Identification of the Functional Binding Site for the Convulsant Tetramethylenedisulfotetramine in the Pore of the \( \alpha_2\beta_3\gamma_2 \) GABA\(_{A}\) Receptor

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ABSTRACT

Tetramethylenedisulfotetramine (TETS) is a so-called “caged” convulsant that is responsible for thousands of accidental and malicious poisonings. Similar to the widely used GABA receptor type A (GABA\(_{A}\)) antagonist picrotoxin, TETS has been proposed to bind to the noncompetitive antagonist (NCA) site in the pore of the receptor channel. However, the TETS binding site has never been experimentally mapped, and we here set out to gain atomistic level insights into how TETS inhibits the human \( \alpha_2\beta_3\gamma_2 \) GABA\(_{A}\) receptor. Using the Rosetta molecular modeling suite, we generated three homology models of the \( \alpha_2\beta_3\gamma_2 \) receptor in the open, desensitized, and closed/resting state. Three different ligand-docking algorithms (RosettaLigand, Glide, and SwissDock) identified two possible TETS binding sites in the channel pore. Using a combination of site-directed mutagenesis, electrophysiology, and modeling to probe both sites, we demonstrate that TETS binds at the T6’ ring in the closed/resting-state model, in which it shows perfect space complementarity and forms hydrogen bonds or makes hydrophobic interactions with all five pore-lining threonine residues of the pentameric receptor. Mutating T6’ in either the \( \alpha_2 \) or \( \beta_3 \) subunit reduces the IC\(_{50}\) of TETS by \~700-fold in whole-cell patch-clamp experiments. TETS is thus interacting at the NCA site in the pore of the GABA\(_{A}\) receptor at a location that is overlapping but not identical to the picrotoxinin binding site.

SIGNIFICANCE STATEMENT

Our study identifies the binding site of the highly toxic convulsant tetramethylenedisulfotetramine (TETS), which is classified as a threat agent by the World Health Organization. Using a combination of homology modeling, ligand docking, site-directed mutagenesis, and electrophysiology, we show that TETS is binding at the same site as picrotoxinin at the so-called T6’ ring, wherein five threonine residues line the permeation pathway of the pentameric receptor channel.

Introduction

GABA receptor type A (GABA\(_{A}\)) receptors are pentameric ligand-gated chloride channels (Olsen and Sieghart, 2008) that are activated by GABA, the major inhibitory neurotransmitter in the mammalian and insect nervous system. GABA\(_{A}\) receptors constitute the targets of many widely used drugs as well as of major household and agricultural pesticides. GABA\(_{A}\) receptor agonists like barbiturates or positive allosteric modulators, as exemplified by the various typical and atypical benzodiazepines, reduce neuronal excitability and are widely used as anesthetics, anxiolytics, and anticonvulsants (Krall et al., 2015; Olsen, 2015). In contrast, compounds that inhibit GABA\(_{A}\) receptor functions increase neuronal firing. Depending on their species specificity, GABA\(_{A}\) antagonists accordingly either constitute useful insecticides like fipronil or afoxalaner (Casida and Durkin, 2015), or, if they inhibit mammalian GABA\(_{A}\) receptors like picrotoxinin or pentylene-tetrazol, induce seizures and, at higher concentrations, death.

The extremely toxic tetramethylenedisulfotetramine (TETS) has been known since the early 1940s and was initially used as an antimold agent for furniture. TETS was subsequently investigated both as a rodenticide and as a pesticide [for an extensive review see Lauková et al. (2020)]. However, since human intoxications resulting in death; seizures; and, if victims survived exposure, neurologic deficits and spontaneous recurrent seizures have repeatedly occurred, the World Health Organization therefore banned the use of TETS (Lauková et al., 2020). But, because of its ease of manufacture and its impressive effectivenes in killing rats and mice, TETS continues to be used as a rodenticide in China and presents a real exposure risk.

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ABBREVIATIONS: AUC, area under the curve; EBOB, 1-(4-ethynylphenyl)-4-n-propyl-2,6,7-trioxabicyclo[2.2.2]octane; EC\(_{90}\), 90% effective concentration; ECD, extracellular domain; EM, electron microscopy; GABA\(_{A}\), GABA receptor type A; NCA, noncompetitive antagonist; PCR, polymerase chain reaction; pdb, Protein Data Bank; RDL, resistant to dieldrin; REU, Rosetta energy unit; TBPS, tert-butylbicyclophosphorothionate; TETS, tetramethylenedisulfotetramine; TMD, transmembrane domain.
risk to humans and a viable threat agent for inciting a mass-casualty incident. Pharmacological experiments performed in the late 1950s demonstrating that sublethal doses of TETS could reverse pentobarbital-induced decreases in blood pressure and respiration in dogs (Haskell and Voss, 1957) suggested that TETS might be targeting GABA<sub>A</sub> receptors. This was later confirmed in binding assays. TETS, which is far more toxic to mice than to insects (Esser et al., 1991), has affinity for both mammalian GABA<sub>A</sub> receptors and the insect RDL receptor. Although the insect RDL receptor is a homopentamer consisting of five identical subunits, mammalian GABA<sub>A</sub> receptors are typically composed of two α, two β, and one γ or δ subunit assembled as a pentamer around a central chloride-conducting pore (Olsen and Sieghart, 2019; García-Nafría and Tate, 2020). Using three recently widely used drugs, such as the benzodiazepines (Masiulis et al., 2019), has affinity for both mammalian GABA<sub>A</sub> receptors and the insect RDL receptor. Although the insect RDL receptor is a homopentamer consisting of five identical subunits, mammalian GABA<sub>A</sub> receptors are typically composed of two α, two β, and one γ or δ subunit assembled as a pentamer around a central chloride-conducting pore (Olsen and Sieghart, 2008). In binding assays with rat brain synaptosomes, TETS displaces GABA<sub>A</sub> receptor blockers like [3H]-1-(4-ethylphenyl)-4-n-propyl-2,6,7-trioxabicyclo[2.2.2]octane (EOB) and [35S] TBPS (Esser et al., 1991; Zhao et al., 2014), most likely by binding to the noncompetitive antagonist (NCA) site in the GABA<sub>A</sub> receptor pore. However, it was not known whether TETS displayed any subtype selectivity and where exactly it was binding. To address the first question, our group recently tested the potency of TETS on the major synaptic and extrasynaptic GABA<sub>A</sub> receptors associated with convulsant activity using whole-cell patch clamp and reported that TETS is most active on α<sub>2</sub>β<sub>3</sub>γ<sub>2L</sub> and α<sub>2</sub>β<sub>3</sub>γ<sub>2L</sub> GABA<sub>A</sub> receptors (Pressly et al., 2018). Based on the observation that α<sub>2</sub>β<sub>3</sub>γ<sub>2</sub> receptors make up 15%–20% of the GABA<sub>A</sub> receptors in the mammalian central nervous system, we suggested that this receptor combination probably constitutes the most important GABA<sub>A</sub> receptor target for the seizure-inducing activity of TETS (Pressly et al., 2018). The results still left the question of the binding site unanswered. 

