

# A Benzodiazepine Ligand with Improved GABA<sub>A</sub> Receptor $\alpha 5$ -Subunit Selectivity Driven by Interactions with Loop C<sup>§</sup>

Xenia Simeone, Filip Koniuszewski, Markus Müllegger, Andreas Smetka, Friederike Steudle, Roshan Puthenkalam,  Margot Ernst, and  Petra Scholze

Center for Brain Research, Medical University of Vienna, Vienna, Austria

Received May 13, 2020; accepted October 20, 2020

## ABSTRACT

The family of GABA<sub>A</sub> receptors is an important drug target group in the treatment of sleep disorders, anxiety, epileptic seizures, and many others. The most frequent GABA<sub>A</sub> receptor subtype is composed of two  $\alpha$ -, two  $\beta$ -, and one  $\gamma 2$ -subunit, whereas the nature of the  $\alpha$ -subunit critically determines the properties of the benzodiazepine binding site of those receptors. Nearly all of the clinically relevant drugs target all GABA<sub>A</sub> receptor subtypes equally. In the past years, however, drug development research has focused on studying  $\alpha 5$ -containing GABA<sub>A</sub> receptors. Beyond the central nervous system,  $\alpha 5$ -containing GABA<sub>A</sub> receptors in airway smooth muscles are considered as an emerging target for bronchial asthma. Here, we investigated a novel compound derived from the previously described imidazobenzodiazepine SH-053-2'-F-R-CH<sub>3</sub> (SH53d-ester). Although SH53d-ester is only moderately selective for  $\alpha 5$ -subunit-containing GABA<sub>A</sub> receptors, the derivative SH53d-acid shows superior (>40-fold) affinity selectivity and is a positive modulator. Using two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes and radioligand displacement assays with human embryonic kidney 293 cells, we demonstrated that an acid group as

substituent on the imidazobenzodiazepine scaffold leads to large improvements of functional and binding selectivity for  $\alpha 5\beta 3\gamma 2$  over other  $\alpha x\beta 3\gamma 2$  GABA<sub>A</sub> receptors. Atom level structural studies provide hypotheses for the improved affinity to this receptor subtype. Mutational analysis confirmed the hypotheses, indicating that loop C of the GABA<sub>A</sub> receptor  $\alpha$ -subunit is the dominant molecular determinant of drug selectivity. Thus, we characterize a promising novel  $\alpha 5$ -subunit-selective drug candidate.

## SIGNIFICANCE STATEMENT

In the current study we present the detailed pharmacological characterization of a novel compound derived from the previously described imidazobenzodiazepine SH-053-2'-F-R-CH<sub>3</sub>. We describe its superior (>40-fold) affinity selectivity for  $\alpha 5$ -containing GABA<sub>A</sub> receptors and show atom-level structure predictions to provide hypotheses for the improved affinity to this receptor subtype. Mutational analysis confirmed the hypotheses, indicating that loop C of the GABA<sub>A</sub> receptor  $\alpha$ -subunit is the dominant molecular determinant of drug selectivity.

## Introduction

GABA<sub>A</sub> receptors are GABA-gated chloride channels that are expressed in neurons, glial cells, and several non-neuronal cell types (Gladkevich et al., 2006; Mizuta et al., 2008; Olsen and Sieghart, 2008; Barragan et al., 2015; Wan et al., 2015) wherein they influence a variety of cellular processes through ligand-gated chloride flux. These receptors are pentamers of subunits that are comprised of different subunit classes. The existence of six  $\alpha$ -, three  $\beta$ -, three  $\gamma$ -, one  $\delta$ -, one  $\epsilon$ -, one  $\theta$ -, one  $\pi$ -, and three  $\rho$ -subunits in mammalian systems gives rise to an enormous theoretical diversity of GABA<sub>A</sub> receptor subtypes with distinct subunit composition and unique pharmacological properties. Because of the presence of multiple GABA<sub>A</sub>

receptor subunits in individual cells, however, an unequivocal identification of a receptor subtype is difficult (Olsen and Sieghart, 2008). So far, the native expression of only 11 subunit combinations has been conclusively demonstrated. In addition, there is strong evidence for the existence of several other subunit combinations, the number of which is slowly increasing (Olsen and Sieghart, 2008).

GABA<sub>A</sub> receptors in the central nervous system (CNS) are the site of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, and anesthetics, that allosterically modulate GABA-induced currents eliciting anticonvulsant, sedative-hypnotic, and anxiolytic effects (Brickley and Mody, 2012; Rudolph and Möhler, 2014; Sieghart, 2015). In addition to these synthetic drugs, a wide range of natural products (Khom et al., 2010; Lorenz et al., 2010; Hanrahan et al., 2011) as well as some endogenous agents, such as neuroactive steroids (Belelli and Lambert, 2005), the endocannabinoid 2-arachidonoyl glycerol (Sigel et al., 2011), histamine (Saras et al., 2008; Fleck et al.,

This study was supported by the Austrian Science fund (FWF), doctoral college W1232 "Molecular drug targets."

<https://doi.org/10.1124/molpharm.120.000067>.

<sup>§</sup> This article has supplemental material available at molpharm.aspetjournals.org.

**ABBREVIATIONS:** ASM, airway smooth muscle; CNS, central nervous system; cryo-EM, cryogenic electron microscopy; EC<sub>3-5</sub>, GABA concentration eliciting 3–5% of the maximal GABA response BMI binding mode one BMII binding mode two HEK, human embryonic kidney; PDB, Protein Data Bank; RMSD, root-mean-square deviation; TC50, 50 mM Tris-citrate; TEV, two-electrode voltage clamp.

2012), and dopamine (Hoerbel et al., 2015), modulate GABA<sub>A</sub> receptors.

The benzodiazepine binding site is located at the extracellular  $\alpha$ - $\gamma$  interface of the GABA<sub>A</sub> receptors (Sigel and Lüscher, 2011; Richter et al., 2012). Thus, the potency and efficacy of benzodiazepine site ligands are dependent on the subtype of both of these subunits. In the CNS, benzodiazepine ligands exert mostly anxiolytic, anticonvulsive, sedative hypnotic, and myo-relaxant properties (Sieghart, 2015; Sieghart and Savić, 2018). The individual effects of benzodiazepines seem to be predominantly mediated by distinct GABA<sub>A</sub> receptor subtypes, and drugs specifically interacting with these subtypes are thus expected to exhibit quite selective pharmacological and behavioral effects (Möhler, 2011).

One of the first relatively  $\alpha 5\beta 3\gamma 2$ -selective positive allosteric modulators reported was the imidazobenzodiazepine SH-053-2'-F-R-CH<sub>3</sub> (SH53d-ester), featuring a window of separation between the modulation of  $\alpha 5\beta 3\gamma 2$  receptors and  $\alpha 1$ ,  $\alpha 2$ , or  $\alpha 3\beta 3\gamma 2$  receptors (Savić et al., 2010). In electrophysiological experiments, SH53d-ester is selective for  $\alpha 5$  receptors at low nanomolar concentration. At 100 nM concentration, this compound markedly enhances GABA-elicited currents at  $\alpha 1\beta 3\gamma 2$ ,  $\alpha 2\beta 3\gamma 2$ ,  $\alpha 3\beta 3\gamma 2$ , and  $\alpha 5\beta 3\gamma 2$  receptors (Fischer et al., 2010; Savić et al., 2010). Concomitantly, this compound has moderate affinity for the benzodiazepine binding site of  $\alpha 5\beta 3\gamma 2$  receptors and low affinity for  $\alpha 1$ ,  $\alpha 2$ , or  $\alpha 3\beta 3\gamma 2$  receptors (Fischer et al., 2010).

Ester-to-amide substitution in SH53d-ester led to MP-III-022 with improved selectivity, efficacy, and kinetic behavior as a positive modulator of GABA<sub>A</sub> receptors containing the  $\alpha 5$  subunit (Stamenić et al., 2016). Accordingly, binding affinities of MP-III-022 (K<sub>i</sub>) for the different receptor subtypes  $\alpha 1\beta 3\gamma 2$ ,  $\alpha 2\beta 3\gamma 2$ ,  $\alpha 3\beta 3\gamma 2$ , and  $\alpha 5\beta 3\gamma 2$  expressed in human embryonic kidney (HEK) 293 cells were 850, 360, 660, and 55 nM, respectively. TEV electrophysiology in oocytes revealed allosteric modulation of MP-III-022 in  $\alpha 5\beta 3\gamma 2$  GABA<sub>A</sub> receptors with efficacies of 300% at 100 nM while being nonmodulatory ( $\alpha 1$ ) or only weakly modulatory at  $\alpha 2$ - and  $\alpha 3$ -containing receptors.

Clinically applied drugs targeting GABA<sub>A</sub> receptors are mainly used for their effects on the human CNS (Sieghart, 2015). However, considerable interest in these receptors expressed in peripheral tissues as potential therapeutic targets has emerged (Gladkevich et al., 2006; Mizuta et al., 2008; Sengupta et al., 2014; Barragan et al., 2015; Gallos et al., 2015; Wan et al., 2015). Of specific interest are GABA<sub>A</sub> receptors expressed in airway smooth muscle (ASM) and their ability to induce relaxation of an established contraction, which could have enormous clinical implications in bronchoconstrictive diseases, such as asthma. It was shown that the  $\alpha 5$ -preferring chiral imidazobenzodiazepine SH53d-ester relaxes ASM and enhances chloride currents in cultured ASM cells (Gallos et al., 2015), thus suggesting that benzodiazepine-sensitive  $\alpha 5\beta 3\gamma 2$ -containing receptors are present.

