsy were discovered in the γ2 subunit (Baulac et al., 2001; Wallace et al., 2001) and since then, a multitude of other mutations have been identified (Hernandez and Macdonald, 2019; Maljevic et al., 2019). Importantly, benzodiazepines remain as frontline drugs in attenuating seizures (Greenfield, 2013; Amengual-Gual et al., 2019) even though a variety of GABA<sub>a</sub>R-independent pathophysiological alterations can contribute to seizure generation (Stafstrom, 2010). Additionally, anxiety-like behaviors have also been observed concomitantly with dysregulation of inhibitory circuits (Smith and Rudolph, 2012; Smith et al., 2012; Nuss, 2015; Engin et al., 2018) and benzodiazepines continue to stand the test of time as effective anxiolytics (Balon and Starcevic, 2020). Apart from benzodiazepines, the therapeutic potential of neurosteroids have also garnered interest following the recent FDA approval of brexanolone (i.e., allopregnanolone; Mody, 2019) for postpartum depression (PPD) (Figure 1). During pregnancy, increased hormone production has been observed along with enhanced neurosteroid levels, such as allopregnanolone, and transient changes in allopregnanolone concentrations are implicated as a contributing factor to temporary changes in GABA-mediated inhibition, which can result in PPD-associated affective behaviors (Frye et al., 2011; Schüle et al., 2014). The therapeutic utility of neurosteroids in other forms of depression, anxiety, and epilepsies are also currently being explored (Lévesque et al., 2017; Czyk, 2019; Walton and Maguire, 2019; Zorumski et al., 2019; Belelli et al., 2020). We acknowledge that a variety of neurological and neuropsychiatric conditions involve GABA dysregulation (Ramamoorthi and Lin, 2011; Mele et al., 2019), but we have chosen to focus on disorders where GABA<sub>a</sub>R pharmacology is currently and commonly used: benzodiazepines for epilepsy and anxiety, as well as neurosteroids for PPD. Individuals suffering from these conditions stand the most to gain from the development of novel therapeutic GABA<sub>a</sub>R-targeting drugs that achieve higher specificity and efficacy, while also mitigating adverse side effects.

BENZODIAZEPINE ACTIONS ON GABA<sub>a</sub>Rs

GABA<sub>a</sub>Rs with high sensitivity to benzodiazepines are typically tri-heteromeric and composed of two α 1-3, 5, two β (1-3), and one γ2 subunit (Farrant and Nusser, 2005). Similar to GABA, subunit composition alters sensitivity to benzodiazepines (Minier and Sigel, 2004). The classical, high-affinity benzodiazepine binding site is present at the extracellular α-γ interface and upon binding, benzodiazepines can increase GABA affinity (Twyman et al., 1989; Lavoie and Twyman, 1996), modulate gating by priming the receptor toward a preactivated step, and affect the rate of desensitization (Twyman et al., 1989; Gielen et al., 2012; Goldschen-Ohm et al., 2014; Jatczak-Sliwa et al., 2018). With respect to single-channel properties, benzodiazepines increase the frequency of channel opening and...
bursting, with no effect on conductance or channel opening duration (Twyman et al., 1989; Rogers et al., 1994). The actions of benzodiazepines observed at the microscopic level continue to shape our understanding of how inhibitory postsynaptic currents (IPSCs) are modulated. Accordingly, in the presence of benzodiazepines, IPSC decay is prolonged and at some synapses, an increase in amplitude has also been observed (Mohler et al., 2002; Mozrzymas et al., 2007; Karayannis et al., 2010). The efficacy of benzodiazepine potentiation ranges from partial to full allosteric modulation, which is dependent on the binding mode of the benzodiazepine ligand and its effects on GABA$_A$Rs gating properties (Elgarf, 2018).

Given that the high affinity binding site exists at the $\alpha\gamma$ interface, a multitude of side effects can occur following administration due to their non-selective targeting of GABA$_A$Rs. Classical benzodiazepines interact non-selectively with all GABA$_A$Rs, but $\alpha$-containing GABA$_A$Rs are expressed with greater confinement to specific brain regions (Engin et al., 2018). Modulation of $\alpha$- and $\gamma$-containing GABA$_A$Rs are associated with the sedative (Rudolph et al., 1999; McKernan et al., 2000; Rowlett et al., 2005) and anxiolytic (Löw et al., 2000) properties of benzodiazepines, respectively. In addition, $\gamma$-containing GABA$_A$Rs have also been implicated with the anxiolytic effects of benzodiazepines (Atack et al., 2005; Dias et al., 2005), but this has also been disputed (Löw et al., 2000; Behlke et al., 2016). Lastly, $\gamma$-containing GABA$_A$Rs may also mediate certain aspects of anxiety behaviors (Botta et al., 2015) and modulation of this subtype can result in anxiolysis (Behlke et al., 2016). As a result of the predominant behavioral effects produced by $\alpha$- and $\gamma$-containing GABA$_A$Rs, there have been attempts to develop novel compounds that target these GABA$_A$R subtypes specifically. Most notably, non-benzodiazepines (also known as $z$-drugs) were developed and are used as sleep aids due to their higher selectivity for $\alpha$-containing GABA$_A$Rs (Atack, 2011; Tan et al., 2011; Cheng et al., 2018; Sieghart and Savić, 2018). However, there are currently no FDA-approved, subtype-selective GABA$_A$R-targeting drugs that function as anxiolytics and are devoid of sedative properties.