The GABA<sub>A</sub> field has recently advanced substantially through the publication of numerous new structures (García-Nafria and Tate, 2020). As of now, 19 GABA<sub>A</sub> receptor structures have been resolved in the closed, open, or desensitized state. Although the question of the binding site unanswered. The GABA<sub>A</sub> field has recently advanced substantially through the publication of numerous new structures (García-Nafria and Tate, 2020). As of now, 19 GABA<sub>A</sub> receptor structures have been resolved in the closed, open, or desensitized state. Although the question of the binding site unanswered. 

Methods

Study Design and Sample Sizes. This is an exploratory study for which we started with the working hypothesis that TETS is binding in the pore of the α<sub>2</sub>β<sub>3</sub>γ<sub>2</sub> GABA<sub>A</sub> receptor based on the fact that it behaves like a noncompetitive antagonist in electrophysiological experiments (Pressly et al., 2018) and displaces known pore blockers in radioligand binding assays (Esser et al., 1991; Zhao et al., 2014). Based on our previous experience with Rosetta modeling (Nguyen et al., 2017), we planned on generating 10,000 models for obtaining full-atom homology models of the α<sub>2</sub>β<sub>3</sub>γ<sub>2</sub> GABA<sub>A</sub> receptor and 50,000 docking trajectories for each ligand before identifying the 50 lowest-energy structures (see below). For the electrophysiological experiments, we also relied on our previous experience in mapping binding sites (Wulff et al., 2001; Zimin et al., 2010; Jenkins et al., 2011; Nguyen et al., 2017) and typically used five to eight independent recordings per data point when screening mutants or obtaining data for concentration-response curves. 

Rosetta Molecular Modeling. We generated three full-atom homology models of the α<sub>2</sub>β<sub>3</sub>γ<sub>2</sub> GABA<sub>A</sub> receptor using the Rosetta molecular modeling suite (Rohl et al., 2004) with membrane environment–specific energy functions (Yarov-Yarovoy et al., 2012). A model of the open state was based upon the X-ray structure of the β<sub>3</sub>-extracellular domain (ECD)–α<sub>5</sub>-transmembrane domain (TMD) chimera, which had been reported in the presence and absence of the neurosteroid pregnanolone (Miller et al., 2017). The pregnanolone-bound structure was chosen as a template (Protein Data Bank (pdb): 5OSF). Two additional models were generated based on the cryo-EM structure of α<sub>2</sub>β<sub>3</sub>γ<sub>2</sub> (Masilis et al., 2019) with alprazolam (pdb: 6HUO) or with picrotoxinin bound (pdb: 6HUG). Before homology modeling, all ligands and nanobody were removed from the templates, and Jalview 2 (http://www.jalview.org/) was used to align the sequences of α<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> with the templates. For the first model based on the β<sub>3</sub>ECD–α<sub>5</sub>TMD chimera, the ECDs of α<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> were aligned with the β<sub>3</sub>ECD, and the TMDs were aligned with the α<sub>5</sub>TMD. The sequence homology between α<sub>2</sub> and α<sub>5</sub> or α<sub>1</sub> is 73% and 70% in the TMD, respectively. The sequence homology between β<sub>3</sub> and α<sub>2</sub> or γ<sub>2</sub> is 33% in the ECD. No additional loop modeling was performed since we were primarily interested in the well resolved TMD domains. All three homology models were refined using RosettaES (Frenz et al., 2017). Ten thousand models were generated, and the top-10 converging, lowest-energy models were selected and subjected to a final round of side-chain relaxation to minimize the energy. Before transferring the models to RosettaLigand, Glide, or Swissdock for ligand docking, posttranslational modified amino acids (e.g., glycosylated or palmitoylated) were converted to standard amino acids to avoid problems with minimization procedures in these programs. Verification of correct residues was performed visually. As described below, we then first probed the TMD with two ligands (picrotoxinin and EBOB) known to bind to the NCA site to verify how suitable our models were for proceeding to dock TETS.

RosettaLigand Docking of NCAs. Docking of picrotoxinin, EBOB, and TETS was performed with the RosettaLigand application (Meiler and Baker, 2006; Davis and Baker, 2009), which is comprised of three stages that progress from low-resolution conformational sampling and scoring to full-atom optimization. The RosettaLigand application with the Talaris2014 energy function was used for all docking procedures. In the first, low-resolution stage, the ligand is placed randomly within the binding site, and its center of mass is constrained to move within a 7-Å diameter sphere. EBOB and picrotoxinin were placed according to their published binding sites (Chen et al., 2006; Masilis et al., 2019) with their center of mass at
the 6' ring in the pore of the GABA_A receptor. For TETS, we made the initial placements at six sites from position 0' to position 20' of the pore. Conformers were generated using OEChem, version 1.7.4 (OpenEye Scientific Software, Inc., Santa Fe, NM; www.eyesopen.com) and were then randomly rotated as a rigid body and scored for shape compatibility with the target protein (Hawkins et al., 2010; Hawkins and Nicholls, 2012). Please note that because of its caged structure, TETS only has one conformer. The best-scoring models were filtered by root-mean-square deviation to eliminate near duplicates, and one of the remaining models was selected at random to continue to the next stage. In the second, high-resolution stage, the Monte Carlo minimization protocol was employed, and ligand position and orientation were randomly perturbed by small 0.1-Å and 3° deviations: receptor side chains were repacked using a rotamer library; the ligand position, orientation, and torsions and protein side-chain torsions were simultaneously optimized using quasi-Newton minimization; and the end result was accepted or rejected based on the Metropolis criterion. Scoring used the full-atom Rosetta energy function with softened van der Waals repulsion. The side-chain rotamers were searched simultaneously during full repack cycles and one at a time in the rotamer trials cycles. The full repack makes ~10^6 random rotamer substitutions at random positions and accepts or rejects each based on the Metropolis criterion. Rotamer trials choose the single best rotamer at a random position in the context of the current state of the rest of the system with the positions visited once each in random order. The ligand is treated as a single residue, and its input conformers serve as rotamers during this stage. During the energy minimization step, the finely sampled rotamer library and soft-repulsive energy function allow access to off-rotamer conformations. In the third and final stage, a more stringent gradient-based minimization of the ligand position, orientation, and torsions as well as receptor torsions for both side chains and backbone were used. Scoring applies the same Rosetta energy function but with a hard-repulsive van der Waals potential, which creates a more rugged energy landscape that is better at discriminating native from non-native binding modes. Fifty thousand docking trajectories were generated for each channel-ligand pair, and the top 50 structures were selected according to the interface scores between the ligand and the protein. When a ligand converged on a common pose in a low-energy state with reoccurring interactions, we considered it converged. Rosetta energies comparing the three channels states on the arbitrary scale employed for the Rosette energy units (REUs) were calculated with the updated RosettaLigand application using the REF2015 energy function.