The derivative of SH53d-ester, which we characterize here (called SH53d-acid, formerly known as compound 2), has also been tested in this assay and shown to be effective (Forkuo et al., 2017), whereas a detailed presentation of the compound's pharmacology was still missing. In the current study we now provide this detailed pharmacological characterization of SH53d-acid and describe its superior (>40-fold) affinity

selectivity for  $\alpha 5$ -containing GABA<sub>A</sub> receptors. Computational docking provides a hypothesis for the improved affinity to this receptor subtype. Using mutational analysis, we were able to identify loop C of the GABA<sub>A</sub> receptor  $\alpha$ -subunit as the dominant molecular determinant of drug selectivity.

## Materials and Methods

**Compounds.** SH-053-2'-F-R-CH<sub>3</sub> = SH53d-ester: (*R*)-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylic acid ethyl ester; SH53d-acid: (*R*)-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylic acid; Fig. 1. All compounds listed above were synthesized at the Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee. For details of SH53d-acid synthesis, see “compound 2” in Forkuo et al. (2017).

[<sup>3</sup>H]flunitrazepam (specific activity 83 Ci/mmol) and [<sup>3</sup>H]Ro 15-4513 (specific activity 49.5 Ci/mmol) were purchased from Perkin Elmer New England Nuclear (Waltham, MA). Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepine-2-one) was from Nycomed (Opfikon, Switzerland). Standard chemicals came from Sigma-Aldrich (St. Louis, MO).

**GABA<sub>A</sub> Receptor Subunits and Mutated Subunits.** cDNAs of rat GABA<sub>A</sub> receptor subunits  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 5$ ,  $\beta 3$ , and  $\gamma 2S$  (GenBank accession numbers: NM\_183326.2, NM\_001135779.2, NM\_017069.3, NM\_017295.1, NM\_017065.1, NM\_183327.1) were cloned into pCI expression vectors. The mutant subunits (for details on the nomenclature of the mutated amino acids see Supplemental Fig. 1 and Supplemental Table 2) were constructed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) following manufacturer's instruction using the primers designed with the NEBaseChanger online tool (<https://nebbasechanger.neb.com>) and listed in Table 1. The mutated subunits were confirmed by sequencing (Table 1).

**Culturing of Human Embryonic Kidney 293 Cells.** HEK 293 cells (American Type Culture Collection ATCC CRL-1574) were maintained in Dulbecco's modified Eagle medium (high glucose, GlutaMAX supplement, Gibco 61965-059; ThermoFisher, Waltham, MA) supplemented with 10% fetal calf serum (F7524; Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Gibco 15140-122; ThermoFisher), and minimum Eagle's medium (Nonessential Amino Acids Gibco 11140-035; ThermoFisher) on 10-cm cell culture dishes (Cell<sup>+</sup>; Sarstedt, Nümbrecht, Germany) at 37°C and 5% CO<sub>2</sub>.

HEK 293 cells were transfected with cDNAs encoding rat GABA<sub>A</sub> receptor subunits subcloned into pCI expression vectors. The ratio of plasmids used for transfection with the calcium phosphate precipitation method (Chen and Okayama, 1987) were 3  $\mu$ g  $\alpha$  (1, 2, 3, or 5): 3  $\mu$ g  $\beta 3$ : 15  $\mu$ g  $\gamma 2$  per 10-cm dish. Medium was changed 4–6 hours after transfection. Cells were harvested 72 hours after transfection by scraping into phosphate-buffered saline. After centrifugation (10 minutes, 12,000g, 4°C) cells were resuspended in 50 mM Tris-citrate (TC50) (pH = 7.1), homogenized with an ULTRA-TURRAX (IKA, Staufen, Germany), and centrifuged (20 minutes, 50,000g). Membranes were washed three times in TC50 as described above and frozen at –20°C until use.

**Radioligand Binding Assay.** Frozen HEK 293 membranes were thawed, resuspended in TC50, and incubated for 90 minutes at 4°C in a total of 500  $\mu$ l of a solution containing 50 mM Tris-citrate buffer, pH = 7.1; 150 mM NaCl; and 2 nM [<sup>3</sup>H]flunitrazepam or 5 nM [<sup>3</sup>H]Ro 15-4513 in the absence or presence of 5  $\mu$ M diazepam or 50  $\mu$ M Ro 15-1788 (to determine nonspecific binding; final DMSO concentration 0.5%). Membranes were filtered through Whatman GF/B filters (GE Healthcare, distributed by VWR Austria) and rinsed twice with 4 ml of ice-cold 50 mM Tris-citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after the addition of 3 ml Rotiszint Eco plus liquid scintillation cocktail (Lactan, Graz, Austria). Nonspecific binding determined in the presence of 5  $\mu$ M diazepam or 50  $\mu$ M Ro 15-1788 was subtracted from total binding to determine specific binding.

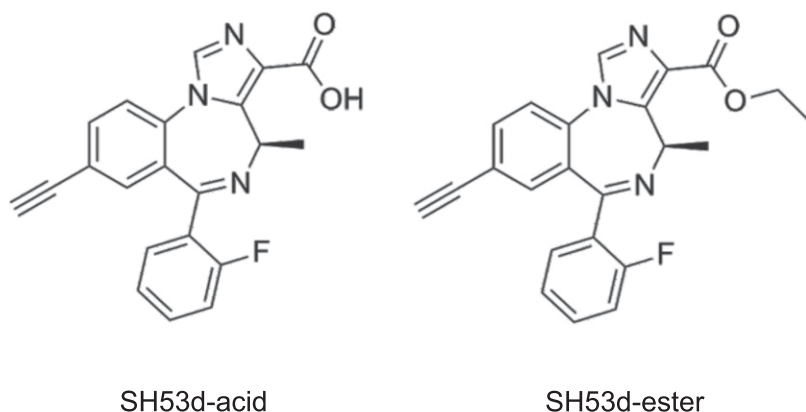


Fig. 1. Chemical structures of the two compounds.

To determine the equilibrium binding constant  $K_D$  for the various receptor subtypes, membranes were incubated with various concentrations of the radioligand in the absence or presence of an inhibitor. Saturation binding experiments were analyzed using the equation  $Y = B_{\max} \times X / (K_D + X)$ . Nonlinear regression analysis of the displacement curves used the equation:  $\log(\text{inhibitor})$  vs. response – variable slope  $Y = 100 / (1 + 10^{(\log IC_{50} - x) \times \text{Hill slope}})$  with Top = 100%; Bottom = 0%; and Hill slope = -1. Both analyses were performed using GraphPad Prism version 8.3.0 for Mac OS X (GraphPad Software, La Jolla, CA; [www.graphpad.com](http://www.graphpad.com)). Drug concentrations resulting in half-maximal inhibition of specific [ $^3\text{H}$ ]-ligand binding ( $IC_{50}$ ) were converted to  $K_i$  values by using the Cheng-Prusoff relationship (Cheng and Prusoff, 1973),  $K_i = IC_{50} / (1 + (S/K_D))$ , with S being the concentration of the radioligand (2 nM for [ $^3\text{H}$ ]flunitrazepam or 5 nM for [ $^3\text{H}$ ]Ro 15-4513) and the measured  $K_D$  values (see Table 4).

**Statistical Analysis.** One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 8.3.0 for Mac OS X; GraphPad Software, [www.graphpad.com](http://www.graphpad.com). The full ANOVA analysis is shown in the Supplemental Data.

**RNA Preparation.** RNA was prepared as described (Simeone et al., 2017): After linearizing the cDNA vectors with appropriate restriction endonucleases, the cDNA was purified and concentrated with the DNA Clean and Concentrator™ Kit (Catalog Number D4005; Zymoresearch). Capped transcripts of the purified cDNA were produced using the mMESSAGE mMACHINE T7 transcription kit (Ambion, TX) and polyadenylated using the Ambion PolyA tailing kit (Ambion). After transcription and polyadenylation, the RNA was purified with the MEGAClear™ Kit (Catalog Number AM1908; Ambion). The final RNA concentration was measured on NanoDrop ND-1000 and finally diluted and stored in diethylpyrocarbonate-treated water at  $-80^\circ\text{C}$ . For the microinjection, the RNA of  $\alpha\beta\gamma$  receptor combinations was mixed at 1:1:5 for  $\alpha x\beta 3\gamma 2$  ( $x = 1, 2, 3$ ) and 3:1:5 for  $\alpha 5\beta 3\gamma 2$ . All receptor combinations had a final concentration of 56 ng/ $\mu\text{L}$ .

