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Direct structural insights into $GABA_A$ receptor pharmacology

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Abstract

$GABA_A$ receptors are pentameric ligand-gated ion channels that mediate most fast neuronal inhibition in the brain. In addition to their important physiological roles, they are noteworthy in their rich pharmacology; prominent drugs used for anxiety, insomnia, and general anesthesia act through positive modulation of $GABA_A$ receptors. Direct structural information for how these drugs work was absent until recently. Efforts in structural biology over the past few years have revealed how important drug classes and natural products interact with the $GABA_A$ receptor, providing a foundation for studies in dynamics and structure-guided drug design. Here, we review recent developments in $GABA_A$ receptor structural pharmacology, focusing on subunit assemblies of the receptor found at synapses.

Keywords

Cryo-EM structure; General anesthetic; Benzodiazepine; Cys-loop receptor structure; bicuculline; picrotoxin

$GABA_A$ receptor physiology and pharmacology

$GABA_A$ receptors (see Glossary) are the principal mediators of fast inhibitory neurotransmission in the brain. Binding of the neurotransmitter **γ-aminobutyric acid (GABA)** to this family of ligand-gated ion channels results in opening of an intrinsic chloride channel. In most adult neurons, the membrane potential at which chloride is at equilibrium is near the resting membrane potential, and thus increasing chloride conductance makes it harder for neurons to fire action potentials and release neurotransmitters.

$GABA_A$ receptors belong to the **Cys-loop superfamily** of neurotransmitter receptors (Box 1). Like other receptors in this family, $GABA_A$ receptors assemble as pentamers, with their five subunits arranged around a central axis that forms the ion permeation pathway through the plasma membrane. Other members of the Cys-loop receptor family include glycine

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receptors, 5-HT_3 receptors, and nicotinic acetylcholine receptors in vertebrate species. Invertebrates have additional Cys-loop receptor families, including chloride channels activated by protons, histamine, and glutamate. Structurally homologous pentameric ligand-gated ion channels (**pLGICs**) are found in prokaryotes; these lack the hallmark Cys-loop disulfide bond, but otherwise recapitulate the core superfamily architecture, and have served as profoundly useful surrogates for structural analysis [1-3]. Recent phylogenetic analysis has identified pLGICs in all kingdoms of life, including metazoan subunits that, like the bacterial orthologs, lack the Cys-loop cysteines [2]. We will use the name pLGIC in this review as it is broadly inclusive.

Nineteen human $GABA_A$ subunits have been described that include α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3 [4-6], and these subunits assemble into a limited number of documented $GABA_A$ receptors [7]. More specifically, most $GABA_A$ receptors in the brain exist as heteropentamers that contain two α and two β subunits, and either one γ or one δ subunit. The subunit stoichiometry of δ-containing receptors is less well defined than for the γ-containing receptors. Subunit composition determines localization and pharmacological and biophysical properties [8, 9]. For example, receptors containing a γ subunit are targeted to synaptic membranes where they mediate fast, phasic responses, while δ subunit containing receptors are localized extrasynaptically and mediate slow, tonic currents. Synaptic and extrasynaptic receptors exhibit different biophysical properties including faster or slower desensitization kinetics, and distinctive pharmacological properties including varying affinities and efficacies for **agonists** and for **positive allosteric modulators** (**PMAs**), such as **benzodiazepines**, anesthetics, neurosteroids, and ethanol [5, 6, 10-12].

$GABA_A$ receptors play an essential role in balancing excitatory signals, and accordingly, their dysfunction results in neurological disorders and mental illnesses including epilepsy, schizophrenia, anxiety, insomnia, and memory impairment [13-16]. Consequently, drugs that modulate $GABA_A$ receptor activity are efficacious as anticonvulsants, anxiolytics, antidepressants, and **general anesthetics** [4, 6]. These diverse drug classes can bind at multiple overlapping and non-overlapping sites on the receptors and can act synergistically to potentiate $GABA_A$ receptor activation. As eluded to above, these drugs also exhibit different specificity for $GABA_A$ receptor subtypes found in distinct brain areas [17]. Among the binding sites for allosteric modulators, the benzodiazepine site in the extracellular domain (ECD) is the best characterized; the $GABA_A$ receptor was first known as the benzodiazepine receptor. Benzodiazepines represent the most successful psychotropic drug class used to treat insomnia [18], and their potency varies among the different receptor subtypes. For example, the α1-3 and α5 subunit-containing receptors are sensitive to potentiation by classical benzodiazepines like diazepam, while the α4 and α6 subunit-containing receptors are not [5]. Moreover, benzodiazepines with α-subunit selectivity connect potentiation of receptors containing specific subunits to clinical effects: α1 subunits are involved in sedation and anti-convulsant action; α2 in anxiolytic action; α2, α3, and α5 in muscle relaxation; and α1 and α5 in amnesia and cognitive impairment [19, 20].

Similar patterns have been observed with other drug classes including intravenous (IV) anesthetics, such as propofol and etomidate, which are currently among the most popular IV general anesthetics in the clinic. A second illustrative example of how different subunit

composition relates to pharmacology of drug action comes from mouse knock-in studies, where binding sites for general anesthetics were mutated in β2 and β3 subunit genes. These experiments revealed that the β2 and β3 subunit-containing receptors are engaged in two distinct physiological components of anesthesia, sedation and immobilization, respectively [21, 22]. These distinctive effects may be related to the site-specific expression of different subtypes of $GABA_A$ receptors in the brain [17]. However, our understanding of which specific subunit stoichiometries are found in which brain circuits is underdeveloped, leaving this area ripe for exploration.

Here, we first provide a recounting of the structures of $GABA_A$ receptors and discuss some of the challenges in obtaining these structures. We then delve into the structural pharmacology of $GABA_A$ receptors, including neurotransmitter and antagonist (bicuculline) binding, benzodiazepine site interactions, and general anesthetic (phenobarbital, etomidate, and propofol) binding. Finally, we discuss potential mechanisms for allosteric potentiation of $GABA_A$ receptors.

A brief history of $GABA_A$ receptor structural biology

Transformative developments in cryo-electron microscopy (cryo-EM) technology enabled a succession of advances in the structural biology of heteromeric $GABA_A$ receptors (Figure 1). Initial $GABA_A$ receptor structural information consisted of the X-ray structure of the β3 homomeric receptor published in 2014 [23], followed by homomeric chimeric receptor structures [24-26]. These structures were foundational in defining, at reliable resolutions, the overall architecture, details of the permeation pathway, a reference point for a desensitized ion channel conformation, and insightful inferences into agonist and neurosteroid binding. These first structures were, however, limited in that GABA does not bind physiologically to a β-β subunit interface, which left much unresolved regarding neurotransmitter recognition and structure-function relationships. Moreover, classical benzodiazepine pharmacology, as well as synaptic trafficking, generally requires the presence of a γ subunit in the pentamer.

Breakthroughs on physiologically relevant $GABA_A$ receptor subunit assemblies, enabled by cryo-EM, came from three independent groups in 2018. These structures revealed the overall architecture of synaptic receptor subtypes: α1β1γ2 in complex with GABA at 3.8 Å overall resolution [27], α1β2γ2 in complex with GABA and flumazenil, a benzodiazepine site antagonist, at 3.9 Å resolution [28], and α1β3γ2 in complex with GABA at 5.2 Å resolution [29] (to be very clear on the nomenclature, each of these structures was of a pentameric receptor comprising two α1 subunits, two β subunits, and one γ2 subunit). Together, these studies provided information on neurotransmitter and benzodiazepine site architecture and revealed a complex network of N-linked glycans in the extracellular channel vestibule. The structures of the α1β1γ2 and α1β2γ2 receptors were at sufficiently high resolution to position amino acid side chains with confidence in the ECD. All these structures, obtained in detergents combined with different lipid and lipid-like additives, suffered from either a collapsed ion-conducting pore or disordered transmembrane domain (TMD) helices, in particular in the γ2 subunits. While it is tempting to chalk up these apparent TMD defects to physiologically irrelevant artifacts caused by mild detergents, it is worth noting that, to our knowledge, these results are unprecedented in the pLGIC superfamily. We suggest that,

while these TMD conformations may not occur physiologically, they hint that the αβγ $GABA_A$ receptor has a fundamentally more dynamic and sensitive TMD than other pentameric channels. This property of heightened TMD dynamics would logically have physiological consequences, and perhaps facilitates the modulation of this subtype by such a diversity of TMD-active drugs.