All molecular graphics were rendered using the UCSF Chimera software (Resource for Biocomputing, Visualization, and Informatics, San Francisco, CA) (Pettersen et al., 2004). Protein Data Bank format files of the closed/resting state of the beta2ECDD-gamma2 TMD chimera (pdb: 5O8F) was loaded into Swissdock (http://www.swissdock.ch) and probed blindly by docking TETS using 5000–15,000 generated iterations. Swissdock uses the Chemistry at Harvard Macromolecular Mechanics force field (CHARMM) for estimating the chemical interactions and treats the protein as a grid. The accurate docking function was used with no flexibility allowed. The top results were examined visually, and only docking poses found in the TMD were further considered and are shown in Fig. 2.

Chemicals. Picrotoxinin and GABA were purchased from MilliporeSigma (St. Louis, MO). Diazepam was purchased from Tocris Bioscience (Bristol, UK). TETS was synthesized at the University of California, Davis as previously described (Zhao et al., 2014). Ten millimolar stocks of GABA were made fresh daily using Ringer's solution (see below for composition). Ten millimolar stocks of picrotoxinin and TETS were prepared in DMSO and diluted down into Ringer's solution only immediately before application onto the cell to avoid any hydrolysis of picrotoxinin (Pressly et al., 2020). The identity of TETS and picrotoxinin was confirmed by 1H- and 13C-NMR; purity of TETS was tested by GC/mass spectrometry or high-pressure liquid chromatography/mass spectrometry and found to be >98% based on total ion fragment analysis. Both TETS and picrotoxinin were prepared with citric acid and disposed of using the waste accumulation program at University of California, Davis. The synthesis and chemical characterization of the TETS haptens used in Fig. 8 was previously described (Barnynch et al., 2017). Hapten 2a is (\(\pm\)-(1R,3S,5R)-2,7-dithia-1,3,6,8-tetraazatricyclo[4.3.1.13,8]undecane 2,2,7,7-tetraoxide); Hapten 4a is (\(\pm\)-(1R,5R)-3,7-dimethyl-1,3,5,7-tetraazabicyclo[3.3.1]nonane 2,2,6,6-tetraoxide); Hapten 6b is ((1R,3S,5r,7r)-7-amino-2-thia-1,3,5-triazaadamantane 2,2-dioxide).

Cell Culture. The human GABA_A receptors alpha_2, alpha_6, beta_3, and gamma_2, cloned into pcDNA3.1 expression vectors were a generous gift from Dr. Robert L. Macdonald, Vanderbilt University, Nashville, TN. L929 cells, a mouse fibroblast cell line (CCL-1), were obtained from American Type Culture Collection (Manassas, VA). L929 cells were cultured in Dulbecco’s modified Eagle’s medium (Lonza, Basel, Switzerland) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen; ThermoFisher, Grand Island, NY) and maintained in humidified 95% air and 5% CO_2 at 37°C. L929 cells were transfected using FuGENE 6 (ThermoFisher) transfection reagent in Opti-MEM reduced serum medium (Life Technologies, Benicia, CA) with an equal amount of each of the subunits (1:1:1) in combination with GFP expressed from the pEGFP-C1 vector (Invitrogen). The ratio of total cDNA to transfection reagent was 2:1. Cells were detached by trypsinization 48 hours post-transfection, washed, and plated onto poly-L-lysine–coated glass coverslips. Transfected cells were identified as GFP-expressing cells using an epifluorescence microscope for electrophysiological whole-cell voltage-clamp studies. Correct incorporation of the gamma subunit was tested by determining sensitivity to diazepam, a GABA_A receptor positive allosteric modulator that binds at the alpha/gamma interface as previously described (Pressly et al., 2018).
Mutagenesis. Mutagenesis primers were designed with PrimerX software (http://www.bioinformatics.org/primerx) and were 20–30 base pairs in length with a five to eight base pair overhang on the 3' end. Appropriate melting temperatures were calculated with NEB Tm Calculator (https://tmcalculator.neb.com/main). The primers were synthesized by ThermoFisher and were diluted to 10 μM from 100 μM stocks for polymerase chain reaction (PCR). NEB Phusion High-Fidelity DNA polymerase (ThermoFisher) was used to amplify the template DNA (10–100 ng). PCR cycling conditions were as follows: initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 10 seconds, annealing at 48–68°C for 30 seconds, and extension at 72°C for 150 seconds; and final extension at 72°C for 10 minutes. One percent DMSO was added to the PCR reactions. After the PCR, the DNA was digested with DpnI (New England Biolabs, Ipswich, MA) overnight at 37°C. Purity of product was assessed on 1% agarose gels. Transformations were performed with 1–5 μl of PCR product for each mutant and 100 μl MAX Efficiency DH10B competent Escherichia coli using heat shock according to the manufacturer’s protocol. Successfully transformed cells gave visible colonies on either ampicillin or kanamycin selection plates. Mutant sequences were confirmed via sequencing using ABI 3730 Capillary Electrophoresis Genetic Analyzers (University of California, Davis DNA Sequencing Facility). Mutants were deemed functional if they produced at least 200 pA of current in response to 100 μM GABA (=EC90 GABA). Since the αβ3γ2L GABA receptor constitutes the most sensitive and important target for the proconvulsant activity of TETS (Pressly et al., 2018), we decided to delineate the TETS binding site in this particular subunit combination. For experimental work and molecular modeling, it should be noted that TETS is neither acidic nor basic and therefore uncharged. It also only has a single conformer. Based on our previously published findings, TETS is a noncompetitive GABA receptor antagonist, which, similar to picrotoxinin, cannot be competed off by raising GABA concentrations (Pressly et al., 2018). As such, TETS could potentially be binding to an open, a postactivation, or a closed state of the αβ3γ2L receptor.

We had previously observed that TETS is more potent when GABA receptors are preincubated with TETS (Pressly et al., 2018) than when TETS and GABA are applied together, suggesting that TETS could be interacting with a closed state of the channel. This experimental setup is illustrated in Fig. 1A; αβ3γ2L receptors were activated by a 5-second application of 100 μM GABA directly to the patch-clamped cell. GABA is then washed away, and TETS is perfused into the recording chamber and allowed to equilibrate for 3 minutes. When GABA is then reapplied to the cell, it induces a much smaller current with virtually no further enhancement of current decay, suggesting that TETS can block closed channels. A 50-second wash of the chamber with Ringer’s solution completely reversed the TETS effect (Fig. 1A). In contrast, if TETS and GABA were perfused together directly onto the cell with no preincubation, the current induced by 100 μM GABA was larger and displayed an accelerated rate of current decay (Fig. 1B), suggesting open-channel block or enhanced desensitization. TETS thus resembles picrotoxinin, which is often described as an open-channel blocker with some additional “allosteric effects” (Xu et al., 1995; Othman et al., 2012; Olsen et al., 2019). Based
Fig. 1. TETS can block closed and open αβγ2L receptors. (A) Chloride currents were activated by a 5-second application of 100 μM GABA directly to the patch-clamped cell. GABA was then washed out by a 50-μs wash of the chamber with Ringer’s solution. One minute later, 50 μM of TETS was perfused into the chamber and allowed to equilibrate for 3 minutes. GABA (100 μM) was then reapplied directly to the cell with TETS in the bath. A subsequent 50-μs wash of the chamber with Ringer’s solution completely reversed the TETS effect. (B) After a control current was elicited by direct application of 100 μM GABA, TETS and GABA were perfused together directly onto the cell with no preincubation.

on this preliminary “look” at the mechanism of TETS blockade, we postulated that TETS is a pore blocker with fast on and off rates that is probably capable of blocking multiple states of GABA<sub>A</sub> receptors.