**Two-Electrode Voltage Clamp Electrophysiology.** Electrophysiological experiments with *X. laevis* oocytes have been described previously (Simeone et al., 2017). Defolliculated cells were obtained from commercial suppliers (EcoCyt Bioscience Europe, Dortmund,

Germany). Healthy oocytes were injected with an aqueous solution of mRNA. A total of 2.5 ng of mRNA per oocyte was injected with a Nanoject II (Drummond). Subunit ratio was 1:1:5 for  $\alpha x\beta 3\gamma 2$  ( $x = 1, 2, 3$ ) and 3:1:5 for  $\alpha 5\beta 3\gamma 2$ . After injection of mRNA, oocytes were incubated at  $18^\circ\text{C}$  in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 5 mM HEPES; pH 7.5) containing penicillin G (10000 IU/100 ml) and streptomycin (10 mg/100 ml) for at least 36 hours before electrophysiological recordings. For current measurements, oocytes were impaled with two microelectrodes filled with 2 M KCl with 1–1.5 M $\Omega$  resistance. The oocytes were constantly washed by a flow of 6 ml/min washing buffer (96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , 5 mM HEPES,  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$  1.8 mM; pH 7.5) that could be switched to the same buffer containing GABA and/or drugs. Drugs were diluted from DMSO solutions, resulting in a final concentration of 0.1% DMSO. The GABA concentration eliciting 3–5% of the maximal GABA response ( $EC_{3-5}$ ) was determined at the beginning of each experiment. Maximum currents measured in mRNA-injected oocytes were in the microampere range for all receptor subtypes. Compounds were coapplied with GABA until a peak response was observed. Enhancement of the chloride current was defined as  $(I_{\text{GABA+Comp}}/I_{\text{GABA}}) - 1$ , wherein  $I_{\text{GABA+Comp}}$  is the current response in the presence of a given compound, and  $I_{\text{GABA}}$  is the control GABA current. Between two applications, oocytes were washed in NDE for up to 15 minutes to ensure full recovery from desensitization. SH53d-acid did not affect the pH of the measuring buffer (7.51–7.52) over the whole concentration range of the compound. pH was measured in the buffer alone, in GABA  $EC_{3-5}$ , and in GABA  $EC_{3-5}$  containing SH53d-acid at 1 nM to 10  $\mu\text{M}$ . Since the measurement buffer keeps the pH constant, we can be sure that over the dose-response curve, the protonation state of the compound did not change. Beyond this observation, we cannot draw any conclusions about the influence of the protonation state on the activity. It seems reasonable to assume that this compound was, as organic acid, deprotonated at physiologic pH. As has been discussed in Forkuo et al., (2018), this is beneficial for topical application. All recordings were performed at room temperature at a holding potential of  $-60$  mV using a Dagan CA-1B Oocyte Clamp or a Dagan TEV-200 A TEV amplifier (Dagan Corporation, Minneapolis, MN). Data were digitized using a Digidata 1322 A or 1550 data acquisition system (Axon Instruments, Union City, CA), recorded using Clampex 10.5 software (Molecular Devices, Sunnyvale, CA), and analyzed using Clampfit 10.5. Data were analyzed using GraphPad Prism v.6 and plotted as concentration-response curves. These curves were normalized and fitted by nonlinear regression analysis to the equation  $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log EC_{50} - X) \times n_H})$ , wherein  $EC_{50}$  is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and  $n_H$  is the Hill coefficient. Data are given as mean  $\pm$  S.D. from at least three oocytes of two or more oocyte batches. Statistical significance was calculated using an extra sum of squares  $F$  test.  $P$  values of  $<0.05$  were accepted as statistically significant.

**Computational Docking.** The experimental structures 6HUO, 6D6T, and 6A96 containing the  $\alpha 1\gamma 2$  interface were employed for this

TABLE 1

Cloning primers

Primer sequences for the cloning of mutated  $\alpha 3$  and  $\alpha 5$  GABA $_A$  receptor subunits. Mutated bases are highlighted in italic. Numbering corresponds to precursor proteins based on the UniProtKB-accession numbers P20236 and P19969.

Q5- $\alpha 3$ -T215P-FW	CTATGCCTAT <sub>2</sub> CCAAAGCTGAAG
Q5- $\alpha 3$ -T215P-RV	CTTCCAAACTTCAGTGGAC
Q5- $\alpha 3$ -S257T-FW	GATAATCCGG <sub>a</sub> CTAGTACAGG
Q5- $\alpha 3$ -S257T-RV	TCTGTCCCAAC <sub>a</sub> ACATGAC
Q5- $\alpha 5$ -197T-FW	TTATGCTTAC <sub>a</sub> CTAATTCCGGAAG
Q5- $\alpha 5$ -197T-RV	CTGCCAAAT <sub>T</sub> TTCAGGGGAC
Q5- $\alpha 5$ -239S-FW	AACATCAGCAG <sub>a</sub> CAGCACAGGT
Q5- $\alpha 5$ -239S-RV	CTCAGTGCCTACTGTCTGC

study. The 6A96 structure does not contain a benzodiazepine site. The  $\alpha 5$  subunit's extracellular domain of 6A96 overlaps well with the  $\alpha 1$  subunits in 6HUO, 6D6T, and 6D6U. Thus, since benzodiazepine-bound states are of interest, we used 6HUO and 6D6T to generate homology models of the extracellular  $\alpha 5/\gamma 2$ - interface on the basis of the alprazolam- and flumazenil-bound experimental structures using Modeller (Sali and Blundell, 1993) for the subsequent docking. Molecular docking was performed with the program GOLD (Jones et al., 1997). The numbering of the amino acids in the figures corresponds to mature human protein UniProtKB-accession numbers P14867 for  $\alpha 1$  and P31644 for  $\alpha 5$  (for comparison between rat and human amino acid numbering, see Supplemental Table 2). The putative binding site was set around the  $\beta$  carbon atom of the  $\alpha 5$ Ser209 that lies on the loop C with a cutoff distance of 12 Å. Five amino acids of the  $\gamma 2$ -subunit and five of the  $\alpha 5$ -subunit were selected to have flexible side chains:  $\gamma 2$ Asp56,  $\gamma 2$ Tyr58,  $\gamma 2$ Phe77,  $\gamma 2$ Thr142, and  $\gamma 2$ Arg144 and  $\alpha 5$ Tyr163,  $\alpha 5$ Thr208,  $\alpha 5$ Ser209,  $\alpha 5$ Thr210, and  $\alpha 5$ Tyr213, respectively. Soft potentials were chosen for  $\alpha 5$ Thr208,  $\alpha 5$ Ser209,  $\alpha 5$ Thr210, and  $\alpha 5$ Gly211 to simulate backbone flexibility of loop C. The ligand was docked with the protonated carboxylic acids group fully flexible and the seven-ring restrained to what is seen in the experimental structures (a control run with a flexible seven-ring gave very similar results). The poses were scored with GoldScore and rescored with ChemScore, as implemented in GOLD. For each ligand and each protein, the top-100 (GoldScore) poses were generated with default settings.

## Results

**SH53d-Acid has Superb Affinity, Efficacy, and Selectivity.** Since the ester-to-amide substitution in SH53d-ester led to MP-III-022 with improved binding and efficacy selectivity, more substituents were explored. Here, we present an acid-substituted compound with superior affinity selectivity. To directly compare the properties of this novel compound SH53d-acid with its parent compound SH53d-ester, both were measured at identical conditions side-by-side in radioligand binding assays as well as in two-electrode voltage clamp experiments. Although SH53d-acid still displays low affinity for  $\alpha 1$ ,  $\alpha 2$ , or  $\alpha 3\beta 3\gamma 2$  receptors (in the micromolar range) in radioligand binding assays, the affinity toward  $\alpha 5\beta 3\gamma 2$  is approximately 10-fold higher than the parent compound, now being in the low nM range (see Fig. 2B; Table 2). SH53d-acid has superior (>40-fold) affinity selectivity for  $\alpha 5$ - over  $\alpha 1$ -containing GABA<sub>A</sub> receptors, which is much more selective than MP-III-022 (15-fold) and SH53d-ester (9-fold). There is also a clear window of separation between  $\alpha 5$ - and  $\alpha 3$ -containing GABA<sub>A</sub> receptors with an affinity selectivity of 16-fold in SH53d-acid compared with 12-fold in MP-II-022, whereas it is only 7-fold in SH53d-ester. Compared with  $\alpha 3$ -containing GABA<sub>A</sub> receptors, the differences in affinities are 13-fold (SH53d-acid), being nearly equal to the parent compound SH53d-ester (15-fold), whereas MP-III-022 has the smallest difference (6-fold). In two-electrode voltage clamp experiments, we not only observed a similar affinity shift but also greatly enhanced efficacy in modulating GABA-induced currents in  $\alpha 5$ -containing receptors compared with the other receptors analyzed (Fig. 2D; Table 3). The SH53d-acid has a wider window of separation, with nearly no modulatory activity in the non- $\alpha 5$  subtypes up to 30 nM (see Table 3). As is also the case for diazepam, the efficacy is higher in  $\alpha 3$ -containing receptors compared with  $\alpha 2$ -containing receptors, but unlike diazepam, it is highest in  $\alpha 5$ -containing receptors (Savić et al., 2010) (Fig. 2; Tables 2 and 3).