The next steps forward involved moving out of detergents into lipid **nanodiscs** and dramatically expanding the repertoire of structural pharmacology. In 2019, structures of the α1β3γ2 receptor, in lipid nanodiscs, were published in complex with GABA with and without the channel blocker picrotoxin, the **antagonist** bicuculline, and GABA plus two benzodiazepines, either alprazolam (Xanax) or diazepam (Valium) [30, 31]. GABA plus picrotoxin, somewhat surprisingly, stabilized a resting-state-like conformation of the receptor akin to that observed in the presence of bicuculline [30] (Box 2). The latter set of structures showed how the classical benzodiazepines bind, which could be compared with the earlier structure in complex with flumazenil [28], the benzodiazepine site antagonist. All agonist and benzodiazepine-bound structures adopted desensitized-like pore conformations, consistent with expectations from electrophysiology experiments [30, 32]. Important additional aspects in these structural studies included the use of full-length receptor constructs and exchanging receptor during purification out of detergent and into lipid nanodiscs. The finding that a well ordered TMD can be recovered upon nanodisc reconstitution suggests that the use of detergent in itself is not harmful; rather, detergents are problematic at the final stage of sample preparation for structural analysis of the αβγ $GABA_A$ receptor (Box 3). This work started to solidify the structural foundation for synaptic $GABA_A$ receptors with a focus on ligands that act mainly through the ECD; major unresolved topics included how general anesthetics work on $GABA_A$ receptors, as well as structure-based functional interrogation and receptor dynamics studies.

At the time of this this review, the most recent study to directly tackle $GABA_A$ receptor structural biology came from our group and sought to build off the lipid reconstitution approach in [31] to address several outstanding questions. First, where do representative general anesthetics bind? Second, can molecular dynamics (MD) simulations shed light on how classical benzodiazepines potentiate GABA activation of $GABA_A$ receptors, as well as clarify the more complex effects of flumazenil? Third, to address the curious result of receptor conformation observed previously in the presence of picrotoxin plus GABA, can a combination of electrophysiology and structural biology make sense of picrotoxin modulation of receptor conformation? And, lastly, how do these different drugs from similar and different classes differentially affect receptor conformation? We addressed these topics by obtaining eight individual α1β2γ2 receptor structures in brain lipid nanodiscs, in complex with different compounds, including GABA alone, GABA plus IV anesthetics (phenobarbital, etomidate and propofol), GABA plus two benzodiazepines (diazepam and flumazenil), and GABA plus picrotoxin, as well as with bicuculline alone [33]. MD and patch-clamp electrophysiology experiments complemented the structural biology. We integrate these findings with other recent structural biology and functional studies below, before considering what is missing and future areas in need of exploration.

$GABA_A$ receptor structural pharmacology

Neurotransmitter-binding site

There are two structurally equivalent and broadly agreed upon neurotransmitter-binding sites found at β-α subunit interfaces in the ECD (Figure 2A and Box 1) [34]. The α1β1γ2 structural study suggested an additional GABA site at an equivalent position in the α-β subunit interface; however this finding has not been supported by the more recent higher resolution structures [30, 31, 33]. The neurotransmitter pocket at β-α interfaces is defined by aromatic residues from discontinuous elements of both subunits, designated loops A-C for the principal (β) subunit and D-F for the complementary (α) subunit. These residues include α1F65, β2Y97, β2Y157, and β2Y205, which form a compact “aromatic box” for GABA binding [30, 33, 35]. The bound GABA forms a cation-π interaction with β2Y205 on loop C, electrostatic interactions with β2Y97 and β2E155 through its amine group, and the GABA carboxylate forms a salt bridge with α1R67 and a hydrogen bond with β2T202. These interactions contribute to GABA binding affinity [36].

Bicuculline is a plant-derived alkaloid convulsant that targets the same site as GABA and acts as a competitive antagonist [37]. Although bicuculline is bulkier than GABA, it fits well in the aromatic box (Figure 2B). However, in both the α1β2γ2 and α1β3γ2 complexes with bicuculline (PDB codes: 6X3S and 6HUK), the α1R67 side chain that formed a salt bridge with GABA rotates away from the membrane to accommodate the bulkier antagonist [30, 33]. αR120 that stabilizes loop C of the β subunit by hydrogen bonding to β2Y205 in the GABA complex rotates away, as well. The phthalide ring system of bicuculline faces loop C and forms π-stacking interactions with β2F200, which stabilizes loop C in a more open conformation, pushed approximately 4.5 Å away from the neurotransmitter site, compared to when GABA is bound. This opening of loop C allows a twist within the β sandwich of the β-subunit ECD that propagates conformational changes in the β1-β2, the Cys and β8-β9 loops and pre-M1 linker, which eventually leads to the M2-M3 loop repositioning to transition from an agonist-bound desensitized conformation to a resting-like, closed-channel state of the receptor.

Benzodiazepine sites

There are four available cryo-EM structures of the $GABA_A$ receptor, in a lipidic environment, in complex with GABA plus benzodiazepines [30, 33]. The GABA plus diazepam-bound α1β2γ2 and α1β3γ2 receptor structures are largely consistent in conformation but differ in the number of the TMD diazepam binding sites between them (PDB codes: 6X3X and 6HUP). The two other structures include alprazolam, a PAM like diazepam, bound to the α1β3γ2 receptor (PDB code: 6HUO), and the benzodiazepine antagonist flumazenil bound to the α1β2γ2 receptor (PDB code: 6X3U). All benzodiazepine-class drugs target the same site at the α-γ subunit interface in the ECD, and classical benzodiazepines have been shown to allosterically stabilize the bound GABA at the β-α interfaces, a mechanism probed by binding assays, electrophysiology and MD simulations [33, 38, 39]. The α-γ ECD locus is known as the high-affinity benzodiazepine site, and consists of several aromatic residues including F100, H102, Y160, and Y210 in the α1 subunit and Y58 and F77 in the γ2 subunit, analogous to the neurotransmitter-binding

site (Figure 2C, 2D). Z-drugs, which are non-benzodiazepine sedatives, also target this site [4, 6].

A key binding determinant for traditional benzodiazepines like diazepam is a histidine that is conserved among α1, α2, α3, and α5 subunits [5]. This histidine (H102 in human α1) is positioned to form a hydrogen bond with the chlorine atom of diazepam and alprazolam, which have the same binding mode in the pocket. Mutagenesis studies revealed that replacing this histidine with an arginine residue found in the α4 and α6 subunits diminished the binding of classical benzodiazepines [40]. In contrast, the benzodiazepine antagonist flumazenil does bind with high affinity to receptors containing α4 and α6 subunits [41]. Flumazenil has a completely different binding mode compared to diazepam [28, 30, 33], even though it contains the benzodiazepine core and halobenzene substituent. The superposition of the two different benzodiazepine bound structures highlights the differences in the chemical structure of these two drug molecules. While diazepam adopts a more three-dimensional structure in solution, flumazenil is more planar (Figure 2C, 2D) [28, 30, 33]. This difference may be the reason why the replacement of histidine with arginine does not affect flumazenil binding, or that of other similarly planar benzodiazepines.