At the time, when we started this work in 2017, the most suitable template that was available for homology modeling was the X-ray structure of β<sub>2</sub>ECD-α<sub>2</sub>TMD chimera (Fig. 2A), which had been reported in the presence and absence of the neurosteroid pregnanolone (Miller et al., 2017). Before homology modeling, pregnanolone and the nanobodies, which had served as crystallization aids, were removed from the 3.2-Å resolution structure (Fig. 2A). We then aligned the sequences of the ECDs of α<sub>2</sub>, β<sub>3</sub>, and γ<sub>2</sub> with the β<sub>3</sub>ECD and the TMD sequences of these subunits with the α<sub>2</sub>TMD part of the chimeric structure and generated a full-atom homology model of the αβγ<sub>2</sub> GABA<sub>A</sub> receptor using the Rosetta molecular modeling suite (Rohl et al., 2004) followed by RosettaES refinement (Frenz et al., 2017). In the resulting homology model of the αβγ<sub>2</sub> GABA<sub>A</sub> receptor shown in Fig. 2B, the two α<sub>2</sub> subunits are colored in blue, the two β<sub>3</sub> subunits are colored in red, and γ<sub>2</sub> is shown in yellow. The pore is formed by five M2 helices and is 4.26 Å–wide at the desensitization gate on the intracellular side (2′ proline) similar to its template, which is 4.3 Å–wide at this position and therefore presumably open. Based on the high degree of homology in M2 between the different GABA<sub>A</sub> subunits (Fig. 2C), the Arg residue at the cytoplasmic end of M2 is typically designated 0′, and the residue at the extracellular end of the pore is designated 20′. Residues below the 0′ Arg are counted with negative numbers (Fig. 2D). This commonly used residue numbering system makes it easy to compare mutagenesis and modeling work across different GABA<sub>A</sub> receptor subtypes, and we are therefore also using it here.

To validate our αβγ<sub>2</sub> homology model, we first docked two widely used GABA<sub>A</sub> antagonists: picrotoxinin and EBOB. Both compounds are known to bind to the NCA site in the pore of the GABA<sub>A</sub> receptor (Chen et al., 2006; Olsen, 2006). The most detailed work on the NCA site has been performed with the sesquiterpene picrotoxinin, which has been suggested to interact with residues in the 2′ and 6′ positions based on mutagenesis in the αβγ<sub>2</sub> and the αβγ<sub>3</sub> GABA<sub>A</sub> receptors (Xu et al., 1995; Erkkila et al., 2008; Ng et al., 2016). Other compounds binding to the NCA site have been explored using the insect RDL channel and the probably unphysiological β<sub>3</sub> homopentamer in [3H]EBOB or [<sup>35</sup>S]TBPS binding assays (Esser et al., 1991; Chen et al., 2006). Both TETS and picrotoxinin displace the larger EBOB, suggesting that all three compounds interact with overlapping sites. Based on mutagenesis experiments, EBOB has been suggested to “sit upright” in the pore, blocking the channel’s ability to conduct chloride by stretching from residue 2′ in the lower pore to residue 9′ in the open homopentameric β<sub>3</sub> GABA<sub>A</sub> receptors (Chen et al., 2006).

Using the RosettaLigand method, we docked EBOB and picrotoxinin into the pore of our open αβγ<sub>2</sub> homology model (Supplemental Fig. 1) at the NCA site in the M2 segment, generated 50,000 docking trajectories, and collected the top 50 models with the energetically most-favorable interface scores between the ligand and the protein. For EBOB, Rosetta converged on a dominant binding pose where it is seen stretching from the 2′ to the 9′ ring in keeping with the previously mapped binding site (Chen et al., 2006). One ether oxygen in EBOB’s trioxabicyclooctane cage is hydrogen bonding to β<sub>3</sub>T6′, whereas α<sub>2</sub>V2′; the methyl group of α<sub>2</sub>T6′, and β<sub>3</sub>L9′ are making van der Walls interactions (Supplemental Fig. 1A). For picrotoxinin, Rosetta converged on a frequently sampled binding pose (Supplemental Fig. 1B) in which picrotoxinin forms one hydrogen bond with γ<sub>2</sub>S2′ and two hydrogen bonds with β<sub>3</sub>T6′ and γ<sub>2</sub>T6′ at the so-called T6′ ring or loop, where five threonine residues encircle the permeation pathway of the channel (Fig. 1C). This binding pose agrees well with previously performed mutagenesis showing that the T6′ ring is crucial for picrotoxinin binding (Erkkila et al., 2008; Ng et al., 2016). The Rosetta model is also in good agreement with the subsequently published α<sub>1</sub>β<sub>3</sub>γ<sub>2</sub> cryo-EM structure with picrotoxinin bound, where picrotoxinin is observed between the L9′ ring and the 2′ ring with major interactions, presumably hydrogen bonds, to the T6′ ring (Masiulis et al., 2019).
The Binding Site of TETS

Searching for the TETS Binding Site in the αβ3γ2 GABA_A Receptor. After finding that the RosettaLigand method converged on reasonable binding poses for picROTOxin and EBOB at the NCA binding site in our αβ3γ2 homology model, we next used RosettaLigand to search for the putative TETS binding site in the lower pore. Since the GABA_A receptor pore is long and narrow, TETS was randomly placed into 7-Å diameter spheres at six sites between residue 0' and 20' of the pore. TETS was minimized using the three stages of the RosettaLigand application in all six positions, and convergence of the top 50 models was used to predict a putative binding site. Figure 3A shows the six sites that were probed with Rosetta. TETS converged in two of these sites, which are highlighted in green as site-A (Fig. 3A) and site-C (Fig. 3A). To the right, close-up views of both sites are shown, which we used to guide further mutagenesis work. Site-A is at the -2' to -3' region of the M2 portion of our αβ3γ2 homology model. Here, as shown in a view from below, one oxygen in one of the sulfonamides of TETS is accepting two hydrogen bonds from the hydroxy group and the NH of the amide backbone of β2S-3', whereas the other sulfonamide oxygen is hydrogen bonding with the NH of the amide backbone of β2P-2'. Site-C is at the T6' ring of our αβ3γ2 homology model. In 21 out of the top 50 energetically most-favorable binding poses, the hydroxy groups of the β2T6' and the γ2T6' hydrogen bond to the sulfonamide group of TETS, whereas one of the tertiary nitrogen atoms in the central cage of TETS accepts an additional hydrogen bond from the γ2S2' (Fig. 3A). In an alternative and also relatively frequently sampled binding pose (8 of 50), TETS hydrogen bonds with α5T6' while maintaining the contact with γ2 (unpublished data). The remainder of the energetically favorable poses show hydrogen bonds with at least one T6' residue indicating the importance of this position.