One concern associated with long-term usage of benzodiazepines is the development of tolerance based on observations of reduced GABAergic transmission along with altered subunit composition following chronic benzodiazepine treatment (Ususi-Oukari and Korpi, 2010; Jacob et al., 2012; Vinkers and Olivier, 2012; Lorenz-Guertin et al., 2019). The development of benzodiazepine tolerance likely results from decreased GABA$_A$R surface availability due to enhanced inhibition from prolonged drug exposure (Gallager et al., 1984). Interestingly, changes in GABA$_A$R expression appear to be subunit-specific (Ususi-Oukari and Korpi, 2010) and are also dependent on cell-type and brain region (Poisbeau et al., 1997; Jacob et al., 2012; Foitzick et al., 2020). For example, prolonged DZ treatment has been observed to reduce total $\gamma_2$ expression due to increased lysosomal degradation of this subunit (Lorenz-Guertin et al., 2019). Phosphorylation of GABA$_A$Rs by specific protein kinase C (PKC) isoforms have been demonstrated to impact benzodiazepine sensitivity in recombinant GABA$_A$Rs (Leidenheimer et al., 1992; Qi et al., 2007; Nakamura et al., 2015). In addition, calcineurin-dependent dephosphorylation following application of DZ has also been shown to induce endocytosis of GABA$_A$Rs (Nicholson et al., 2018). Although benzodiazepines non-selectively target $\alpha$-3,5/$\gamma$-containing GABA$_A$Rs throughout the brain, it is possible that variability in benzodiazepine sensitivity among different cell types arises from changes in GABA$_A$R subunit post-translational modifications (PTMs) and/or their interactions with GABA$_A$R-interacting proteins (Jacob et al., 2012). Together, these features could result in variable GABA$_A$R turnover rate in different neuronal populations and may explain why benzodiazepine tolerance for the sedative-hypnotic effects occur more rapidly in comparison to the anticonvulsant/anxiolytic actions (Bateson, 2002; Vinkers and Olivier, 2012), but this supposition requires further clarification.

Furthermore, although acute treatment with benzodiazepines is generally considered safe, their chronic use can result in physical dependence (Soyka, 2017; Silberman et al., 2020) and potentially drug abuse/misuse (Lalive et al., 2011; Tan et al., 2011), which is a major public health concern across the world (Votaw et al., 2019). In general, drugs of abuse hijack brain reward circuitry leading to enhanced dopamine (DA) release from the ventral tegmental area (VTA) into the nucleus accumbens (NAc) (Kauer and Malenka, 2007). Mechanistically, benzodiazepines activate $\alpha$-containing GABA$_A$Rs on GABAergic interneurons in the VTA, resulting in disinhibition which promotes DA release (Tan et al., 2010). Additionally, activation of $\alpha$-containing GABA$_A$Rs in the NAc have also been shown to mediate reward learning associated with benzodiazepines (Reynolds et al., 2012; Engin et al., 2014). It is important to note that the abuse liability for benzodiazepines is the highest in individuals who use other drugs of abuse (Silberman et al., 2020). Additionally, the development of physical dependence can occur independently of addiction (Silberman et al., 2020). Lastly, how tolerance develops requires further elucidation, due to observations that the development of tolerance differs based on the usage of the benzodiazepine (Vinkers and Olivier, 2012). One potential reason for disparities observed in tolerance and risk of misuse is due to the overall half-life of the type of benzodiazepine (Vinkers and Olivier, 2012). Thus, targeting GABA$_A$Rs based on subunit-specificity continues to remain important not only toward understanding the roles of different subtypes in circuits and behaviors, but also for achieving optimal therapeutic efficacy while mitigating adverse effects.

**NEUROSTEROID ACTIONS ON GABA$_A$Rs**

In contrast to the historical usage of benzodiazepines as pharmacotherapy, neurosteroids are the newest class of GABA$_A$R-targeting drugs, with brexanolone as the first therapy indicated for the treatment of PPD (Cristea and Naudet, 2019; Mody, 2019). Neurosteroids are robust modulators of both synaptic and extrasynaptic GABA$_A$Rs.
Thus, their effects on GABAergic transmission are mediated by prolonging phasic inhibition, as well as increasing tonic conductance. Neurosteroid binding sites on GABA<sub>A</sub>Rs were initially discovered within the transmembrane domains of the α1β2γ2 between the α and β subunit interface, and key residues regulating their binding are largely conserved among α2-5/β3γ2 and α4β3δ subtypes (Hosie et al., 2006, 2009). Additionally, neurosteroids potentiate GABA-mediated currents with higher efficacy in δ-containing GABA<sub>A</sub>Rs compared to γ2-containing GABA<sub>A</sub>Rs (Bianchi and Macdonald, 2003; Belelli et al., 2020). Comparable to benzodiazepines, neurosteroids increase the frequency of single channel openings, but also prolong the channel open duration similarly to barbiturates (Twyman and Macdonald, 1992; Belelli et al., 2020). Furthermore, neurosteroids enhance the duration of IPSCs by prolonging the decay time (Lambert et al., 2003). Interestingly, relatively high concentrations (>100 nM) of neurosteroids can directly activate GABA<sub>A</sub>Rs in the absence of GABA (Belelli and Lambert, 2005). These observations may potentially be clinically relevant given that altered neurosteroid concentrations in the brain have been observed in neuropsychiatric conditions such as PPD, anxiety, and stress (Purdy et al., 1991; Schumacher et al., 2003; Belelli et al., 2020).

