Molecular tools for GABA\textsubscript{A} receptors: High affinity ligands for \(\beta1\)-containing subtypes

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\(\gamma\)-Aminobutyric acid type A (GABA\textsubscript{A}) receptors are pentameric GABA-gated chloride channels that are, in mammalians, drawn from a repertoire of 19 different genes, namely \(\alpha1\)-6, \(\beta1\)-3, \(\gamma1\)-3, \(\delta\), \(\epsilon\), \(\theta\), \(\pi\) and \(\rho1\)-3. The existence of this wide variety of subunits as well as their diverse assembly into different subunit compositions result in miscellaneous receptor subtypes. In combination with the large number of known and putative allosteric binding sites, this leads to a highly complex pharmacology. Recently, a novel binding site at extracellular \(\alpha+\beta\) - interfaces was described as the site of modulatory action of several pyrazoloquinolines. In this study we report a highly potent ligand from this class of compounds with pronounced \(\beta1\)-selectivity that mainly lacks \(\alpha\)-subunit selectivity. It constitutes the most potent \(\beta1\)-selective positive allosteric modulatory ligand with known binding site. In addition, a proof of concept pyrazoloquinolone ligand lacking the additional high affinity interaction with the benzodiazepine binding site is presented. Ultimately, such ligands can be used as invaluable molecular tools for the detection of \(\beta1\)-containing receptor subtypes and the investigation of their abundance and distribution.

GABA\textsubscript{A} receptors are pentameric ligand-gated ion channels that can be opened by GABA and alternative agonists, as well as modulated by multiple endogenous or exogenous allosteric ligands, some of which have high clinical importance\textsuperscript{1}. In the nervous system GABA\textsubscript{A} receptors are, among others, targets of certain sleeping aids, general anesthetics and antiepileptic medications. High affinity ligands of the benzodiazepine binding site of these receptors are also used as versatile CNS imaging tools\textsuperscript{2}. Specific receptor subtypes also occur in diverse peripheral tissues where their function is largely unknown\textsuperscript{3,4}.

A total of 19 genes encode, in mammalian species, GABA\textsubscript{A} receptor subunits (\(\alpha1\)-6, \(\beta1\)-3, \(\gamma1\)-3, \(\delta\), \(\epsilon\), \(\theta\), \(\pi\) and \(\rho1\)-3)\textsuperscript{5}. Specific subunits assemble into homo- or hetero- pentameric arrangements, whereby a given pentamer with defined subunit composition and arrangement is referred to as receptor subtype. The receptor subtype composed of \(\alpha1\), \(\beta3\) and \(\gamma2\) subunits was shown to be arranged as \(3\beta3\)-\(\alpha1\)-\(\beta2\) (\(3\beta3\)-\(\alpha1\), where each subunit interface by definition has a principal (plus) and a complementary (minus) side\textsuperscript{6}. The total number of pentameric arrangements that exist in mammalian species is still unknown\textsuperscript{7}, but given the repertoire of 19 subunits, it could be large.

The conserved cys-loop receptor structure harbors a large number of binding sites, including those for the generic agonist GABA, for channel blockers such as picrotoxin, and for a wide range of allosteric modulators\textsuperscript{8}. Each binding sites’ ligand preferences are determined by the subunits that contribute to it. The ion channel pore is formed by the five transmembrane domain two segments (TM2)\textsuperscript{9,10}. Agonist sites are at extracellular interfaces between specific subunits such as the bicuculline insensitive \(p+/p\) - and the bicuculline sensitive \(\beta+/\alpha-\) sites\textsuperscript{11}. Allosteric sites have been described at interfaces and in other locations in the extracellular and transmembrane domains\textsuperscript{8}.

Together, the staggering variety of receptor subtypes and the large number of binding sites on each subtype results in a highly complex pharmacology\textsuperscript{1}. Specific high affinity ligands of GABA\textsubscript{A} receptor subtypes are invaluable tools to study their abundance and distribution in tissues and to detect them in living organisms. Unselective

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high affinity ligands that can be employed to detect large pools of GABA_A receptors exist for specific applications, such as autoradiography and radioligand binding studies. In contrast, only very few tool compounds exist that display specific high affinity binding at individual subtypes. Selective molecular tools do exist for the widely expressed α2 subunit containing receptors. A number of high affinity benzodiazepine site ligands have been developed that facilitate their selective detection in biological samples and in vivo.22,12. These ligands bind to the high affinity benzodiazepine binding site that is formed by a principal α subunit (α1, α2, α3 or α5) together with a complementary γ2 subunit.13 Among them, ligands that bind with higher affinity to α5+γ2 have been identified, such as Ro 15–4513, which is used as α5-specific PET ligand to detect the receptor subtypes containing α5+γ2 binding sites in humans.14,15