To not solely rely on RosettaLigand, we also used Glide and Swissdock software to predict putative TETS binding sites in the Rosetta-generated homology model. With Glide, a program that, like RosettaLigand, requires the ligand to be randomly placed into predefined 7-Å boxes, we probed the M2 segment of the pore in our homology model using the same six starting positions as in Rosetta. Glide XP identified energetically favorable binding poses for TETS at site-A and site-C (shown in purple in Fig. 3B) but failed to identify possible binding poses in the other positions. At site-C in Glide, TETS is again positioned at the T6' ring, whereas at site-A, TETS sits further away from the pore than in RosettaLigand between the β2 and α5 subunits but again forms a hydrogen bond with β2S-3'. With Swissdock, in contrast, we evaluated the entire αβ3γ2 homology model, including the extracellular domain and the outside of the transmembrane domains because of how Swissdock operates. Modeling jobs are submitted to a server, and the program offers users minimal control of the docking process. Swissdock identified two possible binding sites as shown in cyan in Fig. 3C: one site again in the pore at the T6' ring and a second site in the lipid-exposed region of the γ2 subunit. We discarded the second site as unlikely because it was incompatible with the experimentally observed displacement of picROTOxin and EBOB by TETS.

Since all three molecular modeling programs identified a putative binding site for TETS at the T6' ring, and two of the three programs suggested a potential second site at the lower entrance to the pore, we decided to probe both sites by mutagenesis directed by the binding poses suggested by RosettaLigand (Fig. 3A).

Mutations in the α2 and β3 Subunits of the αβ3γ2 GABA_A Receptor Change TETS Potency in Electrophysiology. To determine whether mutations of any of the identified residues would change the ability of TETS to block chloride currents carried by αβ3γ2 GABA_A receptors, we transiently expressed wild-type and mutant receptors in L929 cells and studied them by whole-cell patch clamp. Correct incorporation of the γ subunit was confirmed by testing diazepam sensitivity as previously described (Pressly et al., 2018). As an initial screen for identifying mutations that substantially affect TETS affinity, we tested TETS at a concentration of 50 μM, which is roughly 100-fold above its IC50 of 480 nM for blocking αβ3γ2 GABA_A receptors at the GABA EC90 (100 μM) for this receptor subtype (Pressly et al., 2018).

Since our model suggested that TETS is interacting either with residues at the -3' and -2' positions (Fig. 3A, site-A) at the entrance to the permeation pathway (Fig. 2D) or, alternatively, with residues at the 2' and 6' rings (Fig. 3A Site-C), we started by introducing mutations into the β3 and γ2 subunits at the positions where RosettaLigand had suggested hydrogen-bonding contacts. In Fig. 4 we showcase cutaways of the transmembrane domains for each individual subunit, rendering the mutated pore-lining residues as stick models. (It should be kept in mind that mutations in the α or β subunit always introduce changes in two of the five subunits of the heteropentameric channel consisting of two α, two β, and one γ subunit.) For the β3 subunit, we studied a total of four mutants, S-3′F, A2′S, T6′C, and L9′Y, thus “walking” through the length of the pore starting from the intracellular side (Fig. 4A). Of these four
mutants, A2'S and T6'C showed appreciable reductions in TETS activity, suggesting that T6' and A2' in the β3 subunit are important for TETS binding. In contrast, there was no change in TETS activity with the L9'Y mutation, suggesting that T6 and A2 in the β3 subunit are important for TETS binding. In contrast, there was no change in TETS activity with the L9'Y mutation, suggesting that TETS is not extending as far up into the pore as picrotoxinin or EBOB. β3 mutants with larger amino acid substitution in the T6' position, T6'F and T6'W, did not express in our hands. For the γ2 subunit, we studied a total of six mutants, P-2' H, P-2' F, S2' G, S2' F, S2' A, and T6' C (Fig. 4B). Of these six mutants, which were primarily designed to closely probe the -2' and 2' site by sterically disturbing it, only the T6'C mutation reduced TETS activity, confirming the RosettaLigand prediction that TETS is probably hydrogen bonding with γ2T6' and β3T6'. Our attempts to obtain a larger effect by mutating the T6' position in γ2 failed because the γ2T6'Y mutant was not functional.

Taken together, these results ruled out site-A as a possible TETS binding site, and we therefore next focused our attention on site-C by mutating the 2' and 6' residues in the α2 subunit as suggested by the alternative, less frequently sampled Rosetta-Ligand poses for site-C. Of the four tested α2 mutants, V2'W, T6'M, T6'D, and T6'S (Fig. 4A), all three T6' mutants notably reduced TETS activity, whereas the mutation in the 2' position had no impressive effect, demonstrating that T6' is a critical residue for TETS binding on the α2 subunit. The α2 V2'A and T6'F mutants did not express in our hands and could not be studied. Similar results were obtained with the α6β3γ2 receptor, which is blocked by TETS with an IC50 of 400 nM (Pressly et al., 2018). Mutating β3 in the -3' position (S-3' F) or γ2 in the 2' position (S2' G) did not notably alter the blocking potency of 50 μM TETS (unpublished data), whereas mutating V2' in α6 reduced the ability of 50 μM TETS to block currents elicited by EC90 GABA to 38% (S.D. 4.8%, n = 5).

A Resting/Inactivated State Model of the α2β3γ2 GABA<sub>A</sub> Receptor Produces a “Tighter,” Energetically More-Favorable Model of the TETS Receptor Site. Although the mutagenesis experiments confirmed that TETS is interacting with site-C and not with site-A, we were somewhat concerned that TETS was so mobile in the pore during energy minimization in our open-state model, resulting in multiple possible binding poses instead of a single predominant pose. Specifically, TETS was seen to be sampling several positions at the T6' ring and was alternatively hydrogen bonding with either β3T6' or α2T6' while maintaining contact with

Fig. 3. Searching for the TETS binding site in the open-state model. The receptor is color-coded as follows: α2 (blue), β3 (red), and γ2 (yellow). (A) "Walk" through the pore of the α2β3γ2 homology model in six boxes of 7-Å diameter in RosettaLigand, which identified site-A and site-C as possible binding sites. (B) TETS binding sites suggested by Glide XP. (C) TETS binding sites suggested by Swissdock.
This made us suspect that the pore in this open-state model was too wide to optimally accommodate TETS. When the cryo-EM structure of the full-length human α1β3γ2L in lipid nanodiscs bound to picrotoxinin, bicuculline, alprazolam, and diazepam (Masiulis et al., 2019) was published, we therefore generated two new α2β3γ2 homology models based on the structures with bound alprazolam and GABA (pdb: 6HUO) and picrotoxinin (pdb: 6HUG).