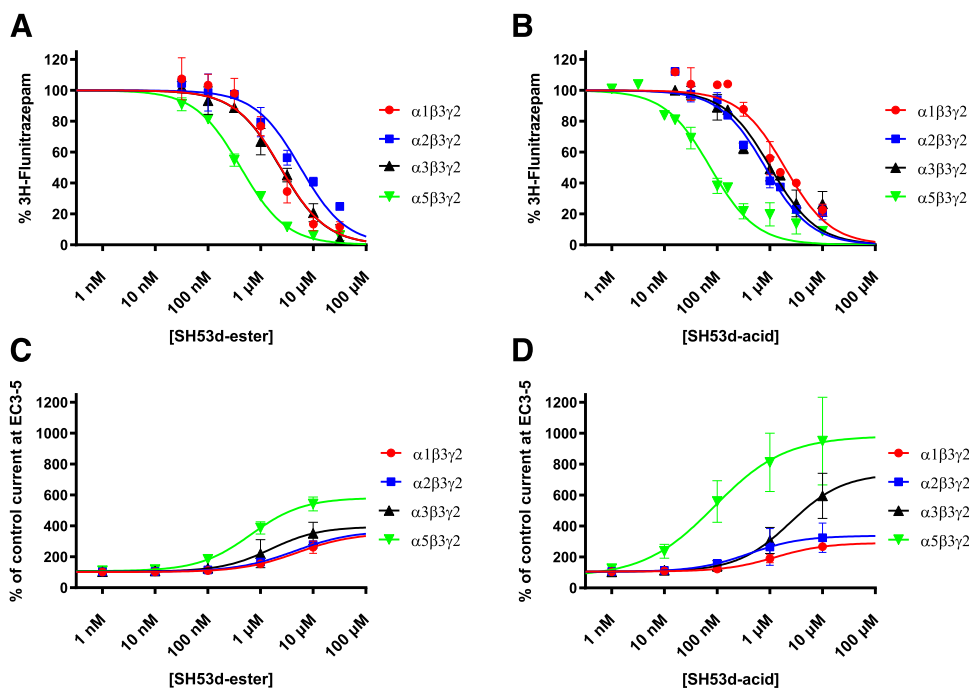
**The Acid Group of SH53d-Acid Is Predicted to Be in Contact with Loop C.** To provide a structural hypothesis for the extraordinary potency preference of the novel ligand SH53d-acid, we performed a computational study utilizing the recently published flumazenil- and alprazolam-bound structures PDB IDs 6D6T and 6D6U (Zhu et al., 2018) and PDB ID 6HUO (Masiulis et al., 2019) together with homology models and computational docking as established in our laboratories (Elgarf et al., 2018; Siebert et al., 2018).

Figure 3 shows the unligated pocket of the recently released heteropentameric cryo-EM structure PDB ID 6D6T (Phulera et al., 2018) and the  $\alpha 5$ -subunits of the PDB ID 6A96 structures (Liu et al., 2018), providing an overview of the pocket contributing amino acids of these two  $\alpha$  isoforms. At the time of writing, no experimental structures of the other  $\alpha$  isoforms were available (Fig. 3).

Computational docking has resulted in two different binding modes as candidates for benzodiazepine- and imidazobenzodiazepine-based ligands, previously termed binding modes one (BMI) and two (BMII) (Richter et al., 2012; Middendorp et al., 2014; Elgarf et al., 2018; Siebert et al., 2018). The R-substituted chiral analogs of SH53d-ester have been proposed previously to use BMI (Elgarf et al., 2018), which later was observed experimentally for flumazenil in the 6D6T and 6D6U structures (Zhu et al., 2018). For the triazolobenzodiazepine alprazolam, on the other hand, a binding mode was observed experimentally, which corresponds to BMII (Richter et al., 2012; Middendorp et al., 2014; Masiulis et al., 2019).

Both the flumazenil- and the alprazolam-bound structures were determined with the  $\alpha 1$ -subunit, whereas for  $\alpha 5$ , so far only an interface with a  $\beta$ -subunit is available (Liu et al., 2018, see Fig. 3). Since the structural similarity between these two  $\alpha$  isoforms is sufficiently high, we took advantage of the induced-fit states of the flumazenil- and alprazolam-bound states and generated the  $\alpha 5$ -containing homologs from these. Subsequent computational docking into the original  $\alpha 1$ -containing structures and the  $\alpha 5$ -containing models produced similar posing space for both isoforms. The results for the  $\alpha 5$ -containing pocket yield a diversity of highly ranked poses without a clear favorite: Docking SH53d-acid into the models of both the flumazenil-bound pocket and the alprazolam-bound pocket yielded among both top-20 ChemScore and GoldScore results both flumazenil-like (BMI) and alprazolam-like (BMII) poses as well as unrelated binding modes. Similarity was assessed by computing pairwise root-mean-square deviation (RMSD) of the common atoms of the three ligands in the superposed pairs of experimental structure with the models. Five poses within ChemScore top 20 and two among GoldScore top 20 are within an RMSD of 0.5–1.5 Å relative to alprazolam. In the comparison with flumazenil, poses with RMSD up to 2 Å contained two in the ChemScore top 20. Thus, the alprazolam-like binding mode was observed more often, but higher level of computational methods would be needed for firm conclusions. Representative poses are depicted in Fig. 4.

Importantly, both binding modes position the acid group in close proximity to segment (loop) C of the pocket. Thus, for both binding modes the structural evidence predicts a strong impact of diverging amino acids in segment C to drive the affinity differences, whereas the difference in segment (loop) B should be of less influence. To test this structural hypothesis, we studied conversion mutants. For the mutational work, we



**Fig. 2.** Affinity and efficacy data in the four diazepam-sensitive GABA<sub>A</sub> receptor subtypes. To directly compare the properties of SH53d-acid and SH53d-ester, both were measured at identical conditions side by side (A and B): Inhibition of binding of [<sup>3</sup>H]flunitrazepam to recombinant  $\alpha\beta\gamma 2$  GABA<sub>A</sub> receptors. Membranes from HEK 293 cells transfected with the GABA<sub>A</sub> receptor subunit combinations were incubated with 2 nM [<sup>3</sup>H]flunitrazepam in the presence of various concentrations of SH53d-ester (A) or SH53d-acid (B). Values are given as mean  $\pm$  S.D. of three experiments performed in duplicates each. (C and D) Concentration-response curves of the compounds SH53d-ester (C) or SH53d-acid (D) in  $\alpha\beta\gamma 2$  GABA<sub>A</sub> receptors expressed in *X. laevis* oocytes using GABA EC<sub>3-5</sub>. Values are given as mean  $\pm$  S.D.,  $n = 3$  or higher from at least two batches of oocytes.

sought to narrow down the influence of amino acids that are different among  $\alpha$  isoforms but do not contribute to the  $\alpha 5$  unique properties. In segment C, the  $\alpha 5$ - and  $\alpha 1$ -subunits differ in two pocket-forming amino acids (see Fig. 3), whereas the  $\alpha 5$ - and  $\alpha 3$ -subunits differ only in one of these. We therefore chose to compare the two subunits that share more amino acids in the pocket and proceeded to the mutational analysis with a side-by-side comparison of the  $\alpha 5$ - and  $\alpha 3$ -subunits as described in the *Materials and Methods*.

**Choice of Radioligand: 3H-Flunitrazepam Proves to Be Unsuitable.** Based on our computational analysis we decided to generate mutated subunits: In the B-loop amino acids, proline and threonine were exchanged between  $\alpha 3$  and  $\alpha 5$ , generating an  $\alpha 3$ T215P- and an  $\alpha 5$ P197T-subunit. Likewise, in the C-loop, amino acids serine and threonine were switched, generating  $\alpha 3$ S257T and  $\alpha 5$ T239S. Those mutated  $\alpha$ -subunits were cotransfected with  $\beta 3\gamma 2$  in HEK 293 cells, and the receptors formed were analyzed via radioligand binding experiments.

Equilibrium binding assays showed that receptors composed of  $\alpha 5$ (P197T) $\beta 3\gamma 2$  bind [<sup>3</sup>H]flunitrazepam with similar high affinity as  $\alpha 5$ (wt) $\beta 3\gamma 2$  ( $K_D$ :  $1.9 \pm 0.2$  and  $2.2 \pm 0.3$  nM, respectively). In contrast,  $\alpha 5$ (T239S) $\beta 3\gamma 2$  receptors lost this high-affinity binding and showed a 10-fold shift in  $K_D$  to  $23.4 \pm 6.1$  nM (see Fig. 5).

Radioligand displacement studies cannot be performed using a low-affinity ligand. Therefore,  $\alpha 5$ (T239S) $\beta 3\gamma 2$  receptors could

not easily have been analyzed using [<sup>3</sup>H]flunitrazepam, and we needed to search for another radioligand: Casula et al. (2001) described similar mutants in their publication and showed that the benzodiazepine Ro 15-4513 had a much higher affinity to the mutated receptors compared with flunitrazepam. We therefore decided to test [<sup>3</sup>H]Ro 15-4513 as possible radioligand, and equilibrium binding assays were performed on transfected HEK 293 cells. As shown in Table 4, [<sup>3</sup>H]Ro 15-4513 exhibited high-affinity binding for all constructs/receptors tested.

**Conversion Mutations Confirm Loop B Is Not the Determinant of the Selectivity.** We transfected HEK 293 cells with various GABA<sub>A</sub> receptor subunit combinations and performed radioligand displacement assays with 5 nM [<sup>3</sup>H]Ro 15-4513 and increasing concentrations of SH53d-acid. The concentrations resulting in half-maximal inhibition of radioligand binding were converted into  $K_i$  values by using the Cheng-Prusoff relationship, and the respective  $K_D$  values were given in Table 4.