It has been previously demonstrated that knock-in mice with α2-containing GABA<sub>A</sub>Rs rendered insensitive to neurosteroid potentiation for this subtype exhibit anxiety-like behaviors without displaying depressive-like phenotypes nor effects on analgesia (Durkin et al., 2018). These observations potentially suggest a specific role for how neurosteroids might be useful as anxiolytics given that α2-containing GABA<sub>A</sub>Rs have already been associated with the anxiolytic effects of benzodiazepines (Löw et al., 2000). While the behavioral effects of neurosteroids can be interrogated based on their interaction with distinct GABA<sub>A</sub>R subtypes (Belelli et al., 2020), achieving subtype-selectivity may prove challenging due to conserved binding sites among synaptic and extrasynaptic GABA<sub>A</sub>Rs (Hosie et al., 2007, 2009). Neurosteroid modulation of GABA<sub>A</sub>Rs are also regulated by phosphorylation status, which affects receptor expression and/or surface trafficking, as well as neurosteroid sensitivity (Smith et al., 2007; Abramian et al., 2014; Comenencia-Ortiz et al., 2014; Nakamura et al., 2015). Distinct neurons have been observed to exhibit differences in their sensitivity to neurosteroids due to changes in phosphorylation mediated by different kinases, such as protein kinase A (PKA) or PKC (Fáncsek et al., 2000; Hodge et al., 2002; Vicini et al., 2002; Harney et al., 2003; Kia et al., 2011). Together, both phosphorylation of GABA<sub>A</sub>R subunits and changes to endogenous neurosteroid levels can have profound effects on phasic and tonic inhibition, contributing to the heterogeneity of inhibition across brain regions.

There has been growing interest in the therapeutic development of neurosteroids for potential application in other forms of depression such as major depressive disorder (zuranolone; Sage Therapeutics) and treatment-resistant depression (zuranolone; Sage Therapeutics, ganaxolone; Marinus Pharmaceuticals). Indeed, it has been observed that neurosteroid levels are reduced in depression and treatment with various antidepressants can normalize these concentrations (Lüscher and Möhler, 2019). Given the comorbidity of anxiety and depression, the potential for neurosteroids being employed as effective anxiolytics are also being explored (Schüle et al., 2014; Czyk, 2019; Gunduz-Bruce et al., 2019; Lüscher and Möhler, 2019; Zorumski et al., 2019). Additionally, neurosteroids are also being considered for use in seizure disorders such as refractory status epilepticus and PCDH19-related epilepsy (ganaxolone; Marinus Pharmaceuticals). Polypharmacy involving administration of both benzodiazepines and neurosteroids together are also being considered for mitigating epileptic seizures (Rogawski et al., 2020). Taken together, there is the possibility that further development of neurosteroids as a therapeutic agent will be applicable to other neurological disorders.

**RECENT ADVANCES IN GABA<sub>A</sub>R BIOLOGY**

Extensive characterization into the physiological and pharmacological properties of distinct GABA<sub>A</sub>R subtypes continues to provide insight toward their relevance at the cellular, circuit, and behavioral level. The past two decades have seen the development of subtype-selective compounds targeting GABA<sub>A</sub>Rs with specific pharmacological and behavioral actions (Rudolph and Knoeflach, 2011; Sieghart and Savić, 2018; Chen et al., 2019). Additionally the recent emergence of structural data revealing GABA<sub>A</sub>Rs bound with different ligands will certainly help refine and guide drug development (Olsen et al., 2019). Differences in expression profiles and receptor function among GABA<sub>A</sub>R subtypes, subunit variants due to alternative splicing (Boileau et al., 2003, 2010; Eom et al., 2011; Miller et al., 2018), and mechanisms that preferentially enrich mRNA transcripts of discrete GABA<sub>A</sub>R subunits at specific subcellular regions (Raigor et al., 2020), continue to highlight the diversity of GABAergic signaling in neuronal inhibition. Furthermore, the discovery of transmembrane proteins that associate with GABA<sub>A</sub>Rs are beginning to shed light on their molecular mechanisms in vivo and may also provide strategies for targeting select GABA<sub>A</sub>R subtypes and in turn, lead to the development of more effective therapeutics for neurological and psychiatric disorders.