The 6HUO structure captured the α1β3γ2 receptor in a desensitized state that is closed down to 1.6 Å at the -2 gate but open to 2.6 Å at the activation-gate level at the T6 Leu. When used as a template for a Rosetta-generated α2β3γ2 homology model, this structure produced a very similar binding model for TETS. RosettaLigand converged on a pose where the two sulfones of TETS are hydrogen bonding with the hydroxy groups of γ2T6 and γ2S2, whereas one of the tertiary nitrogen atoms in the central cage of TETS accepts an additional hydrogen bond from γ2T6 (Supplemental Fig. 2).

We next probed a Rosetta-generated α2β3γ2 model based on the 6HUG template. In this template structure, the α1β3γ2L receptor has been captured at 3.1 Å in what is assumed to be a closed/resting state (Masiulis et al., 2019). Picrotoxinin is positioned between the 2 and 9 rings with its lipophilic isopropenyl moiety surrounded by the L9 ring and its lactone rings forming putative hydrogen bonds with the T6 ring. The conduction pathway in this structure is "narrow" and constricted to ~1.5 Å at both the desensitization gate at -2' and the activation gate at 9'. We removed picrotoxinin from this structure, generated an α2β3γ2 model and then docked TETS into 50,000 random starting positions. The top scoring models in RosettaLigand showed a much "tighter" fit for TETS in this 6HUG-based model than in the β3ECD-α5TMD chimera-based open-state model or the 6HUO-based desensitized state model (Supplemental Fig. 3). When TETS is hydrogen bonding with β3 and γ2 at the T6' ring in the open-state model, there is a considerable amount of empty space between the TETS...
molecule and the other three T6' residues on the second β2 and the two α2 subunits, which often made us wonder whether there could possibly be any water molecules participating in TETS binding at this position. However, in the narrower pore of the closed/resting-state model, TETS is very close to all five T6' residues (Supplemental Fig. 3). In the most frequently occurring low-energy binding pose, TETS accepts hydrogen bonds from the hydroxyl groups of T6' from one of the two β2 subunits and the γ2 subunit as well as from the S2' on γ2 (Fig. 5A). As previously, RosettaLigand again identified alternative poses in which the TETS has slightly turned and is now hydrogen bonding with one of its sulphone groups to one of the α2'T6' positions and the neighboring γ2T6' (Fig. 5B). But in both binding poses, the compact, “caged” TETS molecule shows perfect space complementarity and optimally fills out the pore lumen at the T6' ring of the pentameric receptor as shown in Fig. 5C when viewed from above. In addition to the three or two hydrogen bonds in the two alternative low-energy binding poses (Fig. 5, A and B), TETS is making a network of van der Waals contacts shown as black lines when viewed from the side (Fig. 5D) or from above looking down on the T6' ring (Fig. 5E). Supplemental Fig. 4 shows the dominant TETS binding pose in a 2D ligand-protein interaction diagram, which illustrates both hydrogen bonds and hydrophobic interactions together with the relative polarity of the interacting amino acid residues surrounding the TETS molecule. A comparison of the Rosetta energies for the three states showed the lowest energy for TETS binding in the closed/resting-state model (-10.524 REU) versus -9.562 REU for the desensitized state and -4.955 REU for the open-state model. Because the Rosetta energy function is a combination of physics-based and statistics-based potentials, Rosetta energies are on an arbitrary scale referred to as REUs. Taken together, these results suggest that TETS is more likely to bind to the closed state of the α2β3γ2 GABA_A receptor than to the open state.

The T6' Ring Is Crucial for TETS Activity. We next more closely probed the 6HUG-based resting/closed-state model by obtaining full TETS concentration-response curves for selected mutants. Since RosettaLigand had consistently suggested two hydrogen bonds, multiple van der Waals interactions, and perfect space complementarity for TETS at the T6' ring, we first focused on this position. In keeping with there being two α2 and two β2 subunits in the pentameric receptor, substituting T6' with a smaller but more hydrophobic cysteine or a larger and more hydrophobic methionine residue in these subunits drastically shifted the TETS concentration-response curve to the right and reduced the TETS IC_{50} by ~700 fold (Fig. 6A). In contrast, introducing a T6'C mutation in the γ2 subunit, which is only present once in the channel pentamer, had a less pronounced but still notable effect on TETS activity (Fig. 4B). Please note that we did not test TETS, which has an LD_{50} of 0.1 mg/kg and is an extremely potent convulsant, at concentrations higher than 500 μM because of safety considerations. Coexpressing β2T6'C and γ2T6'C with wild-type α2 did not produce an additional shift in TETS sensitivity compared with the β2T6'C mutation alone (unpublished data). Taken together, the T6' mutation experiments demonstrate that the contacts at the T6' ring are essential for TETS activity. Introducing any residues that change the side-chain volume, charge, or hydropathy index in this position (see Fig. 4; Fig. 6A) is likely to disrupt the hydrogen bonds and van der Waals interactions that TETS is making and prevent TETS from accessing its optimal binding position.

We next turned our attention to the 2' ring, where most models suggested a hydrogen bond (Fig. 3A; Fig. 5A) and van der Waals interactions (Fig. 5D) with the γ2S2'. However, mutating this residue to A, G, or even F did not substantially reduce the blocking potency of 50 μM TETS (Fig. 4B), and we therefore tried to do the opposite and increase the potency of TETS by creating an additional hydrogen bonding opportunity at the 2' ring through the β2A2'S mutation. Interestingly, this mutation drastically reduced TETS potency by shifting the IC_{50} by 620-fold to 300 μM (Fig. 6B). In contrast, a closer look at the α2V2'W mutation with a full concentration-response curve revealed that this mutation, which introduces a large aromatic residue into the 2' ring in two of the five subunits, affects TETS activity and increases the TETS IC_{50} from 480 nM to 5.7 μM (Fig. 6B).

TETS and Picrotoxinin Have Overlapping but Not Identical Binding Sites in the α2β3γ2 GABA_A Receptor. We finally wondered how much the TETS and the picrotoxinin binding sites overlap in the α2β3γ2 GABA_A receptor and therefore docked picrotoxinin into our closed/resting-state homology model. RosettaLigand converged on a dominant binding pose that was very similar to the cryo-EM structure of the picrotoxinin binding site captured in the α1β2γ2L receptor (Masiusis et al., 2019). Supplemental Fig. 5 shows a comparison of the dominant picrotoxinin pose identified by RosettaLigand in the α2β3γ2 model and the picrotoxinin pose observed in the cryo-EM structure of the α1β2γ2L receptor. In both poses, the isopropenyl group is facing upwards toward the L9' ring, and its oxygen-rich lactone rings are centered at the T6' ring. However, in contrast to the cryo-EM structure, which suggested four putative hydrogen bonds for picrotoxinin in the α1β2γ2L receptor (two with the two β2T6' residues, one with α1T6' and one with γ2S2'), RosettaLigand only found three hydrogen bonds and suggested that picrotoxinin is turned and tilted a little differently in the α2β3γ2 homology model where it is hydrogen bonding with two β2T6' residues and γ2T6' but not α2 in the lowest-energy poses (Supplemental Fig. 4). Interestingly, this binding pose with fewer hydrogen bonds in the α2β3γ2 homology model than in the α1β2γ2 receptor structure is in line with picrotoxinin being less potent on α1β2γ2 (IC_{50} 7.5 μM) than on α1β2γ2 (IC_{50} 3.7 μM) in our hands when tested in patch-clamp experiments at EC_{50} GABA (Pressly et al., 2018).