Figure 6B shows the comparison of the  $K_i$  values obtained from the B-loop mutant  $\alpha 5$ P197T and  $\alpha 3$ T215P compared with wild-type receptors (Panel A). As can be seen, the compound SH53d-acid still binds to  $\alpha 5$ -mutant receptors at very high (nanomolar) concentrations and to  $\alpha 3$ -mutant receptors at micromolar concentrations. It seems that proline 197 in the B loop of the benzodiazepine binding pocket does not contribute to the  $\alpha 5$ -selective binding properties of SH53d-acid.

TABLE 2

Potency of [<sup>3</sup>H]flunitrazepam displacement in  $\alpha\beta\gamma 2$  receptors recombinantly expressed in HEK 293 cells

The concentrations resulting in half-maximal inhibition of specific binding from the experiment shown in Fig. 2, A and B were converted to  $K_i$  values using the Cheng-Prusoff relationship (see *Materials and Methods*). Data are reported as mean  $\pm$  S.D. from three displacement curves performed in duplicates each

Compound	Ki $\pm$ S.D. in $\mu$ M				
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 1/\alpha 5$
SH53d-ester	$1.9 \pm 0.5$	$3.3 \pm 0.6$	$1.6 \pm 0.4$	$0.22 \pm 0.03$	9
SH53d-acid	$1.6 \pm 0.44$	$0.53 \pm 0.14$	$0.65 \pm 0.22$	$0.039 \pm 0.003$	41

TABLE 3  
Efficacy at  $\alpha\beta\gamma 2$  receptor expressed in *X. laevis* oocytes given as % of control current at 30 nM concentration of the compound  
Subunit combinations expressed in *X. laevis* oocytes were analyzed as shown in Figs. 1, C and D. The currents at 30 nM were extrapolated via nonlinear regression analysis from the curves shown. Respective  $EC_{50}$  values are given below.

Compound	% of Control Current at 30 nM ( $EC_{50}$ )				
	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 5$	$\alpha 1/\alpha 5$
SH53d-ester	105 (5.0 $\mu$ M)	108 (4.4 $\mu$ M)	112 (1.6 $\mu$ M)	138 (0.7 $\mu$ M)	1.3
SH53d-acid	112 (1.3 $\mu$ M)	127 (0.4 $\mu$ M)	120 (2.4 $\mu$ M)	380 (0.09 $\mu$ M)	3.3

**Affinity Change Induced by Conversion Mutations Confirm Loop C as Dominant Molecular Determinant of the Selectivity.** Figure 6C shows the  $K_i$  values obtained from the C-loop mutants  $\alpha 3S257T$  and  $\alpha 5T239S$ . Exchanging the amino acid sequence from a threonine to a serine at position  $\alpha 5\ 239/\alpha 3\ 257$  in the C loop of the subunits changed the binding properties of SH53d-acid in gaining nanomolar binding to the  $\alpha 3$  mutant while shifting  $\alpha 5$ -mutant binding into the micromolar range. It can therefore be concluded that threonine 239 in the C loop of the benzodiazepine binding pocket contributes heavily to the  $\alpha 5$ -selective binding properties of SH53d-acid (for detailed statistical analysis see Supplemental Fig. 2). For the parent compound SH53d-ester, the influence of threonine 239 in the C loop is much less pronounced (see Supplemental Fig. 2).

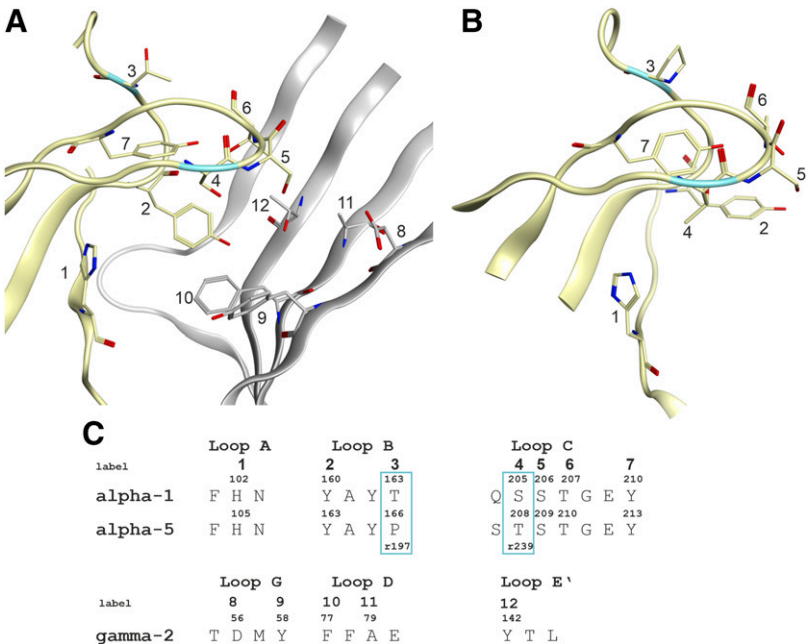
In line with the  $K_i$  values, we also observe a right-shifted dose response in the modulation of  $\alpha 5T239S$  ( $EC_{50}$  of 555 vs. 60 nM in the wild type) and no change in  $EC_{50}$  for the B-loop mutant (see Fig. 6). Both mutants do not strongly impact efficacy (Fig. 6).

Discussion

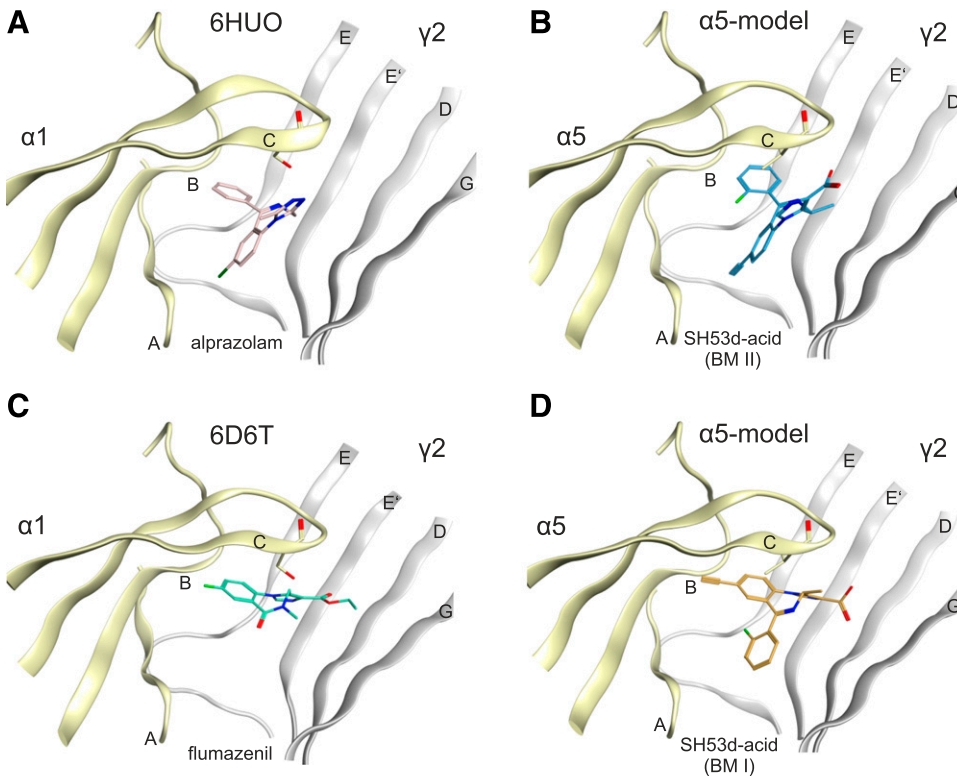
Subtype-selective ligands of the benzodiazepine binding site of GABA<sub>A</sub> receptors are highly desirable both as research tools to study the role of individual receptor subtypes in neuronal circuits and CNS function (Drexler et al., 2013; Sieghart, 2015)

and to selectively modulate these receptors in experiments investigating animal models of diseases as well as GABA effects in non-neuronal cells that express GABA<sub>A</sub> receptors. Because of the high homology of the six  $\alpha$  subunits and particularly of the four diazepam-sensitive  $\alpha 1$ -,  $\alpha 2$ -,  $\alpha 3$ -, and  $\alpha 5$ -subunits, compounds with pronounced selectivity are still very rare (Rudolph and Möhler, 2014). One of the first  $\alpha 5\beta\gamma 2$ -selective compounds reported was L-655,708 (Quirk et al., 1996), a benzodiazepine negative allosteric modulator, which exerts its subtype selectivity via high-affinity binding while showing no change in efficacy (Casula et al., 2001). The imidazobenzodiazepine SH-053-2'F-R-CH<sub>3</sub> (SH53d-ester) proved to be the first  $\alpha 5\beta\gamma 2$ -selective positive allosteric modulator (Savić et al., 2010). Here, we present the pharmacology of a derivative of the  $\alpha 5$ -preferring compound SH53d-ester with improved GABA<sub>A</sub>  $\alpha 5$ -subunit-selective properties. This novel compound SH53d-acid displays a 40-fold-higher affinity toward  $\alpha 5$ -containing receptors. In addition, it shows pronounced efficacy selectivity: The maximal modulation of GABA  $EC_{3-5}$  achieved at high concentrations is 3-fold higher in  $\alpha 5\beta\gamma 2$  compared with  $\alpha 1\beta\gamma 2$ . This combination of affinity and efficacy selectivity enables a specific concentration range (up to ~30 nM) in which other  $\alpha$ -containing receptors are not yet modulated, whereas GABA currents in  $\alpha 5$  receptors are markedly enhanced.