**Structural Insights Into GABA<sub>A</sub>Rs and Signaling Mechanisms**

Molecular modeling of drug-receptor interactions relies on high-resolution structures and aims to characterize not only the spatial architecture of the receptor, but how ligand binding induces changes in receptor conformation and influences channel gating (Olsen et al., 2019). Several structures of AMPA receptors (AMPARs) bound to ligands and/or in complex with auxiliary subunits help exemplify how these interactions are critical for modulating AMPAR function (Herguedas et al., 2019; Nakagawa, 2019; Zhao et al., 2019; Kamalova and Nakagawa, 2020). In contrast, until recently, there has been a lack of structural data regarding GABA<sub>A</sub>Rs, which has limited our understanding of how these receptors structurally interact with their ligands. New studies using cryogenic electron microscopy (cryo-EM)
to examine full-length, synaptic GABA<sub>Α</sub>Rs are now available and will be important for bridging the link between receptor architecture and their pharmacological signaling mechanisms (Laverty et al., 2019; Masiulis et al., 2019; Kim et al., 2020). In addition to high affinity benzodiazepine sites at the α-γ interface, other benzodiazepine binding sites on GABA<sub>Α</sub>Rs have also been inferred (Walters et al., 2000; Baur et al., 2008; Ramerstorfer et al., 2011; Wongsamitkul et al., 2017; Sigel and Ernst, 2018; Lian et al., 2020). Recently, cryo-EM studies confirmed the presence of a low-affinity binding site present within the transmembrane domain of the αβ interface as described in earlier studies (Walters et al., 2000; Ramerstorfer et al., 2011; Sigel and Ernst, 2018; Masiulis et al., 2019; Lian et al., 2020). Moreover cryo-EM studies have identified the presence of other distinct benzodiazepine binding sites, specifically within the β-α and γ-β interfaces (Masiulis et al., 2019; Kim et al., 2020). It has been shown that DZ concentration-response curves exhibit biphasic responses (Walters et al., 2000; Baur et al., 2008; Ramerstorfer et al., 2011; Wongsamitkul et al., 2017). Potentiation of GABA by high concentrations of DZ (>20 µM) is thought to be mediated by a low-affinity binding site and submaximal GABA responses from αβ2 receptors lacking the γ2 subunit have been shown to be potentiated by high concentrations of DZ, which were not blocked by flumazenil (Walters et al., 2000; Baur et al., 2008; Ramerstorfer et al., 2011; Wongsamitkul et al., 2017). Although the amount of DZ used in this particular study far exceeds the concentration needed to sit deeper within the α-γ interface, other unique benzodiazepine binding sites, there are currently no studies that have demonstrated that these unique, low-affinity binding sites can mediate the anesthetic effects of benzodiazepines in isolation. In the future, it would be interesting to explore the conditions that facilitate the activity of GABA<sub>Α</sub>Rs through these low-affinity sites and whether these sites can be selectively targeted. It also is possible that future cryo-EM structures may reveal subtle differences in the benzodiazepine binding pocket among distinct, benzodiazepine-sensitive GABA<sub>Α</sub>Rs, which could help in the development of more subtype-selective compounds.

Structural data has also highlighted the importance of key residues (Hosie et al., 2006) and the binding modes of distinct neurosteroid ligands on GABA<sub>Α</sub>Rs (Laverty et al., 2017; Miller et al., 2017; Sugasawa et al., 2020). Previously, it was thought that the allosteric modulation and direct activation of GABA<sub>Α</sub>Rs by neurosteroids were due to binding on distinct sites, at the α subunit and the αβ interface, respectively (Hosie et al., 2006). However, structures of GABA<sub>Α</sub>R chimeras bound with tetrahydroxyoctocorticoesterone (THDOC) later revealed that both actions resulted from binding at the same site (Laverty et al., 2017). It was also shown that neurosteroids, such as pregnanolone sulfat, occupy a separate site within the intra-subunit transmembrane domain of the α subunit distinct from THDOC (Laverty et al., 2017). Accordingly, it has recently been shown that different neurosteroid ligands can promote either the activation or desensitization of αβ3 GABA<sub>Α</sub>Rs by binding to the intersubunit interface of the β-α subunits or within the intrasubunit on β3, respectively (Laverty et al., 2017; Miller et al., 2017; Sugasawa et al., 2020). Currently, it remains unknown whether neurosteroid binding sites among synaptic and extrasynaptic GABA<sub>Α</sub>R are structurally distinct when ligand bound and if these differences can be exploited for the development of novel neurosteroids with varying efficacy and specificity. However, to date, there are a lack of neurosteroid-based ligands designed for subtype-selectivity (Althaus et al., 2020), such as exclusively targeting α2-containing GABA<sub>Α</sub>Rs for their anxiolytic properties without modulating α4-containing GABA<sub>Α</sub>Rs. Similar to benzodiazepines, future cryo-EM studies may also uncover unique differences among neurosteroid binding sites in different GABA<sub>Α</sub>R subtypes that can be therapeutically leveraged.

**Spatial Expression Profiles of GABA<sub>Α</sub>R Subtypes and GABA<sub>Α</sub>R Subunit Variants**

The circuit and behavioral roles mediated by specific GABA<sub>Α</sub>R subtypes are dependent on their abundance in precisely defined regions. High GABA<sub>Α</sub>R sensitivity to benzodiazepines is conferred by a histidine residue in the N-terminal extracellular region (Wieland et al., 1992) and is specific to GABA<sub>Α</sub>Rs containing α1-, α2-, α3-, and/or α5 subunits. However, GABA<sub>Α</sub>Rs containing these subunits can be found across the brain and overall modulation of these GABA<sub>Α</sub>Rs contributes to both the desired therapeutic effect, but also some of the side effects. Therefore, it is important to understand how brain regions are modulated in a circuit-specific manner and the physiological role of GABA<sub>Α</sub>Rs subtypes within brain regions.