In contrast, no high affinity ligands exist that are selective for receptors containing a specific β isoform. Fragrant dioxane derivatives (FDDs) have been described as a novel structural class of GABA_A receptor positive allosteric modulators with β1-subunit selectivity and have been proven useful for functional studies. However, the relatively low micromolar potency prevents their use as radioligands. Furthermore, salicylidene salicylhydrazide (SCS) has been described as potent partial and selective antagonist of β1-containing receptors and has been used successfully for the functional identification of β1-containing receptors. Nevertheless, it has not been developed into a tool for binding studies so far. For both FDDs and SCS binding sites are unknown. In contrast, a number of modulators for the GABA_A receptor with enhanced selectivity for the β2/β3 subunits over the β1 subunit have been reported, e.g. etomidate, loreclezole and a valerenic acid derivative.24–26 Studies in transgenic animals have shown that compounds that target individual β isoforms selectively would be highly useful, as different effects of sedative and anesthetic compounds could be separated.

We have recently described allostERIC modulation of diverse GABA_A receptors by several pyrazoloquinolines (PQs) that use a binding site at extracellular α+β− interfaces. Since all six α isoforms and all three β isoforms contribute unique amino acid residues to that binding site, it should be possible to identify highly selective ligands for any αk+/βl− (k = 1–6, l = 1–3) combination. We have previously studied 32 pyrazoloquinolines and pyrazolopyridinones at the α1+/β3− binding site,23 and 16 of those were investigated for possible α subtype selectivity. Among the compounds that up until now were only studied as ligands of the α1+/β3− binding site, we selected for this follow up study three analogues which modulated α1β3 receptors with efficacy higher than 300%25 and three analogues thereof (see Fig. 1). Here possible potency selectivity for either α isoforms, or β isoforms was investigated.

We identified, and present here, a highly potent ligand (1) of the α+β− sites featuring an EC_{50} of 130 nM at α1+/β3− interfaces. This ligand is also a benzodiazepine site ligand, and thus not a selective tool for α1+/β1− interfaces. Consequently, we also generated an analogue (7) that lacks benzodiazepine site interaction while largely retaining the desired activity at the homologous α1+/β1− interface site. These studies pave the way towards high affinity molecular tools for the selective detection of receptor subtypes that contain specific αk+/βl− (any of k = 1–6, l = 1–3) interfaces.
Results

Mini library of compounds aimed at studying potency driving ligand features. In our previous work we identified compounds 1–3 to be efficacious modulators of the extracellular α1+/β3− interface site23, and thus selected these for a follow up study to investigate potential potency preferences for any subtype. Due to the strong impact of the R8 substituent on compound efficacy 23, we added three more analogues (compounds 4–6) with another residue in this position. Ligand 7 (see Fig. 1) was added later to confirm the observation that bulk in R8 interferes with the unwanted benzodiazepine site affinity while, at least for some ligands, retaining modulatory action at the extracellular α1+/β3− interface site23.

Compound 1 exerts very similar effects in α1β3, α1β3γ2 and α1β3δ receptors. As we have described previously, many R8 and R4 di-substituted pyrazoloquinolines not only interact with the α+/β− interfaces, but also bind with very high affinity to α+/-β− interfaces (benzodiazepine binding sites) 22, 23, 26. For a library screen, binary αβ receptors offer the advantages that they lack the high affinity benzodiazepine binding site, and express robustly, quickly and consistently in the Xenopus laevis oocyte. To clarify whether the use of binary receptors gives satisfactory results, we carefully investigated the modulatory effects of compound 1 in α1β3, α1β3γ2 (diazepam sensitive, see methods) and α1β3δ (DS2 sensitive, see methods) expressing oocytes as shown in Fig. 2.

Since the modulatory effects that are exerted from the α+/β− interface are nearly unaffected by the presence of a γ2 or a δ subunit, we proceeded to screen our mini library in binary receptors. At the experimental conditions used in this study, the α1β3 (l = 1, 2, 3) receptors formed in the oocyte are thought to be of α1(2)/β3(3) stoichiometry22.