SH53d-acid has already been shown to be effective as an airway smooth muscle relaxant (Forkuo et al., 2017). Airway



**Fig. 3.** The benzodiazepine pocket of the cryo-EM structure (6D6T) and the human  $\alpha 5$  subunit in 6A96. Color coding: Amino acids differing between  $\alpha 5$  and  $\alpha 1$  are marked in cyan.  $\gamma 2$ -Subunit is light gray.  $\alpha 5$ - and  $\alpha 1$ -subunits are in pale yellow. Consecutive numbers are used in the images and the partial alignment to identify the depicted side-chains. (A) The benzodiazepine pocket of the  $\alpha 1$  (pale yellow)/ $\gamma 2$  (gray) GABA<sub>A</sub> receptor with the pocket-forming amino acids in stick rendering. (B) The principal subunit of the benzodiazepine pocket of the  $\alpha 5$ -subunit. (C) Partial alignment of the  $\alpha 1$ - and  $\alpha 5$ -subunits, with the mutated amino acids marked by cyan boxes, pocket-forming amino acids from the  $\gamma 2$ -subunit. For comparison with the rat amino acid numbering, see Supplemental Table 2.



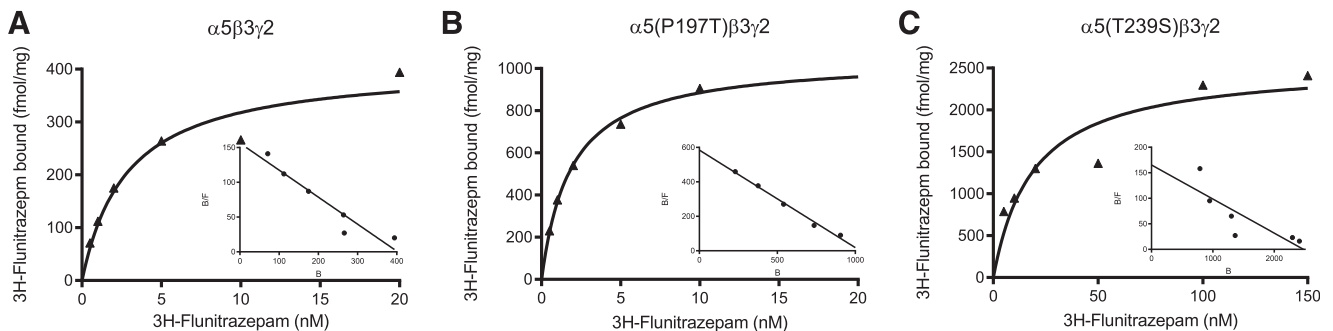
**Fig. 4.** Comparison of alprazolam and flumazenil binding modes with representative results from the top-20 SH53d-acid poses. (A) 6HUO with alprazolam; (B) representative alprazolam-like (BMII) pose; (C) 6D6T with flumazenil; (D) representative flumazenil-like (BMI) pose. Color coding: pale yellow ribbons:  $\alpha 1/\alpha 5$ -subunits; gray ribbons:  $\gamma 2$ -subunit; stick rendering: O is red, N is blue. The Ser/Thr position in which  $\alpha 5$  sequence is uniquely featuring Thr is also shown in stick representation.

smooth muscle cells contain several GABA<sub>A</sub> receptor subunits, in which, among the  $\alpha$  subunits, only  $\alpha 4$  and  $\alpha 5$  are expressed (Mizuta et al., 2008; Gallos et al., 2015; Yocum et al., 2016). It has been demonstrated previously that targeting GABA<sub>A</sub> receptors containing either one of these subunits leads to relaxation of precontracted ex vivo ASM from guinea pig, mouse, and human (Gallos et al., 2015; Yocum et al., 2016) and, indeed, so does SH53d-acid (Forkuo et al., 2017).

A combination of computational modeling, mutagenesis, and radioligand binding assays has been used to determine the amino acids responsible for the exceptional  $\alpha 5$ -subtype selectivity of SH53d-acid. The benzodiazepine ligand binding site is situated at the extracellular interface between adjacent  $\alpha$ - and  $\gamma$ -subunits and is being lined by amino acids located in seven so-called “loops.” Loops A, B, and C are on the principal (+) side of the  $\alpha$ -subunit, whereas loops D, E, F, and G are on the complementary (–) side of the  $\gamma 2$ -subunit (Richter et al.,

2012; Middendorp et al., 2014). The amino acids of loop B (GSYAYT in the subunits  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ; GSYAYP in  $\alpha 4$ ,  $\alpha 5$ , and  $\alpha 6$ ) have been studied previously to understand how they are involved in ligand binding. Especially  $\alpha 1$ -alanine 160 seems to contribute to the benzodiazepine binding site, whereas  $\alpha 1$ -threonine 162 seems to be of less importance (Morlock and Czajkowski, 2011). In our experiments, this region in loop B did not contribute to the  $\alpha 5$ -selective binding properties of SH53d-acid. Our findings are in line with Moody and Jenkins (2018), who describe that mutations in loop B made no significant shifts in affinity and only modest changes in maximum whole-cell current modulation to benzodiazepine (such as midazolam) binding.

Loop C, on the other hand, has long been known to be important for ligand binding since it is more variable than the other loops (Michałowski et al., 2017).  $\alpha 1$ -Serine 206 (neighboring the serine mutated in our study) seems to interact



**Fig. 5.** [<sup>3</sup>H]flunitrazepam equilibrium binding assays. Membranes from transfected HEK 293 cells were incubated with 1–20 nM (A and B) and 5–150 nM (C) [<sup>3</sup>H]flunitrazepam in absence or presence of 5  $\mu$ M diazepam (to determine nonspecific binding). Radioactivity bound to the membranes was determined after rapid filtration. Inserts show the Scatchard transformation of the results. Data represent a single experiment performed in duplicates each. Experiments were repeated three to four times with similar results.

TABLE 4  
Equilibrium binding constant ( $K_D$ ) for [ $^3$ H]Ro 15-4513 binding to the different receptor subtypes  
Membranes from HEK 293 cells transfected with the subunit combinations as indicated were incubated with various concentrations of [ $^3$ H]Ro 15-4513 in either the absence or presence of 50  $\mu$ M Ro 15-1788 (to determine unspecific binding). Results were analyzed using the equation  $Y = B_{max} \times X/(K_D + X)$ .  $K_D$  values are presented as mean  $\pm$  S.D. from three to five independent experiments performed in duplicates.

Subunit combination	$\alpha 3(\text{wt})\beta 3\gamma 2$	$\alpha 3(\text{T215P})\beta 3\gamma 2$	$\alpha 3(\text{S257T})\beta 3\gamma 2$
$K_D \pm \text{S.D. (nM)}$	$10.45 \pm 4.5$	$2.85 \pm 1.12$	$13.57 \pm 0.73$
Subunit combination	$\alpha 5(\text{wt})\beta 3\gamma 2$	$\alpha 5(\text{P197T})\beta 3\gamma 2$	$\alpha 5(\text{T239S})\beta 3\gamma 2$
$K_D \pm \text{S.D. (nM)}$	$0.16 \pm 0.09$	$0.23 \pm 0.04$	$0.47 \pm 0.13$

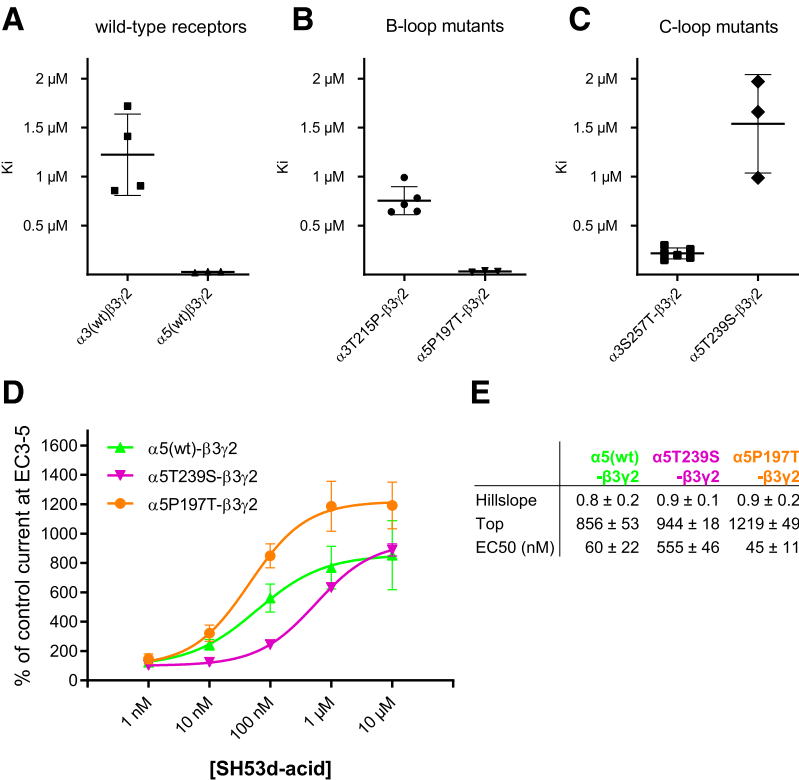
physically with diazepam (Lüscher et al., 2012). Both  $\alpha 1$ -serine 206 as well as  $\alpha 1$ -tyrosine 209 are important in determining the binding affinities for ligands of the benzodiazepine binding (Buhr et al., 1997), and S206 has been shown to influence the efficacy of midazolam to modulate GABA-induced currents (Moody and Jenkins, 2018) as well as the affinities for  $\beta$ -carboline binding (Derry et al., 2004). The neighboring amino acid  $\alpha 1$ -threonine 207 specifically alters benzodiazepine affinity while leaving binding unaffected (Morlock and Czajkowski, 2011).