With respect to the anxiolytic effects of benzodiazepines, the amygdala has received prominent attention due to its role in mediating emotional responses and this structure can be divided into discrete subdivisions based on cell-type, circuit, and physiological role (Ehrlich et al., 2009). Differences in GABA<sub>Α</sub>R subtypes expressed in subdivisions of the amygdala have been observed (Fritschy and Mohler, 1995; Pirker et al., 2000; Kaufmann et al., 2003; Fujimura et al., 2005). Although nearly all GABA<sub>Α</sub>Rs are expressed throughout the amygdala, the region and cellular localization of these GABA<sub>Α</sub>R subtypes can impact anxiety behaviors (Engin et al., 2018). For example, in the central amygdala (CeA), α5-containing GABA<sub>Α</sub>Rs are associated with anxiety-like behaviors in a cell-specific manner (Houbensak et al., 2010; Bottla et al., 2015). Specifically, knockdown (KD) of α5-containing GABA<sub>Α</sub>Rs in PKCδ+ neurons results in anxiogenesis, highlighting the
importance of subtype- and cell-specific regulation of anxiety-like behaviors (Haubensak et al., 2010; Herman et al., 2013; Botta et al., 2015). Additionally, α1-containing GABA_A Rs are localized on corticosterone releasing factor neurons and have been shown to contribute to anxiety-like phenotypes possibly through the regulation of neuronal excitability (Gafford et al., 2012; Herman et al., 2013). However, the specific role of α1-containing GABA_A R activity within the CeA with respect to control over anxiety-like behaviors has yet to fully be determined (Engin et al., 2018). In line with these observations, benzodiazepines have also been shown to impact CeA activity and anxiety-like behaviors (Carvalho et al., 2012; Botta et al., 2015; Griessner et al., 2018). Although this could also be due to a higher degree of α2-containing GABA_A R expression (Fritschy and Mohler, 1995; Pirker et al., 2000), the cell-type and circuit-specific role of α2-containing GABA_A Rs in CeA is not completely understood (Engin et al., 2018). Further complications teasing out the effects of benzodiazepines come from the fact that benzodiazepines non-selectively modulate all α1-3, 5-containing GABA_A Rs distributed across the entire amygdala (Fritschy and Mohler, 1995; Pirker et al., 2000; Engin et al., 2018). Taken together, the complexity regarding the microcircuitry governing anxiety and how both region- and cell-specific expression of GABA_A R subtypes can impact anxiety behaviors still requires further investigation.

Furthermore, subcellular localization of GABA_A Rs also profoundly determines the type of neuronal inhibition exhibited and how GABA_A R-targeting drugs will modulate neuronal activity (Kerti-Szigeti and Nusser, 2016; Nathanson et al., 2019; Kramer et al., 2020). The mobility and diffusion of GABA_A Rs between synaptic and extrasynaptic regions are dependent on subunit composition, specific motifs within the intracellular domain of certain subunits, and the interaction of GABA_A Rs with scaffolding partners such as gephyrin or radixin (Jacob et al., 2005; Thomas et al., 2005; Loebrich et al., 2006; Bannai et al., 2009; Mukherjee et al., 2011; Tyagarajan and Fritschy, 2014; Haurat et al., 2015; Hanner et al., 2019; Davenport et al., 2020). Recently, there have been reports of differences in synaptic and extrasynaptic GABA_A R subunit localization, as well as neuron-specific differences (Schulz et al., 2018; Magnin et al., 2019). For example, it is well-established that in hippocampal pyramidal neurons, the α5 subunit is predominantly expressed at extrasynaptic regions and mediates the majority of tonic inhibition (Crestani et al., 2002; Caraiscos et al., 2004; Farrant and Nusser, 2005; Glyks and Mody, 2007; Glyks et al., 2008). Interestingly, α5-containing GABA_A Rs can also localize synaptically and contribute to phasic inhibition (Brady and Jacob, 2015; Schulz et al., 2018; Davenport et al., 2020). Furthermore, in hippocampal somatostatin interneurons, α5-containing GABA_A Rs appear to be localized synaptically as they co-localize with VGAT and are targeted by vasoactive intestinal polypeptide (VIP)- and calretinin-expressing (Schulz et al., 2018; Magnin et al., 2019), but not parvalbumin interneurons. In addition, α5-GABA_A Rs targeted by VIP interneurons appear to be involved in anxiety-like behaviors (Magnin et al., 2019). More work is needed to understand how distinct GABA_A Rs are targeted by different inhibitory inputs and how differences in compartment localization can dynamically regulate neuronal activity. For example, it is currently unknown whether pharmacological targeting of perisomatic or dendritic inhibition exclusively can result in better therapeutic efficacy. Although currently not feasible, future endeavors examining the specific roles of GABA_A Rs and their contributions to perisynaptic and/or dendritic inhibition will provide a more mechanistic understanding of input-specific inhibition and could perhaps allow these mechanisms to be manipulated pharmacologically.