Additional lower affinity diazepam binding sites in the TMD were suggested by earlier electrophysiology experiments and were mapped in the recent structures [42]. The α1β3γ2 receptor structure uncovered two equivalent sites at the TMD β-α interfaces [30], while the α1β2γ2 receptor structure identified those as well as an additional site at the TMD γ-β interface [33] (Figure 3A). Interestingly, the diazepam bound at the γ-β interface is an enantiomer of that in the β-α interfaces (Figure 3B, 3C). This finding suggests that adding a chiral center to the diazepine ring to prevent the enantiomeric conversion in solution would generate two stereoisomers: one selective for the single γ-β interface, and the other selective for the pair of β-α interfaces. This observation of diazepam binding to a combination of ECD and TMD sites occurs for multiple classes of modulators [43]. It is not immediately clear why diazepam is observed in the TMD γ-β interface in the α1β2γ2 structure but not in the α1β3γ2 structure. One explanation stems from differences in sample preparation: a 2-fold higher concentration of diazepam was used to obtain the α1β2γ2 structure (200 μM versus 100 μM), and diazepam was included throughout the purification of the α1β2γ2 receptor but added just at the end for the α1β3γ2 structure. A second possible explanation is that subunit interfaces are more tightly packed overall in the α1β3γ2 structures, which may stem from delipidation during exchange into lipid nanodiscs [33]. There are additional differences in the sample preparations including the expression constructs (Box 2), however these construct modifications were not found to have an effect on drug response or on subunit conformations [33].

After determining the structure of the receptor bound by GABA alone, with GABA plus diazepam, or with GABA plus flumazenil, we observed that diazepam binding potently stabilized the receptor, while flumazenil had the opposite effect [33]. Briefly, the GABA and diazepam-bound structure reached the highest resolution and had the most stable TMD. In contrast, the GABA and flumazenil-bound and GABA alone complexes exhibited less stable TMDs. In the flumazenil complex, a gap is present at the γ-β interface in the TMD. The size of this gap becomes gradually smaller in the order of the flumazenil-bound > GABA alone >

diazepam-bound complexes. The relative stability of the γ-TMD correlates with the tightness of the γ-β interface in this series, and moreover the γ-TMD is the most dynamic component of all αβγ $GABA_A$ receptor structures (assessed by relative atomic B factors and local map resolution). This combination of locally high flexibility in the γ-TMD and its sensitivity to drug binding is consistent with recently proposed asymmetric contributions of the γ2 subunit to $GABA_A$ receptor desensitization [44].

Flumazenil is commonly referred to as an inert benzodiazepine antagonist and is used clinically as an antidote for benzodiazepine overdose. However, the activity of flumazenil varies as a function of receptor subunit composition, in some cases acting at high doses as either a positive or a **negative allosteric modulator (NAM)** [45]. Its effects are thus more complex than its binding having no effect on receptor conformation. Furthermore, flumazenil has been used to hasten reversal of anesthesia induced by non-benzodiazepine class drugs, such as propofol or isoflurane, which only target the TMD [46, 47]. This anesthetic reversal cannot be explained by simple competitive antagonism at the α-γ ECD benzodiazepine site. MD simulations together with the recent structural analysis suggested that flumazenil binding at the ECD benzodiazepine site of α1β2γ2 destabilizes the receptor ECD and TMD [33]. In MD simulations, placement of flumazenil at the α-γ site allosterically destabilized the bound GABA at the β-α sites. In contrast, diazepam stabilized the bound GABA, consistent with its PAM activity. Moreover, flumazenil binding at the ECD site also destabilized the binding of TMD modulators, in particular at the γ-β interface. The combined early and recent studies thus suggest that flumazenil globally destabilizes the receptor with consequences on TMD conformation and drug binding, while diazepam stabilizes the receptor and thereby potentiates GABA activation.

We and others have assigned agonist plus modulator-bound structures to desensitized states based on electrophysiological experiments and the observation that the ‘desensitization gate’ at the 2’ position is closed. Do the structures inform on how the modulators potentiate channel activity? Earlier functional studies suggested that diazepam enhances agonist activity by increasing agonist affinity [48, 49]. However, this mechanism became difficult to rationalize after finding that the response to saturating concentrations of partial agonists could also be potentiated by diazepam [50-52]. It is currently thought that diazepam potentiates receptor activation by agonists through increasing occupation of a pre-activated “flipped” or “primed” conformation [53-55]. The diazepam-bound α1β2γ2 receptor structures revealed a pore expansion at the 9’ activation gate, which could intuitively represent a shift away from a resting or a desensitized state toward an open channel state. The pre-active states are very short lived [53, 54] and not conceptually straightforward to trap at equilibrium for structural analysis. We favor a conclusion that the diazepam-bound structure, and moreover all the GABA plus PAM bound structures represent potentiated desensitized states.

Anesthetic binding sites in the TMD

IV anesthetics, such as barbiturates, propofol, and etomidate, target the TMD of the $GABA_A$ receptor and potentiate GABA activation [56-58]. High concentrations of these drugs can directly activate the receptor in the absence of GABA [59, 60]. Photochemical labeling

[61-63] and mutagenesis experiments [64-66], together with mouse knock-in studies [21, 22], indicated binding sites for IV anesthetics at subunit interfaces in the TMD. There are five homologous cavities at these TMD subunit interfaces that can potentially be targeted by small anesthetic molecules. These cavities result from a short **π-helix** in M1 that creates a bulge, which in turn creates a pocket of space at its interface with M3 from the adjacent subunit. The recent structural studies on αβγ receptors build off the mutagenesis, labeling, and animal work to better define which benzodiazepine and anesthetic drugs bind in which of these cavities, what the specific interactions are, and which conformational states of the receptor they stabilize [30, 33].

We recently reported three anesthetic-bound α1β2γ2 receptor structures in complex with phenobarbital, etomidate, and propofol in lipidic nanodiscs (PDB codes: 6X3W, 6X3V, and 6X3T) [33]. All three compounds bind in the predicted TMD interfacial cavities, in specific interfaces shared in part with those found for diazepam (Figure 3A). Two phenobarbital molecules were observed, one at the γ-β and the other at the α-β interface. While the complementary β subunit provides the same mainly hydrophobic interactions to each of the two bound phenobarbitals, the principal sides of the two sites are different, although the M2 15’ position serine residue is conserved in both pockets (Figure 3D, 3E). Besides the hydrophobic interactions to phenobarbital, a barbituric acid nitrogen is positioned to form a hydrogen bond with the backbone carbonyl oxygen of β2L223. Interestingly, no barbiturate molecules were observed at the two β-α and single α-γ interfaces. This finding is superficially inconsistent with the β3 knock-in study where a single mutation of asparagine at the 15’ position of the β3 subunit to methionine caused a partial loss of pentobarbital response [67]. Interpretation of mutagenesis results from this cavity must be interpreted with caution, however, as the mutations are known to, in the absence of modulators, alter GABA potency [66, 68]. More broadly, β3 15’ could form part of a barbiturate site in a subunit assembly other than α1β2γ2.

Etomidate and propofol, which were developed more recently than phenobarbital, were only observed to bind at the β-α TMD cavities in the $GABA_A$ receptor complex structures (Figure 3F, 3G) [33]. Distinctive features in the β-α site, compared to other sites, include the presence of an asparagine residue (β2N265) at the M2 15’ position and a methionine residue (β2M286) on the M3 helix of the β subunit that reaches across the subunit interface. The subunit interfaces involved in binding of etomidate and propofol are shared, but the receptor conformations, in particular the M2 orientations, are distinct. The etomidate imidazole ring sits between α1P233 and β2F289, while its phenyl ring points toward the ECD site almost perpendicular to the cell membrane (Figure 3F). This phenyl ring likely forms a π-electron mediated interaction with β2N265, consistent with mutation of β2N265M resulting in a complete loss of potentiation by etomidate [64]. Affinity labeling and further mutagenesis studies are consistent with the localization and binding pose of etomidate [22, 61, 64].