In our study mutating the conserved serine **SxTGEY** in the subunits  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ , and  $\alpha 6$  versus **TSTGEY** in  $\alpha 5$  had a huge influence in shifting binding affinities, proving to be the dominant molecular determinant of drug selectivity. Although the S > T mutation is conserved, the threonine side chain has an additional methyl group and thus a larger volume and hydrophobic surface. It could be speculated that ligand burial is improved, leading to a more efficient change in conformation and higher affinity of binding.

Two recently released cryo-EM structures feature binding modes of flumazenil and alprazolam that are otherwise quite different but share the positioning of the imidazole ring, which bears the acid group. Computational docking of the

SH53d-acid results in both predictions (i.e., the compound can assume a flumazenil-like or an alprazolam-like position in the pocket). In both cases, the acid group is in the vicinity of loop C. Thus, our docking results correlate well with experimental evidence but do not differentiate between these two known binding modes. The cryo-EM structures were obtained with human subunits, whereas our experimental data were obtained with rat subunits. The  $\alpha$  isoforms are highly conserved across mammalian species (%ID between rat and human subunits is 98% for  $\alpha 1$  and  $\alpha 3$  and 96% for  $\alpha 5$ , respectively; the binding sites are 100% conserved), and both rodent and human subunits are broadly used to characterize benzodiazepines. Thus, the use of the human structural data is valid for the interpretation of our experimental data.

In the current study we provide the detailed pharmacological characterization of SH53d-acid and describe its superior (>40-fold) affinity selectivity for  $\alpha 5$ -containing GABA<sub>A</sub> receptors. This makes SH53d-acid a potentially very useful research tool for applications, such as slice electrophysiology, because of its much wider window of selectivity. In addition, in the current study we were able to identify the molecular basis for drug selectivity: Computational docking combined with mutational analysis identified loop C of the GABA<sub>A</sub> receptor



**Fig. 6.** Inhibition constants ( $K_i$ ) of SH53d-acid competition for [ $^3$ H]Ro 15-4513 binding and TEV functional data in  $\alpha 5\text{T239S}\beta 3\gamma 2$ - and  $\alpha 5\text{P197T}\beta 3\gamma 2$ -injected oocytes. (A–C) Membranes from transfected HEK 293 cells were incubated with 5 nM [ $^3$ H]Ro 15-4513 in the presence of various concentrations of SH53d-acid. The concentrations resulting in half-maximal inhibition of radioligand binding were converted into  $K_i$  values by using the Cheng-Prusoff relationship, and the respective  $K_D$  values were given in Table 4. For detailed statistical analysis, see Supplemental Fig. 3. (D and E) Concentration-response curves and respective fitting parameters of SH53d-acid in mutated  $\alpha 5\beta 3\gamma 2$  GABA<sub>A</sub> receptors expressed in *X. laevis* oocytes. Values are given as mean  $\pm$  S.D.,  $n = 4$  to 5 for at least two batches of oocytes.

$\alpha$ -subunit as the dominant molecular determinant of drug selectivity. Thus, we characterize a promising novel  $\alpha 5$ -subunit-selective drug candidate and suggest that this atom-level structural hypothesis can now be used toward a structure-guided design of further novel compounds with similarly pronounced selectivity and otherwise improved properties. As suggested based on in vivo data from murine asthma models (Forkuo et al., 2017), this compound might be suitable for clinical development as a topical asthma medication. Compared with the -ester or -amide analogs, it is more selective and, as an acid, is expected to be very inefficient in passing the blood-brain barrier.

Additionally, our results may pave the way to improved  $\alpha 5$ -subunit-selective drug candidates useful also for CNS applications based on screening compounds into pharmacophore models based on the SH53d-acid-bound state model. There is still big interest in  $\alpha 5$ -subunit-selective CNS-permeant positive modulators to alleviate, for example, certain symptoms of schizophrenia (Gill and Grace, 2014; Jacob, 2019).

#### Acknowledgments

We thank Sabah Rehman for the support with the electrophysiological measurements and the data analysis. We acknowledge the help of Florian D. Vogel with the sequence comparisons.

#### Authorship Contributions

*Participated in research design:* Ernst, Scholze.

*Conducted experiments:* Simeone, Koniuszewski, Müllegger, Smetka, Steudle, Puthenkalam.

*Performed data analysis:* Simeone, Koniuszewski, Steudle, Ernst, Scholze.

*Wrote or contributed to the writing of the manuscript:* Simeone, Koniuszewski, Ernst, Scholze.