In addition, GABA_A R splice variants with differences in function and pharmacology continue to further enhance the diversity and classification of GABA_A R subtypes (Whiting et al., 1990; Boileau et al., 2010; Miller et al., 2018; Smart and Stephenson, 2019; Rajgor et al., 2020). For example, the γ2 subunit can exist as either a long (γ2L) or short (γ2S) variant, with the latter missing eight amino acids in the long intracellular loop (Whiting et al., 1990; Boileau et al., 2010). Functionally, γ2S differs from γ2L in zinc sensitivity and kinetics, and the surface expression of γ2S alone is possible even when co-transfected with γ and β subunits (Boileau et al., 2003, 2010). Additionally, γ2L and γ2S expression changes over the course of development and their expression is confined to different brain regions (Wang and Burt, 1991; Gutiérrez et al., 1996). Noteworthily, it has also been observed that in schizophrenia, γ2S is decreased (Huntsman et al., 1998) which elicits the question as to whether γ2L and/or γ2S are differentially involved in other neurological and neuropsychiatric conditions. Lastly, PTMs can influence GABA_A R properties and phosphorylation of the serine site S343 which is exclusive to the γ2L variant and not in γ2S (Lorenz-Guertin et al., 2018) has been documented (Nakamura et al., 2015). However, more research is required in order to identify other residues within the γ2L and γ2S variants that are subject to phosphorylation or other PTMs, as well as how these PTMs modulate GABA_A R properties. With newer tools that can achieve greater drug targeting specificity (Shields et al., 2017; Atasoy and Sternson, 2018; Rao et al., 2019; Crocetti and Guerrini, 2020) along with rational drug design (Antkowiak and Rammes, 1999; Scott and Aricescu, 2019), it may be possible in the near future for drug development strategies to exploit these differences in order to maximize the therapeutic efficacy of newer compounds while also decreasing the likelihood of unwanted side effects by “sparing” other GABA_A R subtypes.

**GABA_A R-Associated Transmembrane Proteins**

Although a majority of compounds have been developed that target the pore-forming subunits of GABA_A Rs, recent advances in the field of GABA_A R biology have illuminated that native GABA_A R exist as a complex with other accessory proteins rather than in isolation (Khayenko and Maric, 2019). Within the past decade (Figure 1), novel transmembrane proteins have been discovered that associate with native GABA_A Rs and have distinct effects on receptor trafficking, kinetics, and/or pharmacology (see Han et al., 2020 for an in-depth review). Therefore, these proteins present themselves as potentially novel sites for new GABA_A R drug development.
Lipoma HMGIC Fusion Partner-Like 4

Lipoma HMGIC fusion partner-like 4 (Lhfp4, LH4; also referred to as GABA_3-R) is a four-pass transmembrane protein that was recently shown to critically regulate GABA_3-R anchoring at inhibitory synapses and thus impact the strength of fast inhibitory synaptic transmission. Indeed, both KD (Yamasaki et al., 2017) and knockout (KO) (Davenport et al., 2017; Wu et al., 2018) of LH4 resulted in decreased GABA_3-R clustering, as well as diminished GABA_3-ergic synaptic transmission and GABA_3-ergic synapse density (Davenport et al., 2017; Yamasaki et al., 2017; Wu et al., 2018). Additionally, LH4 forms a tripartite complex with GABA_3-As and neuroligin-2 (NL2) (Davenport et al., 2017; Yamasaki et al., 2017; Wu et al., 2018), a postsynaptic inhibitory cell adhesion molecule (Pouloupolos et al., 2009; Li et al., 2017b; Lu et al., 2017). Interestingly, the δ subunit plays an important role in the regulation of GABA_3-R assembly within the cerebellum by preventing incorporation of γ2 and LH4 (Martenson et al., 2017). Incorporation of the δ subunit prevented assembly of γ2 into GABA_3-Rs, as well as the interaction with LH4 (Martenson et al., 2017). In this manner, δ-containing GABA_3-Rs became extrasynaptically localized whereas γ2-containing GABA_3-Rs were localized at synapses via their interaction with both LH4 and NL2 (Davenport et al., 2017; Martenson et al., 2017; Yamasaki et al., 2017; Wu et al., 2018). Lastly, although LH4 did not alter sensitivity to endogenous GABA, THIP, or picrotoxin (Yamasaki et al., 2017), LH4-dependent modulation of other GABA_3-R-targeting compounds still requires further investigation.

Cleft Lip and Palate Transmembrane Protein 1

Abnormal trafficking of GABA_3-Rs can involve a variety of mechanisms, leading to a lack of GABA_3-R availability at the neuronal surface. Specifically, a novel transmembrane protein, cleft lip and palate transmembrane protein 1 (Cclptm1) was identified as a negative regulator of GABA_3-R forward trafficking of both synaptic and extrasynaptic GABA_3-Rs through receptor confinement primarily in the ER and reduced the surface availability of GABA_3-Rs (Ge et al., 2018). Importantly, this modulation of GABA_3-R forward trafficking impacted inhibitory transmission bi-directionally; overexpression and KD of Cclptm1 resulted in diminished and enhanced postsynaptic inhibitory responses, respectively. Additionally, this bi-directional effect was similarly observed in tonic currents generated from extrasynaptic GABA_3-Rs. Mechanistically, these data suggest that Cclptm1 is a pan-GABA_3-R regulator and thus impacts both synaptic and tonic inhibition through restriction of receptor forward trafficking.