Propofol is the smallest anesthetic examined and binds to the same interfacial cavities as etomidate; however, its binding mode is completely different [33]. Propofol’s phenyl ring is sandwiched between β2M286 and β2T262, oriented with its face parallel to the membrane normal (Figure 3G). The hydroxyl moiety forms a hydrogen bond with the backbone carbonyl oxygen of α1I228 in the M1 helix, which is important for the high affinity of

propofol. Both isopropyl groups are wedged tightly between the β2N265 (M2) and β2F289 (M3) residues. This interaction stabilizes M2 in a more extended conformation, resulting in a shifting up of β2N265, toward the ECD, approximately 2 Å (Cα) compared to that in the etomidate-bound model (Figure 4A, 4B). Moreover, the M2 helix rotates in a clockwise direction near the propofol binding site (viewed from the synapse), which may be stabilized by the tight interactions between the sandwiched phenyl ring and β2M286 and β2T262 residues. Together, these changes position the 9'Leu residue approximately 2 Å deeper in the subunit interface, away from the channel lumen, compared to the complexes with phenobarbital and etomidate. This distinct M2 helix conformation explains why the propofol-bound structure exhibits such a wide pore diameter at the 9' activation gate (Figure 4C, 4D).

While all anesthetic-bound structures exhibit a desensitized-like conformation with a closed desensitization gate at the −2' position, they show variable expansions of the ion channel at the 9' activation gate (Figure 4D). The pore diameter analyzed by Hole2 [69] is the largest (over 10 Å) in the propofol-bound model, although the diameter in the etomidate and barbiturate models also exhibit expansion at this 9'Leu position (~7-9 Å in diameter), compared to that in the GABA alone bound model (4.6 Å) [33]. Anesthetics are known to increase the open probability of the $GABA_A$ receptor to potentiate GABA activation [70, 71]. These three anesthetic-bound complexes illustrate how these anesthetics mechanistically contribute to the opening of the channel by stabilizing the activation gate in a widely open conformation.

Etomidate and propofol bind in the β-α pockets, while phenobarbital binds at two different interfaces, in the γ-β and α-β pockets. This distinction raises the topic of the selectivity of each binding cavity. IV anesthetics at high concentrations can directly activate the $GABA_A$ receptor. Does this mean that they could bind to cavities other than those we have observed structurally? Barbiturates have a notoriously narrow therapeutic index compared to etomidate and propofol; their effective dose is near their lethal dose, and varies among patients, thus requiring careful patient monitoring. Compared to the two more recently developed anesthetics, which only bind specifically at the β-α pockets, phenobarbital molecules were observed in two different interfaces, suggesting their selectivity is less strict. This lower selectivity among sites may contribute to its narrow therapeutic index compared to propofol and etomidate, which, while they have their own risk profiles, are generally much safer for use in anesthesia [72]. The results further highlight that each TMD cavity has different drug selectivity, and therefore should be able to be targeted specifically. This set of anesthetic-bound structures, in concert with the enantiomeric conversion observed for diazepam, provide a foundation for design of TMD modulators with improved selectivity.

Recent structures provide touchstones in the gating cycle of $GABA_A$ receptors

The gating mechanism of $GABA_A$ receptors is incompletely mapped at the level of 3D structure, with missing pieces inferred from work on other Cys-loop family receptors, especially glycine and 5-HT_3 homomeric receptors [73-76]. A simplified view of gating

includes a resting, closed-channel state, an agonist-bound, activated, open-channel state, and an agonist-bound, desensitized, closed-channel state [3, 77]. Detailed analysis of single channel records reveals more complexity, including pre-active states and multiple kinetically distinct desensitized states [54, 78]. At the level of structure, the gating of $GABA_A$ receptors is explained by a two-gate model shared with other Cys-loop superfamily proteins [77, 79]. Transition from resting to an activated state involves opening the activation gate, which always includes the 9'Leu residues from the M2 pore-lining helix. Desensitization, in contrast, involves closure of the intracellular mouth of the pore, forming a desensitization gate near the 2', and −2' (or −1' in cationic channels) positions on the M2 helices. In $GABA_A$ receptors, both gates are largely hydrophobic, while in cation-selective 5-HT_3 and nicotinic acetylcholine receptors, the desensitization gate is formed by polar side chains [80, 81].

No open-state structure for a $GABA_A$ receptor has been reported yet, and as such, channel activation and desensitization mechanisms remain speculative. We can however summarize observations from recently published structures. Bicuculline [30] and its quaternary derivative [33] were used to obtain structures likely representative of resting states (PDB codes: 6HUK and 6X3S, respectively); these show nearly identical ligand poses and overall structures. In the resting-like state, loop C at the orthosteric GABA binding site is propped open by the antagonist, which results in an expanded ECD conformation. The M2 helices orient normal to the membrane plane and pack tightly together, with both activation and desensitization gates closed. The 9'Leu residues face the channel lumen, which creates a hydrophobic barrier for ion permeation. In contrast, upon GABA binding, loop C packs down around the bound agonist. This change transmits to the TMD through the coupling loops located at the ECD-TMD junction, which include the β1-β2, Cys, β8-β9, pre-M1 and M2-M3 loops. The recent structure in complex with GABA alone is likely representative of a slow desensitized state, where the −2' desensitization gate at the intracellular side end of the M2 helices is closed and the 9' activation gate is partially open [33]. The upper pore expansion increases in the presence of IV anesthetics, where the M2 helices rotate more compared to the GABA alone complex structure, resulting in the 9'Leu residues rotating away from the pore axis and toward adjacent subunits. While the desensitization gate remains closed in the GABA plus anesthetic complexes, this opening of the activation gate may serve to lower the barrier to subsequent activation. The rotation of M2 thus may relate to the mechanism by which the anesthetics increase channel open probability (Figure 4D). It is noteworthy that each ligand or ligand combination stabilizes receptor conformations that distinctively affect access to the TMD interfacial cavities.

Concluding remarks

Recent progress in structural biology of the heteromeric $GABA_A$ receptor has resolved several fundamental questions in biochemical and pharmacological properties of the receptor, including the lipid sensitive TMD with an especially flexible γ-TMD, and has provided detailed views of how GABA, modulators and antagonists bind and affect receptor conformation. Many pieces remain missing that structural biology, at least in part, can address (see also Outstanding Questions). First, an open state structure of the receptor is necessary to architecturally map the basic gating cycle of the $GABA_A$ receptor. Second,

evidence for additional native subunit assemblies is continually being discovered [82], however all structural information available is limited to the α1β(x)γ2 synaptic receptors. Structural information on additional receptor subtypes is needed to help answer why distinct receptor subunit assemblies exhibit different pharmacological and physiological properties. Third, densities presumed to be ordered lipids have been observed in the recent receptor structures, but biophysical and physiological elucidation of their roles remains speculative and unexplored. Lastly, the distribution and proportion of $GABA_A$ receptor subtypes in the brain is poorly understood, and recombinant approaches may not accurately define the predominant native assemblies. Functional approaches can report well on how specific subunits contribute to specific synapses and circuits [83], but the absolute subunit compositions of native receptors remain ill-defined. Development of new approaches to map regional and circuit specific $GABA_A$ receptor assemblies will be essential to understand how combined receptor ensembles and their modulation by drugs integrate to tune brain function.

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Glossary:

Agonist
a molecule that binds to a receptor to stimulate a physiological response.

Antagonist
a molecule that binds to a receptor to prevent the agonist from stimulating a physiological response. The inhibition of activity can be achieved through direct competition with agonist or through allosteric inhibition.