An overlay of the dominant TETS and picrotoxinin binding poses in the α2β3γ2 receptor model (Fig. 7A) shows that both TETS and picrotoxinin are centered at the T6' ring but that the larger picrotoxinin extends further up into the L9' ring where the lipophilic isopropenyl group makes hydrophobic contacts with the lipophilic leucine residues as visualized in the space-filled renderings of picrotoxinin and TETS (Fig. 7A). When obtaining picrotoxinin concentration-response curves for the same two mutants (α2T6'M and β2T6'C), which had practically made the α2β3γ2 receptor insensitive to TETS (Fig. 6A), we found that the α2T6'M also rendered the α2β3γ2 receptor insensitive to picrotoxinin, whereas the β2T6'C mutant remained as sensitive to picrotoxinin as the wild-type channel (Fig. 7B). Taken together, these modeling and
mutagenesis findings suggest that the picrotxoxin and the TETS binding sites are overlapping but not identical on α2β3γ2 GABA<sub>A</sub> receptors.

**Confirmation of the Mutagenesis with a Chemical Biology Approach.** To not solely rely on mutagenesis for identification the TETS binding site, we decided to confirm our results by altering the structure of the ligand instead of mutating the receptor site. Our group had previously synthesized a library of TETS derivatives for use as haptens for the development of an immunoassay for the detection of TETS (Barnych et al., 2017). We selected three of these TETS haptens (Fig. 8) and measured their ability to block chloride currents elicited by 100 μM GABA (EC<sub>90</sub>) through the α2β3γ2 GABA<sub>A</sub> receptor. Two TETS derivatives that enlarge (Hapten 2a) or open the tetraazaadamantane cage (Hapten 4a) were found to be roughly 15-fold less potent than TETS, whereas a derivative lacking one of the two hydrogen bond–accepting sulfone groups (Hapten 6b) was 180-fold less potent than TETS. To confirm that all three TETS haptens were still interacting with the T6' ring and that we were indeed investigating a structure-activity relationship, we also tested the three haptens at a concentration of 1 mM on the β3T6'C mutant (see gray box in Fig. 8). Although Hapten 2a and 4a still exhibited some effect that was dramatically reduced, Hapten 6b was found to be ineffective on the β3T6'C at 1 mM (Fig. 8).

**Discussion**

We here used atomistic scale molecular modeling, site-directed mutagenesis, and whole-cell voltage-clamp experiments to map the binding site of the convulsant agent TETS in the α2β3γ2 GABA<sub>A</sub> receptor. Like other “caged” convulsants, TETS interacts with residues in the M2 segment lining the chloride permeation pathway at the NCA site. That TETS binds to this site was suspected since the early 1990s when the Casida laboratory reported that TETS displaced [35S]TBPS from what was then termed the mammalian-brain GABA-gated chloride channel in rat brain synaptosomes (Esser et al., 1991). However, since the first insect and mammalian GABA<sub>A</sub> receptors were cloned and recombinantly expressed,
the question of the putative TETS binding site became somewhat confused. Although TETS was found to displace [3H]EBOB and [3H]dihydropicrotoxinin from Drosophila receptors or human α3β2γ2 receptors, it did not inhibit GABA-stimulated chloride fluxes through the human β3 homopentamer (Ratra et al., 2001). To determine whether TETS indeed directly binds to GABA1 receptors, the Casida group synthesized [14C]TETS in 2014 and used accelerated mutagenesis data to map the binding site of TETS on the ECD of the human GABA1 receptor using Rosetta (Fig. 3A), we first generated a homology model of the α1β2γ2 GABA1 receptor based on the open-state structure of the Caenorhabditis elegans glutamate-gated chloride channel (Hibbs and Gouaux, 2011) and used molecular dynamics simulations to propose a putative TETS binding site in the lower pore of the α1β2γ2 GABA1 receptor where TETS was predicted to make three hydrogen-bonding interactions with two α1T1' residues and γ2S2'. However, although appearing reasonable at the time, this prediction, which was never confirmed by mutagenesis, was called into question when the subsequently solved GABA1 receptor structures showed that T1' was not a pore-lining residue and that GABA1 receptors in various states are narrower at 2'. (Miller et al., 2017; Laverty et al., 2019; Masiulis et al., 2019) and not splayed open as widely as the ECD of C. elegans GluCl channel, making it impossible for TETS to hydrogen bond with T1'.

When determining the GABA1 receptor subtype selectivity of TETS, we observed that TETS inhibits α2β3γ2 and α6β3γ2 GABA1 receptors with submicromolar IC50 values in whole-cell patch-clamp experiments while it blocks α1, α4, β2, γ1, or δ containing GABA1 receptors roughly 5–10-fold less potently and basically has no effect on β1-containing receptor combinations (Pressly et al., 2018), suggesting a preference for the α3 or α6β3γ2 combination. We therefore here set out to map the binding site of TETS on the α2β3γ2 receptor, which we believe is the pharmacologically most-relevant GABA1 receptor for the seizure-inducing activity of TETS. Since we had previously successfully used the Rosetta molecular modeling suite to make a homology model of the pore region of the calcium-activated potassium channel KCa3.1 (Nguyen et al., 2017) that agreed well with the subsequently solved cryo-EM structure (Lee and MacKinnon, 2018) and that allowed us to correctly predict the binding sites of several KCa3.1 inhibitors in either the pore or the fenestration region of KCa3.1 with RosettaLigand, we again used the Rosetta Membrane method (Rohl et al., 2004; Yarov-Yarovoy et al., 2012) for homology modeling of the α3β2γ2 receptor but this time in combination with the more recently developed RosettaES refinement approach (Frenz et al., 2017), which is particularly useful for “cleaning up” and relaxing cryo-EM structures before docking ligands. When we started this work, the structure that seemed most attractive to us as a template was the X-ray structure of a chimeric channel in which the ECD of the human β3 subunit had been fused to the TMD of the human α3 subunit (Miller et al., 2017). Although the ECD of this chimera, which can only be activated by histamine and not by the natural ligand GABA because it is lacking the GABA binding site in the αβ interface, might be of questionable value for modeling the ECD, the membrane-embedded part of this chimera was appealing because its pore showed two constriction sites at the intracellular end at the 2' and -2' ring, and binding of the positive allosteric modulator pregnanolone enlarged the pore diameter at the -2' proline ring. The β2ECD-α3TMD chimera was further capable of producing picrotoxin-sensitive chloride currents (Miller et al., 2017), suggesting that it contained an NCA site in the TMD. After generating what we here assume to be an open-state homology model of the α3β2γ2 receptor using Rosetta (Fig. 3A), we first probed the putative NCA site of our model by docking picrotoxinin and EBOB. For both these “classical” antagonists, RosettaLigand identified binding poses that were in good agreement with literature and existing mutagenesis data (Esser et al., 1991; Xu et al., 1995; Chen et al., 2006; Erkkila et al., 2008; Rozycka et al., 2010; Yarov-Yarovoy et al., 2012).
Encouraged by this validation, we next used three different ligand-docking algorithms, Rosetta-Ligand, Glide, and Swissdock, with different energy functions, different parametrizations, and different requirements for initial ligand placements to search for the TETS binding site (Fig. 3). Specifically, although RosettaLigand and Glide require ligands to be placed into predefined boxes for which the diameter can be chosen, which somewhat biases the search toward potential binding sites selected by the user, Swissdock requires no such assumption. The different programs found two possible binding sites for TETS: one site at the T69 ring, which was identified by all three programs, and a second site lower down at the entrance to the permeation pathway. Site-directed mutagenesis experiments (Fig. 4) ruled out the lower site and confirmed that TETS interacts with the T69 ring where threonine residues from all five subunits line the channel pore.