Shisa7

Members of the Shisa family of proteins are single-pass transmembrane proteins containing both cysteine and proline rich domain on the N- and C-terminus, respectively (Pei and Grishin, 2012). Specifically, Shisa6-9 are referred to as cystine knot AMPAR membrane proteins (CKAMP) (Farrow et al., 2015) due to the presence of an AMPAR interacting domain in the C-terminus (von Engelhardt, 2019). Notably, Shisa7 (CKAMP59) has emerged as an interesting member of the Shisa family in that unlike other CKAMP counterparts, Shisa7 has a direct role in GABA_3-R regulation at inhibitory synapses (Han et al., 2019). While other CKAMPs are localized at glutamatergic synapses (von Engelhardt et al., 2010; Klaassen et al., 2016; Peter et al., 2020), we observed that Shisa7 co-localizes specifically with gephyrin and GABA_3-Rs in hippocampal neurons (Han et al., 2019) and not at excitatory synapses as reported in an earlier study (Schmitz et al., 2017). Functionally, Shisa7 regulated GABA_3-R trafficking and inhibitory synaptic transmission without affecting excitatory synaptic transmission (Han et al., 2019). Strikingly, Shisa7 also modulates GABA_3-R kinetics and pharmacological properties. Indeed, in heterologous cells, Shisa7 decreased the deactivation time constants of α1β2γ2 and α2β3γ2 receptors, and conversely Shisa7 KO prolonged the decay time constant of GABA_3-ergic transmission in hippocampal neurons (Han et al., 2019). Lastly, Shisa7 increased DZ-induced potentiation of GABA_3-Rs in heterologous cells and Shisa7 KO significantly reduced DZ actions in vivo (Han et al., 2019). Taken together, this is the first documentation of a transmembrane auxiliary subunit unique to GABA_3-Rs that can influence receptor trafficking, kinetics, and pharmacology.

Targeting Transmembrane Accessory Molecules That Interact With Native GABA_3-Rs

Although 19 different GABA_3-R subunits have been identified, there are a multitude of different possible subunit combinations that can occur. Thus, one potential obstacle to overcome regarding the development of subtype-specific GABA_3-R-targeting drugs is achieving better therapeutic efficacy and selectivity. One strategy is to “think outside the receptor” and evaluate whether there are “druggable” targets that coexist with native GABA_3-Rs independent of the pore-forming subunits. For example, gephyrin is a well-known postsynaptic scaffolding protein that associates with GABA_3-Rs at inhibitory synapses and dysregulation of gephyrin possibly contributes to disrupted GABA_3-R signaling in disease (Tyagarajan and Fritschy, 2014). A recent preliminary study identified that artemisinin, an anti-malarial compound, could bind to gephyrin and subsequently affect GABA_3-R-mediated signaling in pancreatic cells, suggesting within this context that artemisinins could prove useful in treating diabetes (Li et al., 2017a). Additionally, crystallography studies identified that artemisinin and its derivatives bind to the GABA_3-R binding pocket in gephyrin and resulted in destabilization of gephyrin, as well as α1- and α2-containing GABA_3-Rs (Kasaragod et al., 2019). This exciting piece of evidence suggests that proteins that interact with GABA_3-Rs can be targeted and subsequently impact GABA_3-R signaling, making them ripe candidates for new drug development.

In addition to GABA_3-R-associated scaffolds and molecular adaptor proteins (Khayenko and Maric, 2019), newly identified transmembrane proteins that interact with GABA_3-Rs are additional targets that can potentially be exploited in drug development. In fact, transmembrane AMPAR regulatory proteins (TARPs), which are auxiliary subunits of AMPA receptors (Ziff, 2007; Milstein and Nicoll, 2008), are
currently being evaluated for the treatment of epilepsy and pain (Maher et al., 2017). Thus, the discovery of novel GABA\(_{A}\)-R-associated transmembrane proteins (LH4, Clptm1, and Shisa7) as discussed above provide a potentially exciting opportunity for drug development. For example, Shisa7 can modulate GABA\(_{A}\)-R kinetics and pharmacology (Han et al., 2019). Thus, it will be interesting to investigate whether there are any compounds that can interact with Shisa7 and/or other transmembrane proteins, or their interfaces with GABA\(_{A}\)-Rs, to produce clinically relevant effects. In terms of selectivity, LH4 could potentially offer an opportunity to selectively target γ2-containing GABA\(_{A}\)-Rs while “sparring” δ-containing GABA\(_{A}\)-Rs. Collectively, these initial characterizations of transmembrane GABA\(_{A}\)-R regulators have provided the foundation for a new understanding of GABA\(_{A}\)-R-mediated mechanisms of inhibitory control (Han et al., 2020), and present new potential targets for GABA\(_{A}\)-R drug screening.