Benzodiazepine
a class of psychoactive drugs whose core structure includes benzene fused to a diazepine ring (7-membered ring comprising two nitrogens). Classical benzodiazepines, including diazepam (Valium) and alprazolam (Xanax), are positive allosteric modulators of $GABA_A$ receptors.

Cys-loop receptor
a superfamily of pentameric ligand-gated ion channels that in mammals includes anion-selective $GABA_A$ and glycine receptors, and cation-selective nicotinic acetylcholine and 5-HT_3 serotonin receptors.

Desensitization
a biophysical process observed in most ligand-gated ion channels where in the sustained presence of agonist, the ion channels becomes non-conductive and refractory to further stimulation.

γ-aminobutyric acid (GABA)

an amino acid that functions as the main inhibitory neurotransmitter in the brain via stimulation of GABA receptors. $GABA_A$ receptors are ion channels and $GABA_B$ receptors are heterodimeric 7-TM G protein-coupled receptors.

$GABA_A$ receptor
a Cys-loop receptor family member that is activated by the neurotransmitter GABA. $GABA_A$ receptors assemble physiologically as heteropentamers from a panel of 19 mammalian subunits, with pharmacology, localization and channel biophysics dependent upon subunit composition.

General anesthetic
a drug class that causes sedation at low dose and a loss of consciousness at higher doses. These drugs are generally divided into volatile (inhalational) anesthetics and intravenous (IV) anesthetics. Inhalational and IV anesthetics are often used in combination to induce and maintain anesthesia during surgery.

Nanodisc
a combination of an amphipathic membrane scaffold molecule (usually a protein) and lipids that can stabilize membrane proteins in an environment that better mimics the cell membrane than detergents.

Negative allosteric modulator (NAM)
a molecule that binds to a receptor in a site distinct from where agonists bind and interferes with the ability of agonists to stimulate the receptor.

pLGIC
Pentameric ligand-gated ion channel is a more inclusive name for the superfamily of channels that includes the Cys-loop receptors as well as bacterial orthologs that lack the hallmark cysteines in the Cys-loop.

Positive allosteric modulator (PAM)
a molecule that binds to a receptor in a site distinct from where agonists bind and potentiates the ability of agonists to stimulate the receptor. Many PAMs can directly activate the receptor at high concentrations; these are called allosteric agonists or ago-PAMs.

π-helix
a type of secondary structure found in proteins. It is a less tightly wound helical conformation than observed in the common α-helix. In an α-helix, the backbone amide N-H forms hydrogen bonds with the backbone carbonyl O at the n+4 position. In π-helices the bonding occurs at the n+5 position.

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Box 1

General receptor architecture

The $GABA_A$ receptor adopts an overall cylindrical shape, with five subunits arranged in a pseudo five-fold symmetric manner (Figure I). The subunits in a classical synaptic receptor are ordered β-α-β-α-γ in a counter-clockwise fashion around the central permeation pathway, from the perspective of outside the cell looking in. Each subunit shares a conserved domain organization, at least in the extracellular domain (ECD) and transmembrane domain (TMD), with other **Cys-loop receptors** and homologous prokaryotic pentameric ligand-gated ion channels (pLGIC). In addition to $GABA_A$ receptors, this superfamily includes the homologous glycine receptors, nicotinic acetylcholine receptors, serotonin ($5\text{-}HT_3$) receptors, the Zn^{2+}-activated ion channel (ZAC), and several receptors found specifically in invertebrates and in prokaryotes [3]. In the common heteromeric $GABA_A$ receptors, GABA generally binds to β-α interfaces in the ECD; in the homomeric $GABA_A$-ρ receptors, GABA binds at ρ-ρ interfaces. The TMD contains the anion channel. The intracellular domain (ICD) is poorly conserved among Cys-loop receptors as well as within the $GABA_A$ receptor branch. Cation-selective Cys-loop receptors (nicotinic acetylcholine and $5\text{-}HT_3$ receptors) have conserved and structurally ordered helices in the ICD that play roles in ion permeation. Anion-selective Cys-loop receptors ($GABA_A$ and glycine receptors), in contrast, have fewer predicted or as-yet observed secondary structural features in the ICD [74].

The ECD comprises an N-terminal α-helix followed by a sandwich of ten β strands. The signature Cys-loop necessary for transducing the neurotransmitter binding signal to the TMD sits between the β6 and β7 strands, in a hinge region connecting the ECD to TMD. The TMD is composed of four transmembrane α helices (M1-M4), with the M2 helices from each of the 5 subunits lining the ion-conducting pore. The M2 helices are responsible for two gates that block ion flux: an activation gate near the midpoint of the pore and a **desensitization** gate near the cytosolic mouth of the pore. The loop between the M2 and M3 helices directly interacts with the Cys-loop and the β1-β2 loop, and forms a major bridge linking ECD and TMD conformations. The TMD also contains small cavities within the subunit interfaces created by a conserved proline residue in the first half of the M1 helix of all five subunits. These cavities allow the binding of chemically diverse small molecule anesthetics [84]. The ICD lies in primary sequence between the M3 and M4 TMD helices, and is known to be important for tuning channel kinetics as well as mediating interactions with synaptic anchoring proteins such as gephyrin [85, 86], GABARAP [87, 88], and LH4 [89, 90], and other cellular proteins including kinases [91].

Box 2

Picrotoxin

Picrotoxin, or more precisely its active component picrotoxinin, is a poisonous convulsant from the *Anamirta cocculus* plant. This alkaloid toxin is a channel blocker of anionic Cys-loop receptors; it has been used historically to characterize channel physiology, as an antidote for barbiturate overdose, and more recently to assign physiological states to receptor structures [79, 92]. There are now several pairs of α1β2γ2 and α1β3γ2 structures in lipid nanodiscs bound to the same or very similar compounds; most of these structures are consistent in ligand position and receptor conformation, even though the α1β2γ2 structures have truncated ICDs [30, 33].

The picrotoxin complexes are a curious exception [33]. Expectations from electrophysiology experiments are as follows. Activation of the receptor by GABA or another agonist facilitates picrotoxin binding; picrotoxin can also bind to spontaneously activated receptors. Picrotoxin can then be trapped in a closed pore [93]. Dissociation of picrotoxin requires channel re-opening. Thus, in the presence of saturating GABA, picrotoxin is expected to stabilize an open state, or a desensitized state, or something intermediate, but not a resting state. In the absence of GABA, picrotoxin, trapped in a closed pore, is thought to stabilize a resting-like state [77, 79]. The α1β2γ2 receptor structure bound to GABA and picrotoxin (PDB code: 6X40) adopts a conformation that appears intermediate: the extracellular domain (ECD) adopts a conformation identical to that when only GABA is bound, while the TMD adopts a conformation with the resting gate more open than in the antagonist bound state, and the desensitization gate closed [33]. These structural observations are consistent with the historical physiology as well as voltage-clamp fluorometry experiments showing that picrotoxin does not affect the conformation of the ECD [94].

In contrast, the α1β3γ2 receptor structure in the presence of GABA plus picrotoxin (PDB code: 6HUJ), and with picrotoxin alone (PDB code: 6HUG), adopt an overall resting-like conformation, nearly identical to the bicuculline complex, varying only locally in the position of Loop C [30]. These strikingly different findings lack a clear physiological explanation. Based on the structural comparisons, as well as principal component analyses, molecular dynamic (MD) simulations, new electrophysiology experiments, and pairwise superpositions of benzodiazepine and antagonist-bound structures, we proposed the hypothesis that delipidation during sample preparation, and subsequent condensation of the nanodisc belt, constrains the TMD conformations observed in the α1β3γ2 receptor structures [30]. Consistent with this hypothesis, all matched complexes have narrower ion conducting pores in the α1β3γ2 structures, compared to the α1β2γ2 receptor structures [30, 33]. The differences are systematic but subtle, except in the case of the picrotoxin complexes.