#### References

- Barragan A, Weidner JM, Jin Z, Korpi ER, and Birnir B (2015) GABAergic signalling in the immune system. *Acta Physiol (Oxf)* **213**:819–827.
- Bellesi D and Lambert JJ (2005) Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat Rev Neurosci* **6**:565–575.
- Brickley SG and Mody I (2012) Extrasynaptic GABA(A) receptors: their function in the CNS and implications for disease. *Neuron* **73**:23–34.
- Buhr A, Schaerer MT, Baur R, and Sigel E (1997) Residues at positions 206 and 209 of the  $\alpha 1$  subunit of gamma-aminobutyric acid A receptors influence affinities for benzodiazepine binding site ligands. *Mol Pharmacol* **52**:676–682.
- Casula MA, Bromidge FA, Pillai GV, Wingrove PB, Martin K, Maubach K, Seabrook GR, Whiting PJ, and Hadingham KL (2001) Identification of amino acid residues responsible for the  $\alpha 5$  subunit binding selectivity of L-655,708, a benzodiazepine binding site ligand at the GABA(A) receptor. *J Neurochem* **77**:445–451.
- Chen C and Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7**:2745–2752.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K<sub>i</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Derry JMC, Dunn SMJ, and Davies M (2004) Identification of a residue in the gamma-aminobutyric acid type A receptor  $\alpha 5$  subunit that differentially affects diazepam-sensitive and -insensitive benzodiazepine site binding. *J Neurochem* **88**:1431–1438.
- Drexler B, Zinser S, Huang S, Poe MM, Rudolph U, Cook JM, and Antkowiak B (2013) Enhancing the function of  $\alpha 5$ -subunit-containing GABA<sub>A</sub> receptors promotes action potential firing of neocortical neurons during up-states. *Eur J Pharmacol* **703**:18–24.
- Elgarf AA, Siebert DCB, Steudle F, Draxler A, Li G, Huang S, Cook JM, Ernst M, and Scholze P (2018) Different benzodiazepines bind with distinct binding modes to GABA<sub>A</sub> receptors. *ACS Chem Biol* **13**:2033–2039.
- Fischer BD, Licata SC, Edwankar RV, Wang ZJ, Huang S, He X, Yu J, Zhou H, Johnson EM Jr, Cook JM, et al. (2010) Anxiolytic-like effects of 8-acetylene imidazobenzodiazepines in a rhesus monkey conflict procedure. *Neuropharmacology* **59**:612–618.
- Fleck MW, Thomson JL, and Hough LB (2012) Histamine-gated ion channels in mammals? *Biochem Pharmacol* **83**:1127–1135.
- Forkuo GS, Nieman AN, Kodali R, Zahn NM, Li G, Rashid Roni MS, Stephen MR, Harris TW, Jahan R, Guthrie ML, et al. (2018) A novel orally available asthma drug candidate that reduces smooth muscle constriction and inflammation by targeting GABA<sub>A</sub> receptors in the lung. *Mol Pharm* **15**:1766–1777.
- Forkuo GS, Nieman AN, Yuan NY, Kodali R, Yu OB, Zahn NM, Jahan R, Li G, Stephen MR, Guthrie ML, et al. (2017) Alleviation of multiple asthmatic pathologic features with orally available and subtype selective GABA<sub>A</sub> receptor modulators. *Mol Pharm* **14**:2088–2098.
- Gallós G, Yocum GT, Siviski ME, Yim PD, Fu XW, Poe MM, Cook JM, Harrison N, Perez-Zoghbi J, and Emala CW Sr (2015) Selective targeting of the  $\alpha 5$ -subunit of GABA<sub>A</sub> receptors relaxes airway smooth muscle and inhibits cellular calcium handling. *Am J Physiol Lung Cell Mol Physiol* **308**:L931–L942.
- Gill KM and Grace AA (2014) The role of  $\alpha 5$  GABA<sub>A</sub> receptor agonists in the treatment of cognitive deficits in schizophrenia. *Curr Pharm Des* **20**:5069–5076.
- Gladkevich A, Korf J, Hakobyan VP, and Melkonian KV (2006) The peripheral GABAergic system as a target in endocrine disorders. *Auton Neurosci* **124**:1–8.
- Hanrahan JR, Chebib M, and Johnston GA (2011) Flavonoid modulation of GABA(A) receptors. *Br J Pharmacol* **163**:234–245.
- Hoerbel P, Lindsley TA, and Fleck MW (2015) Dopamine directly modulates GABA<sub>A</sub> receptors. *J Neurosci* **35**:3525–3536.
- Jacob TC (2019) Neurobiology and therapeutic potential of  $\alpha 5$ -GABA type A receptors. *Front Mol Neurosci* **12**:179.
- Jones G, Willett P, Glen RC, Leach AR, and Taylor R (1997) Development and validation of a genetic algorithm for flexible docking. *J Mol Biol* **267**:727–748.
- Khom S, Strommer B, Ramharther J, Schwarz T, Schwarzer C, Erker T, Ecker GF, Mulzer J, and Hering S (2010) Valerianic acid derivatives as novel subunit-selective GABA<sub>A</sub> receptor ligands - in vitro and in vivo characterization. *Br J Pharmacol* **161**:65–78.
- Liu S, Xu L, Guan F, Liu YT, Cui Y, Zhang Q, Zheng X, Bi GQ, Zhou ZH, Zhang X, et al. (2018) Cryo-EM structure of the human  $\alpha 5\beta 3$  GABA<sub>A</sub> receptor. *Cell Res* **28**:958–961.
- Lorenz M, Kabir MS, and Cook JM (2010) A two step synthesis of BzR/GABAergic active flavones via a Wacker-related oxidation. *Tetrahedron Lett* **51**:1095.
- Lüscher BP, Baur R, Goeldner M, and Sigel E (2012) Influence of GABA(A) receptor  $\alpha$  subunit isoforms on the benzodiazepine binding site. *PLoS One* **7**:e42101.
- Masiulis S, Desai R, Uchanski T, Serna Martin I, Laverty D, Karia D, Malinauskas T, Zivanov J, Pardon E, Kotecha A, et al. (2019) GABA<sub>A</sub> receptor signalling mechanisms revealed by structural pharmacology. *Nature* **565**:454–459.
- Michalowski MA, Kraszewski S, and Mozrzymas JW (2017) Binding site opening by loop C shift and chloride ion-pore interaction in the GABA<sub>A</sub> receptor model. *Phys Chem Chem Phys* **19**:13664–13678.
- Middendorp SJ, Puthenkalam R, Baur R, Ernst M, and Sigel E (2014) Accelerated discovery of novel benzodiazepine ligands by experiment-guided virtual screening. *ACS Chem Biol* **9**:1854–1859.
- Mizuta K, Xu D, Pan Y, Comas G, Sonett JR, Zhang Y, Panettieri RA Jr, Yang J, and Emala CW Sr (2008) GABA<sub>A</sub> receptors are expressed and facilitate relaxation in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **294**:L1206–L1216.
- Möhler H (2011) The rise of a new GABA pharmacology. *Neuropharmacology* **60**:1042–1049.
- Moody OA and Jenkins A (2018) The role of loops B and C in determining the potentiation of GABA<sub>A</sub> receptors by midazolam. *Pharmacol Res Perspect* **6**:e00433.
- Morlock EV and Czajkowski C (2011) Different residues in the GABA<sub>A</sub> receptor benzodiazepine binding pocket mediate benzodiazepine efficacy and binding. *Mol Pharmacol* **80**:14–22.
- Olsen RW and Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* **60**:243–260.
- Phulera S, Zhu H, Yu J, Claxton DP, Yoder N, Yoshioka C, and Gouaux E (2018) Cryo-EM structure of the benzodiazepine-sensitive  $\alpha 1\beta 1\gamma 2S$  tri-heteromeric GABA<sub>A</sub> receptor in complex with GABA. *eLife* **7**:e39383.
- Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, and McKernan RM (1996) [3H]L-655,708, a novel ligand selective for the benzodiazepine site of GABA<sub>A</sub> receptors which contain the  $\alpha 5$  subunit. *Neuropharmacology* **35**:1331–1335.
- Richter L, de Graaf C, Sieghart W, Varagic Z, Mörzinger M, de Esch IJ, Ecker GF, and Ernst M (2012) Diazepam-bound GABA<sub>A</sub> receptor models identify new benzodiazepine binding-site ligands. *Nat Chem Biol* **8**:455–464.
- Rudolph U and Möhler H (2014) GABA<sub>A</sub> receptor subtypes: therapeutic potential in Down syndrome, affective disorders, schizophrenia, and autism. *Annu Rev Pharmacol Toxicol* **54**:483–507.
- Sali A and Blundell TL (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* **234**:779–815.
- Saras A, Gisselmann G, Vogt-Eisele AK, Erkkamp KS, Kletke O, Pusch H, and Hatt H (2008) Histamine action on vertebrate GABA<sub>A</sub> receptors: direct channel gating and potentiation of GABA responses. *J Biol Chem* **283**:10470–10475.
- Savić MM, Majumder S, Huang S, Edwankar RV, Furtmüller R, Joksimović S, Clayton T Sr, Ramerstorfer J, Milinković MM, Roth BL, et al. (2010) Novel positive allosteric modulators of GABA<sub>A</sub> receptors: do subtle differences in activity at  $\alpha 1$  plus  $\alpha 5$  versus  $\alpha 2$  plus  $\alpha 3$  subunits account for dissimilarities in behavioral effects in rats? *Prog Neuropsychopharmacol Biol Psychiatry* **34**:376–386.
- Sengupta S, Weeraratne SD, Sun H, Phallen J, Rallapalli SK, Teider N, Kosaras B, Amani V, Pierre-Francois J, Tang Y, et al. (2014)  $\alpha 5$ -GABA<sub>A</sub> receptors negatively regulate MYC-amplified medulloblastoma growth. *Acta Neuropathol* **127**:593–603.
- Siebert DCB, Bampali K, Puthenkalam R, Varagic Z, Sarto-Jackson I, Scholze P, Sieghart W, Mihovilovic MD, Schnürch M, and Ernst M (2018) Engineered flumazenil recognition site provides mechanistic insight governing benzodiazepine modulation in GABA<sub>A</sub> receptor. *ACS Chem Biol* **13**:2040–2047.
- Sieghart W (2015) Allosteric modulation of GABA<sub>A</sub> receptors via multiple drug-binding sites. *Adv Pharmacol* **72**:53–96.
- Sieghart W and Savić MM (2018) International union of basic and clinical pharmacology. CVI: GABA<sub>A</sub> receptor subtype- and function-selective ligands: key issues in translation to humans. *Pharmacol Rev* **70**:836–878.
- Sigel E, Baur R, Rácz I, Marazzi J, Smart TG, Zimmer A, and Gertsch J (2011) The major central endocannabinoid directly acts at GABA(A) receptors. *Proc Natl Acad Sci USA* **108**:18150–18155.

- Sigel E and Lüscher BP (2011) A closer look at the high affinity benzodiazepine binding site on GABAA receptors. *Curr Top Med Chem* **11**:241–246.
- Simeone X, Siebert DCB, Bampali K, Varagic Z, Treven M, Rehman S, Pyszkowski J, Holzinger R, Steudle F, Scholze P, et al. (2017) Molecular tools for GABA<sub>A</sub> receptors: high affinity ligands for  $\beta$ 1-containing subtypes. *Sci Rep* **7**:5674.
- Stamenić TT, Poe MM, Rehman S, Santrać A, Divović B, Scholze P, Ernst M, Cook JM, and Savić MM (2016) Ester to amide substitution improves selectivity, efficacy and kinetic behavior of a benzodiazepine positive modulator of GABA<sub>A</sub> receptors containing the  $\alpha$ 5 subunit. *Eur J Pharmacol* **791**:433–443.
- Wan Y, Wang Q, and Prud'homme GJ (2015) GABAergic system in the endocrine pancreas: a new target for diabetes treatment. *Diabetes Metab Syndr Obes* **8**:79–87.
- Yocum GT, Gallos G, Zhang Y, Jahan R, Stephen MR, Varagic Z, Puthenkalam R, Ernst M, Cook JM, and Emala CW (2016) Targeting the  $\gamma$ -aminobutyric acid A receptor  $\alpha$ 4 subunit in airway smooth muscle to alleviate bronchoconstriction. *Am J Respir Cell Mol Biol* **54**:546–553.
- Zhu S, Noviello CM, Teng J, Walsh RM Jr, Kim JJ, and Hibbs RE (2018) Structure of a human synaptic GABA<sub>A</sub> receptor. *Nature* **559**:67–72.

---

**Address correspondence to:** Petra Scholze, Medical University of Vienna, Center for Brain Research, Spitalgasse 4, A-1090 Vienna, Austria. E-mail: [petra.scholze@meduniwien.ac.at](mailto:petra.scholze@meduniwien.ac.at); or Margot Ernst, Medical University of Vienna, Center for Brain Research, Spitalgasse 4, A-1090 Vienna, Austria. E-mail: [margot.ernst@meduniwien.ac.at](mailto:margot.ernst@meduniwien.ac.at)

---