**SUMMARY AND FUTURE OUTLOOK**

Characterization and investigation of the various binding sites on GABA\(_{A}\)-Rs have provided invaluable data for the development of pharmaceuticals that are used to treat a wide variety of neurological conditions and psychiatric disorders. However, there are still many challenges and issues to be faced with the current state of available GABA\(_{A}\)-R-targeting drugs. One of the biggest challenges comes from ubiquitous expression of GABA\(_{A}\)-R subtypes throughout the brain. This can prove to be problematic when considering off-target drug effects and unforeseen complications from drug administration due to drug binding across many GABA\(_{A}\)-R subtypes in different brain regions. Considering the nature of current GABA\(_{A}\)-R pharmaceuticals, the design of these drugs is based on previously characterized binding sites, such as the high affinity benzodiazepine binding site which exists between the α and γ subunits (Moody and Jenkins, 2018). However, there is still a lack of new and clinically relevant subunit-specific GABA\(_{A}\)-R-targeting drugs despite scientific success in furthering our understanding of GABA\(_{A}\)-R structure and function (Figure 1). The need for subtype-specific GABA\(_{A}\)-R-targeting drugs is not solely confined to clinical applications, but is also needed in biomedical research. The expression of specific GABA\(_{A}\)-Rs in unique cell populations and the lack of compounds for certain subunits, such as a commercially available δ subunit-selective antagonist, only highlights the importance for the future development of more highly selective compounds and will help address the role of specific GABA\(_{A}\)-Rs at the cellular, circuit, and behavioral level.

Although initial studies have characterized the role of transmembrane GABA\(_{A}\)-R accessory proteins within the hippocampus and cerebellum (Martenson et al., 2017; Yamasaki et al., 2017; Ge et al., 2018; Wu et al., 2018; Han et al., 2019), it has not yet been investigated whether these observations apply to other brain regions. Therefore, the possibility exists that these proteins might differentially impact GABA\(_{A}\)-R function within discrete brain regions which contributes to their impact on physiology and behavior, as well as the pharmacological effect of drugs. While GABA\(_{A}\)-Rs are expressed throughout the brain, the potential region-specific distribution of GABA\(_{A}\)-R-associated transmembrane proteins could enhance the selectivity for future GABA\(_{A}\)-R-targeting drugs. Considering this, further investigation requires cell- and circuit-specific interrogation to define how these accessory proteins function in different brain regions. Understanding how these transmembrane proteins associate with GABA\(_{A}\)-R and their role within defined brain structures could potentially allow for the development of drugs that target these GABA\(_{A}\)-R-associated transmembrane proteins directly or compounds that work synergistically with other GABA\(_{A}\)-R-targeting drugs to enhance their therapeutic efficacy and limit unwanted side effects.

To complement structural and binding studies, genetic studies have also proved invaluable in our understanding of GABA\(_{A}\)-Rs. For example, knockin mice harboring histidine-to-arginine mutations in α1-α2-α3-, or α5- GABA\(_{A}\)-R subunits render them insensitive to benzodiazepines (Rudolph et al., 1999; Löw et al., 2000; McKernan et al., 2000; Rudolph and Möhler, 2004) and have provided critical insight and direction toward the development of several subtype-selective ligands (Rudolph and Möhler, 2006; Rudolph and Knoflach, 2011; Richter et al., 2012; Forman and Miller, 2016; Yamaura et al., 2016; Solomon et al., 2019). However, genetic approaches also have limitations and can pose a challenge in studying specific contributions of discrete GABA\(_{A}\)-R subtypes, as well as modeling alterations to GABA\(_{A}\)-R function in disease. For example, GABA\(_{A}\)-R expression and subunit composition change throughout development and there are differences in GABA\(_{A}\)-R subtypes within various brain regions (Luscher et al., 2011), which creates difficulty in studying the role of GABA\(_{A}\)-R in KO models. Genetic deletion of subunits can also create complications when studying discrete subunit contributions to GABA\(_{A}\)-R function. For example, complete KO of the γ2 subunit results in death shortly after birth ( Günther et al., 1995), preventing a functional analysis of γ2 subunit in vivo at later time points. Furthermore, genetic deletion of α subunits can result in compensatory effects. Indeed, deletion of the α1 subunit can promote upregulation of other α-containing subtypes in response (Sur et al., 2001; Kralic et al., 2002a,b, 2006). Additionally, deletion of the γ2 subunit can lead to compensation through replacement of synaptic GABA\(_{A}\)-Rs with the γ3 subunit (Kerti-Szigeti et al., 2014). Thus, the development of more selective drugs would allow for precise investigation as to the role of subtype-selective GABA\(_{A}\)-Rs and further elucidates the role of GABA\(_{A}\)-Rs in health and disease.

A major goal in GABA\(_{A}\)-R drug discovery has been to discover compounds that are more selective and efficacious with less side effects. Although classical benzodiazepines have been successfully used to treat a wide variety of conditions (Chen et al., 2019), their non-selective binding to essentially all γ2-containing GABA\(_{A}\)-Rs can account for many of their side effects (Engin et al., 2018; Chen et al., 2019). The recent emergence of several high-resolution structures of GABA\(_{A}\)-Rs (Laverty et al., 2017, 2019; Miller et al., 2017; Phulera et al., 2018; Zhu et al., 2018;
Masulis et al., 2019) will be important for precision-based drug design that enhances drug selectivity for discrete receptor subtypes. Excitingly, the discovery of transmembrane GABAAR accessory proteins will likely provide further opportunities to develop compounds that can target GABAARs in complex with other accessory proteins, but not bind and modulate GABAARs in isolation. In summary, there is a need for future interrogation of GABAAR pharmacology which takes into account distinct subunit compositions, discrete brain region localizations, and associated GABAAR proteins that better mimic native receptor complexes when designing future pharmaceuticals.

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