Box 3

Challenges for $GABA_A$ receptors in structural biology

Beyond obtaining sufficient sample amount, purity, and biochemical quality, there are additional challenges to consider for $GABA_A$ analysis by single particle cryo-electron microscopy (EM). Pseudosymmetry like that observed in the $GABA_A$ receptor presents a problem when aligning particles; a feature visible at low resolution is needed to properly register the subunits, or alignment will fail and only low resolution, symmetry-averaged maps will be obtained. To date, all heteromeric $GABA_A$ receptor structural biology studies have used antibody-like fragments, Fabs or an engineered variant [27-31, 33, 95], to break the pseudosymmetry.

A second challenge in studying Cys-loop receptor structural biology by cryo-EM arises from an orientation bias in the vitreous ice. To our knowledge, all Cys-loop receptors, in the absence of detergent, exhibit a severe orientation bias in the holes of EM grids, with their channel axes oriented normal to the air-water interface. This preferred orientation prevents high-resolution 3D reconstruction without tilting the grid. Two methods have been used in the field to achieve random orientations of the receptor. Adding small amounts of fluorinated detergents right before freezing grids works, and does not appear to have an effect on receptor conformation [96-98]; however, addition of any detergent results in the vast majority of the receptor molecules sticking to the carbon grid material and avoiding the imaging holes. Increasing the protein concentration into the crystallization regime (4-10 mg/ml) overcomes this problem, and this approach was used for structures of the α1β2γ2 receptor [33]. The second successful approach was used for the α1β3γ2 receptor structures , and involved binding of a single copy of an α1 subunit-specific nanobody that was enlarged by fusion to a scaffolding protein to generate a megabody [99]. A concern with all binding partners like Fabs and megabodies is an effect on protein conformation and/or function; both the megabody (for α1β3γ2) and the Fabs (for α1β2γ2 and α1β1γ2) were found to be weak positive modulators [27, 28, 31]. Comparison of these two structures revealed no apparent conformational changes induced by the EM “chaperones,” providing confidence that their effects in this case are negligible.

Outstanding questions

- What is the structure of the receptor in an activated, open-channel state?
- How do specific lipids modulate the structure and function of the receptor?
- How do the receptor structures and structure-based mechanisms of drug modulation differ among synaptic and extrasynaptic receptor subtypes?
- How do the native receptor structures differ from those obtained in recombinant preparations?
- What are the architectural details of larger $GABA_A$ receptor signaling complexes, at and outside of synapses?
- What are the distributions and proportions of specific $GABA_A$ receptor subunit assemblies in different regions of the brain?

Highlights

- $GABA_A$ receptors are ion channels important in brain function and are the target of chemically diverse and clinically important drugs for insomnia, epilepsy and anesthesia.
- Recent structures of $GABA_A$ receptors have revealed detailed interactions of the neurotransmitter GABA, benzodiazepines, general anesthetics, and antagonists.
- Intravenous anesthetics bind in both common and distinctive membrane sites.
- Electrophysiological and molecular dynamics studies build off the static structures to probe mechanisms of potentiation and inhibition by chemically diverse compounds.

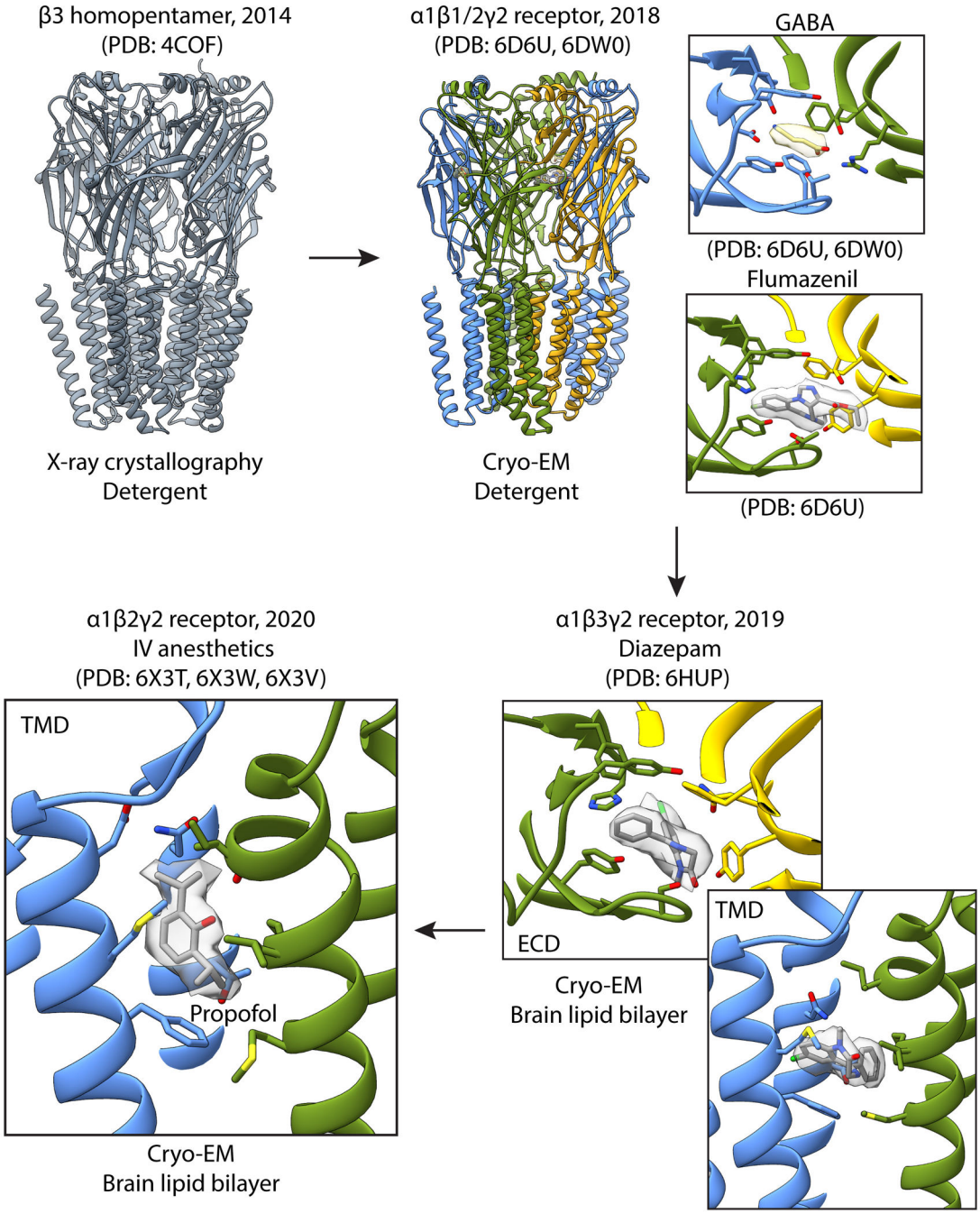

Figure 1: Historical progression of $GABA_A$ receptor structural biology.

Top left, side view of the X-ray structure of the β3 homomer (PDB code: 4COF). Top right, side view of the cryo-EM structure of the α1β2γ2 heteropentameric receptor and the binding sites for GABA (top) and flumazenil (bottom) (PDB code: 6D6U). Bottom right, the extracellular domain (ECD) and transmembrane domain (TMD) binding sites for diazepam in the α1β3γ2 receptor (PDB code: 6HUP). Bottom left, the binding site for propofol in the α1β2γ2 receptor (PDB code: 6X3T).