Although the predominant TETS binding poses identified by RosettaLigand at the T69 ring of our open-state model agreed with the mutagenesis, TETS was only partially

Fig. 7. (A) Overlay of the lowest-energy binding poses of TETS and picrotoxinin in stick representation in the closed/resting-state homology model of the a2β3γ2 GABA_A receptor. The receptor is color-coded as follows: a2 (blue), β3 (red), and γ2 (yellow). One a2 subunit is removed for clarity. Picrotoxinin is shown in black. Hydrogen bonds are shown in green. The panels on the side show transparent molecular surfaces of picrotoxinin (black) and TETS (green) with pore-lining residues in the L9', T6', and 2' ring rendered as spheres. (B) Concentration-response curves for picrotoxinin inhibition of currents evoked by EC50 GABA (100 μM) comparing wild-type a2β3γ2 receptors with T6' mutations: a2β3γ2M (IC50 6.8 μM, 95% CI 4.5–8.4 μM), a2T6'Mβ3γ2L (no meaningful IC50 can be determined since the maximal effect is drastically reduced, and we therefore consider this channel insensitive to picrotoxinin), and a2β3T6'Cγ2L (IC50 4.2 μM, 95% CI 1.2–6.9 μM, P = 0.2). Individual data points are presented as mean ± S.D. from five to eight independent recordings. Concentration-response curves were compared using an extra sum-of-squares F test (GraphPad Prism8; GraphPad Software). The reported P-values test the null hypothesis that the concentration-response curves for wild-type and mutant channels are identical. CI, confidence interval.
occluding the pore (Supplemental Fig. 3), raising the question of why this position was so sensitive to relatively small changes in side-chain volume in the mutagenesis and making us wonder whether we could be missing water molecules, which are not explicitly modeled by Rosetta. Fortunately, while we were working on this project, the cryo-EM structure of the human α2β3γ2 receptor in a lipid environment was published in both the closed and the desensitized states (Laverty et al., 2019; Masiulis et al., 2019). Using these new templates, we generated two additional homology models of the α2β3γ2 receptor in the desensitized and closed/resting states and again docked TETS. Although the docking poses in the desensitized state somewhat reduced the space at the T6’ ring (Supplemental Fig. 3), the dominant docking poses of TETS in the closed/resting model had the comparatively lowest Rosetta energy and showed a much “tighter” fit for TETS (Fig. 5), which now perfectly “snuggled” into the T6’ ring by making three hydrogen bonds and literally a net of van der Waals interactions in its dominant low-energy binding pose (Fig. 5). The perfect space complementarity and the multitude of interactions observed in this pose also provided a much better explanation for why the introduction of any residue that changes side-chain volume, charge, or hydrophobicity index in the T6’ ring reduces TETS activity (Fig. 4; Fig. 6A). In contrast, the results of mutating the 2’ ring, where TETS is again seen to hydrogen bond with γ2S2 in our closed/resting-state model, were not as straightforward to interpret. Introducing a large, aromatic Trp in α2 shifted TETS potency, as would be expected for introducing steric bulk into the 2’ ring, but mutations of the γ2S2’ residue itself did not have the expected effects. However, when we then replaced Ala with Ser in β3 in an attempt to potentially pick up an additional hydrogen bonds, we saw a dramatic reduction in TETS potency (Fig. 6B). One explanation for this finding could be that maybe we did create an additional hydrogen-bonding opportunity and were now “catching” TETS at the 2’ ring and preventing it from accessing the T6’ ring. Interestingly, our finding that an A2’S mutation reduces TETS potency ~600-fold is in line with reports that in the insect RDL receptor, the A2’S mutation confers resistance to cyclodiene insecticides and picrotoxin (FFrench-Constant et al., 1993; Zhang et al., 1994) and reduces fipronil activity (Zhang et al., 2016).

Based on our molecular modeling results, which showed a “tighter” fit and a relatively lower energy in a resting/closed state than in an open or desensitized state model of the α2β3γ2 receptor, we suggest that TETS preferentially binds to the closed state. This assumption is in line with our observations that in electrophysiological experiments current inhibition by TETS shows no delays and that TETS produces more block if GABA receptors are preincubated with TETS before channels are activated with GABA (Fig. 1A) than if GABA and TETS are applied simultaneously (Fig. 1B). The template for our closed-state model was the cryo-EM structure of the α2β3γ2L receptor (Masiulis et al., 2019) in complex with picrotoxinin (pdb:6HUG), which, based on the pore radius at the -2’ and 9’, is closed at both the desensitization and the activation gate and therefore assumed to have captured the channel in the closed/resting state. Picrotoxinin, which has been reported to be an open-channel blocker while also sometimes displaying some competitive inhibitor effects (Xu et al., 1995; Mehta and Ticku, 2001; Wang et al., 2006; Olsen et al., 2019), is believed to bind initially to an open-channel pore and then to stabilize a closed/resting receptor state (Masiulis et al., 2019). We here show that the TETS and the picrotoxinin binding sites in the α2β3γ2L receptor are overlapping but not identical. TETS is centered at the T6’ ring with an additional contact at the 2’ ring, whereas the larger picrotoxinin extends further up into the pore to the L9’ ring (Fig. 7). It therefore is possible that TETS differs from picrotoxinin, and it would be desirable in the future to determine its structure in complex with the α2β3γ2L receptor. TETS could be binding directly to the closed state or like picrotoxinin initially interact with the open state, enhance the rate of current decay, and then stabilize the closed state.
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References


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The Binding Site of TETS 91