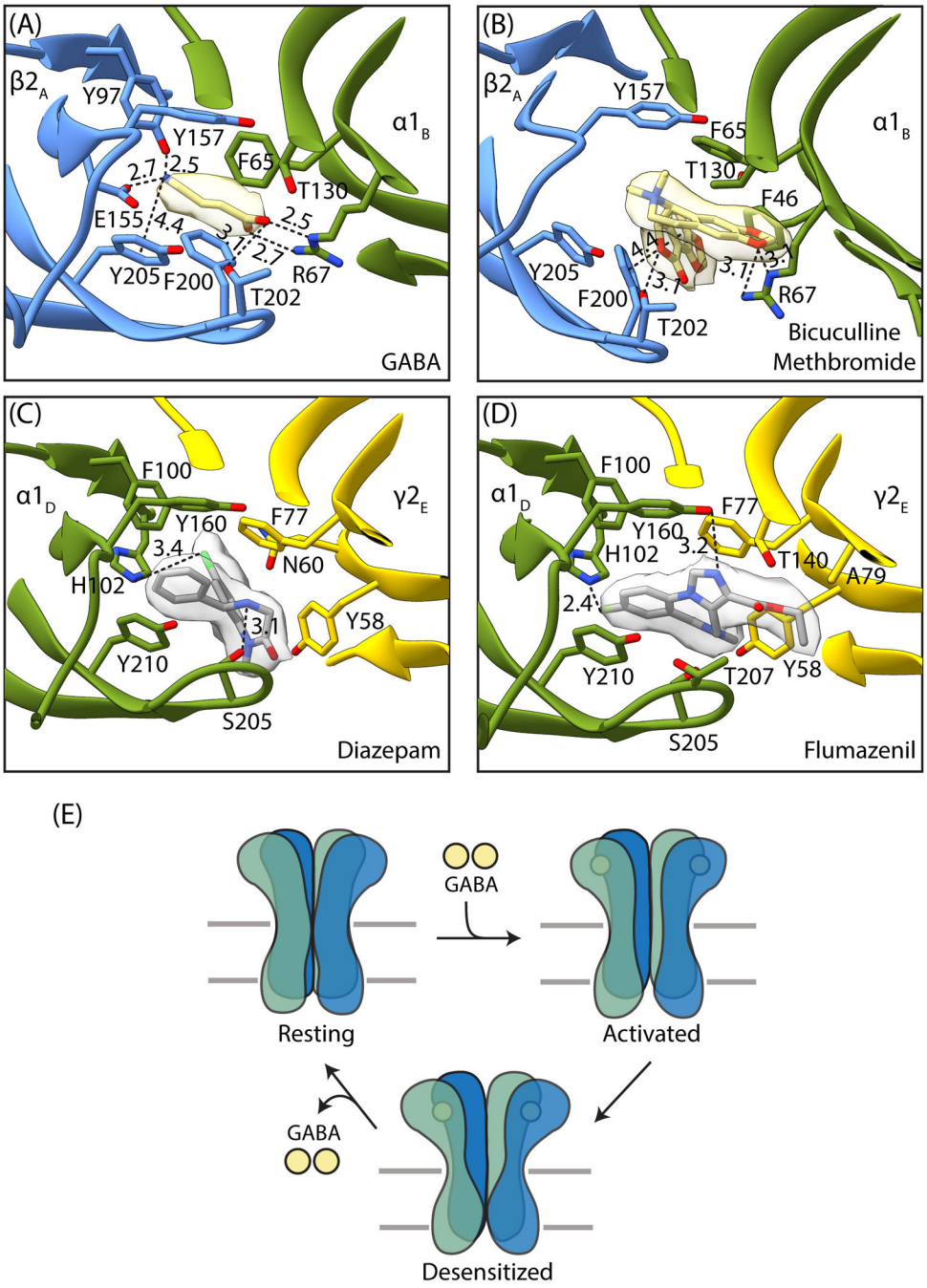

Figure 2: Neurotransmitter and benzodiazepine binding sites in the ECD.

Panels A and B show the orthosteric neurotransmitter binding sites with the bound GABA (PDB code: 6X3Z) and bicuculline methbromide (PDB code: 6X3S), respectively. Panels C and D show the bound diazepam (PDB code: 6X3X) and flumazenil (PDB code: 6X3U), respectively, in the high affinity benzodiazepine site. Semitransparent surface is the experimental density map for the ligand. Panel E presents a cartoon schematic of the three principal conformational states of the receptor during its gating cycle.

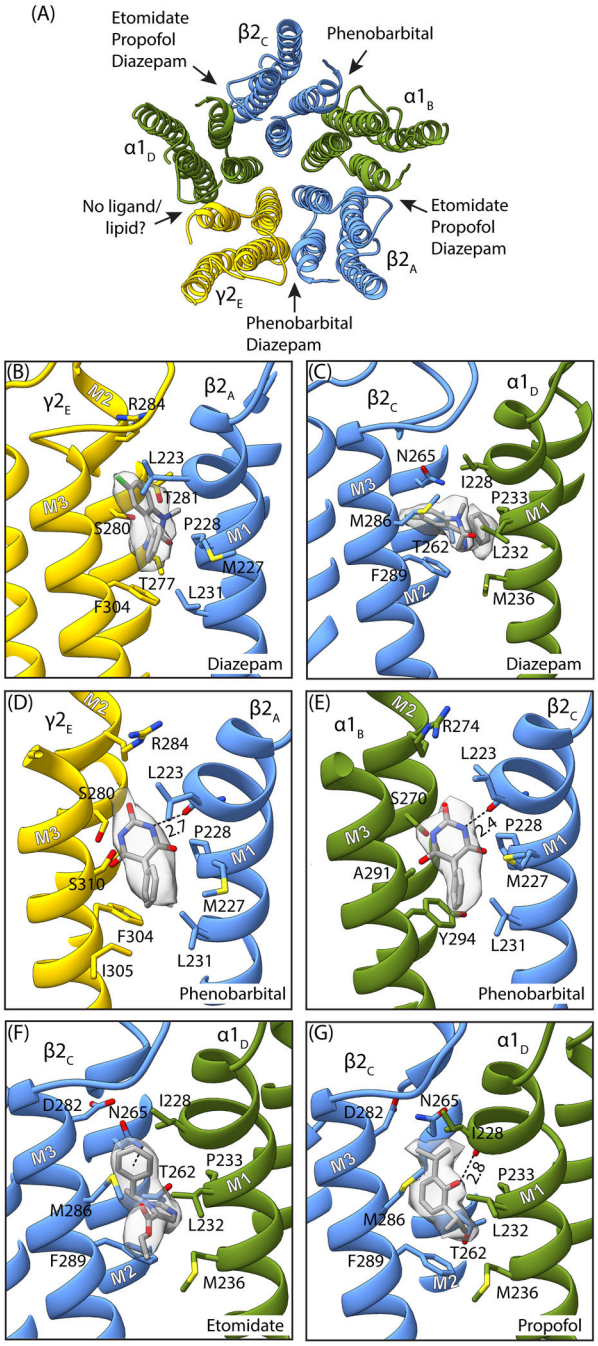

Figure 3: Anesthetic and benzodiazepine binding sites in the TMD.

Panel A, synaptic perspective of the transmembrane domain (TMD) showing an overview of where different drugs were found to bind in the α1β2γ2 receptor structures. Panels B and C show the two distinct binding sites of TMD binding sites and ligand conformation for diazepam (PDB code: 6X3X). Panels D and E show the two binding sites identified for phenobarbital (PDB code: 6X3W). Panels F and G show representative binding sites for the IV anesthetics etomidate and propofol (PDB code: 6X3V and 6X3T), respectively.

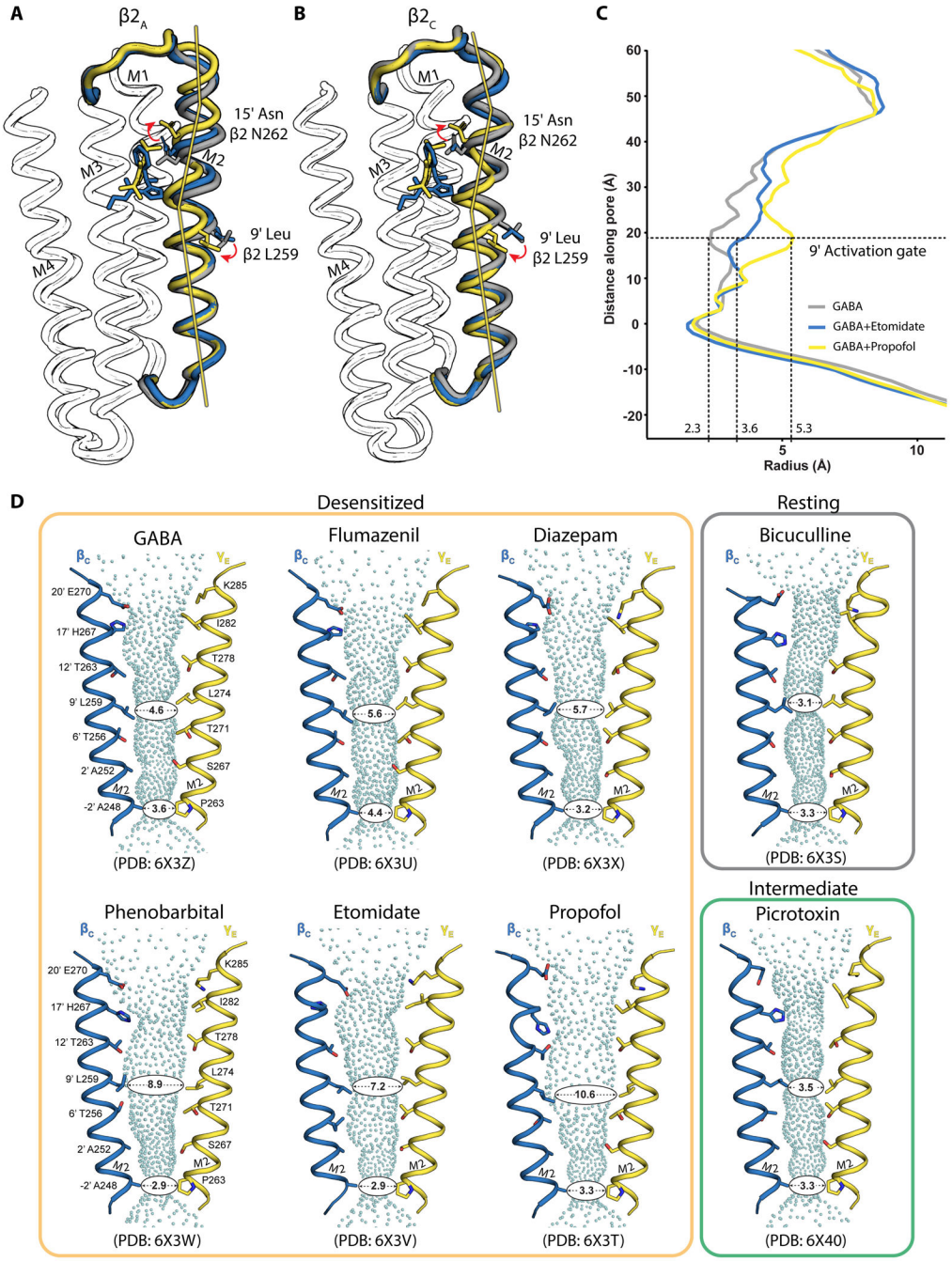

Figure 4: TMD pore profiles for recent structures of the α1β2γ2 receptor.

Panels A and B show the two binding sites for propofol (yellow) and etomidate (blue) in the context of the 4-helix bundle from individual β2 subunits. The emphasis is on how the conformation of the M2 helix differs among the GABA alone (grey), GABA + propofol, and GABA + etomidate structures. Panel C shows how pore diameter varies along the pseudo-5-fold channel axis: all three structures have a closed desensitization gate at the bottom of the pore, and variable constrictions at the 9' activation gate. Panel D shows a pair of opposing M2 α-helices for each ligand complex; all structures include GABA bound except for

bicuculline. Blue-green spheres and diameters illustrate shape of permeation pathway analyzed by HOLE (modified from Kim *et al.* 2020 [33]).

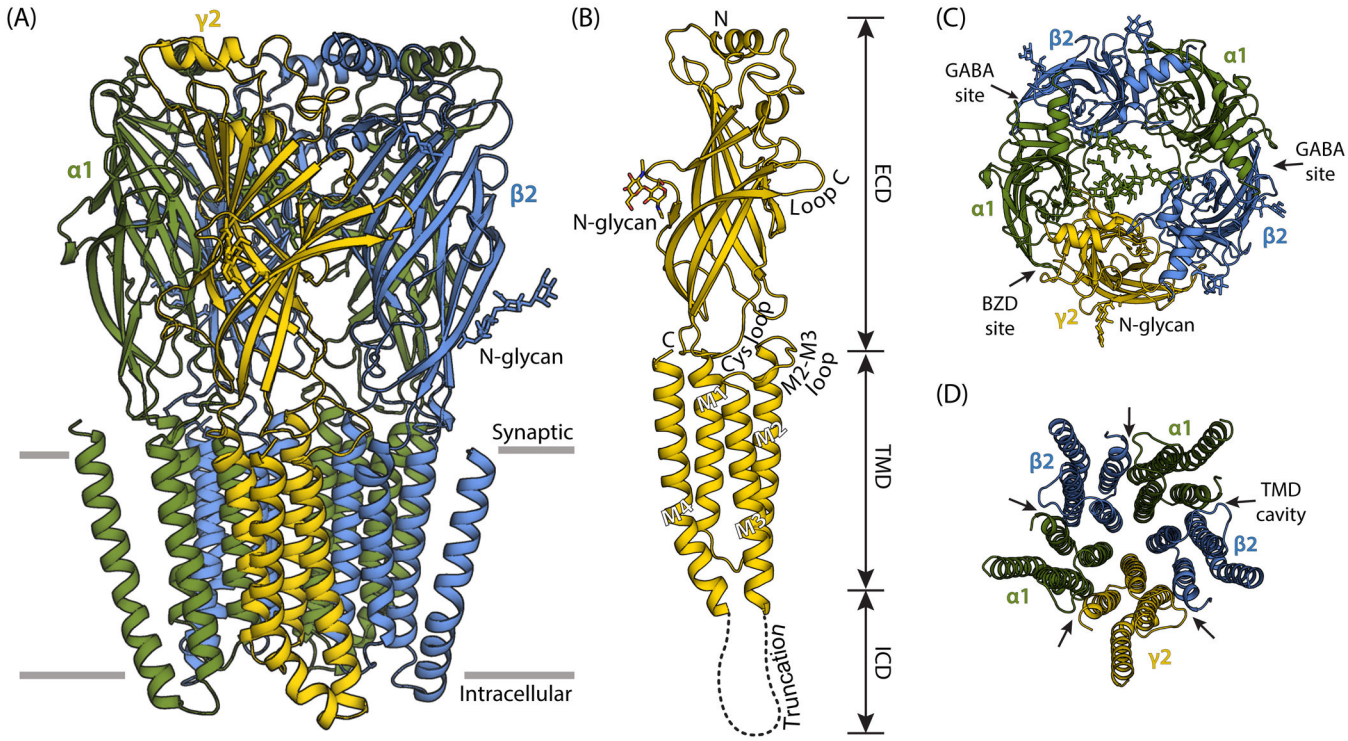

Figure I: General architecture of a synaptic $GABA_A$ receptor.

Panel A, side view of the α1β2γ2 subunit assembly in complex with GABA (PDB code: 6X3Z). Panels B and C show the same structure from the perspective of the synapse of the extracellular domain (ECD) and transmembrane domain (TMD), respectively, highlighting binding sites for different small molecules.