Potency selectivity for β3-containing receptors. Next, we searched for selectivity in α1β3 (l = 1, 2, 3) receptors (see Fig. 3a–f). First, compounds 1–6 were investigated without GABA using α1β3 (l = 1, 2, 3) expressing oocytes. None of the compounds displayed any GABA independent effects at 10 and 30 μM. Compound modulatory effects were then investigated at low GABA concentrations (corresponding to EC 3–5), where all compounds showed to be positive modulators, and all of them displayed higher potency in α1β3 receptors than in α1β2 or α1β3 receptors (see Fig. 3). The most potent ligand in α1β3 is compound 1 (EC 50: 130 nM). Moreover, compounds 3 and 4 also display high potencies (~200 nM) for α1β3. Potency differences between α1β3 and α1β2 are statistically significant for compounds 1, 2, 4 and 5 (p < 0.001, p < 0.01, p < 0.01 and p < 0.05, respectively). Furthermore, compounds 1, 3 and 4 (p < 0.0001, p < 0.01, and p < 0.05, respectively) also show statistically significant potency differences between α1β3 and α1β3.

All compounds have approximately the same efficacy in α1β3 and enhance the GABA EC 3–5 currents in this subtype up to ~400%. On the other hand, the efficacy in β2– and β3-containing receptors varies widely: 1 and 4 have much higher efficacy in α1β2 and α1β3 compared to α1β3, 2 and 5 modulate all three receptors to the same degree, while 3 and 6 display reduced efficacy in α1β2 and α1β3 compared to α1β3 (see Fig. 3).

We observed that all six ligands influenced receptor kinetics in a way such that at low compound concentrations, the current rise was delayed compared to the reference GABA trace (see Fig. 3g and Supplementary Fig. S7 panel f). Interestingly, compounds 1 and 3, as well as 4 and 6 also accelerate current decay at high concentrations (see panel g in Fig. 3 and Supplementary Fig. S7), while 2 and 5 do not. A similar phenomenon has been observed and reported previously for an unrelated allosteric modulator28. We explain the apparent drop in efficacy at high concentrations of compounds 4 and 3 by the accelerated current decay. At very high concentrations, the current decay is so fast that the peak amplitude of the initial current enhancement drops (see Fig. 3g). Thus, to obtain
Figure 3. Compounds 1–6 show potency selectivity for β1-containing receptors. Dose-response data of compounds 1–6 at α1β1, α1β2 and α1β3 subunit combinations; (a–c) Left, aggregate dose-response curves of R^0 = chloro compounds 1–3 co-applied with GABA EC_{3–5}. Right, EC_{50} values obtained by fitting data of each cell individually; (d–f) Left, aggregate dose-response curves of R^0 = methoxy compounds 4–6 co-applied with GABA EC_{3–5}. Right, EC_{50} values obtained by fitting data of each cell individually. Highest potency was consistently observed at α1β1 receptors. Compound 6 (f) lacked efficacy at α1β2 and α1β3, therefore EC_{50} values could not be obtained. In those instances where high compound concentrations elicited substantial desensitization (see panels a, c, d, f and sample traces in (g,h)), the highest compound concentration was excluded from the fit. Statistically significant differences were assessed by one-way ANOVA with Tukey’s multiple comparison test; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. = not significant, n.d. = not determined. n = 3–8. (g–i) Sample traces obtained with compound 1. Note the desensitization in α1β1 (g) at 10µM and 30µM, increasingly limiting maximum current amplitudes. Tabulated data corresponding to panels a–f are provided in Supplementary Tables S1–S6. Additional sample traces are provided in Supplementary Fig. S7.
compound EC₅₀ values, only the data points that fall on the sigmoidal phase of the curve were utilized (see Supplementary Tables S1–S6).

The Hill slopes of the compound dose-response curves range from 1 to 3 (see Supplementary Tables S1–S6). This is consistent with the recently proposed view that some pyrazoloquinolinolones may have additional binding sites in the transmembrane domain of certain specific subunit combinations.

**Mutational analysis supports the main site of action to be at the extracellular minus side of the β subunit.** As additional binding sites for pyrazoloquinolinolones have been proposed, we aimed to investigate the molecular determinants which lead to the potency preference of our test ligands for the β₁ isoform. We compared the different extracellular minus sides utilizing homology models based on the recently published β₃-homopentameric crystal structure. These models (see Supplementary Fig. S8) indicate that the amino acids corresponding to β₁R41 and β₃N41 (numbering according to mature rat protein without signal peptide) on segment (or “loop”) G, which is structurally a strand within a beta-pleated sheet, are in the variable position most central in the pocket. These are in close interaction with the predicted ligand occupied space (see Supplementary Fig. S8). In the benzodiazepine binding site, the homologous sub-domain has been shown to impact on ligand binding. To test the influence of this amino acid on potency and efficacy of our ligands, two “conversion” mutants were generated. By the point mutations β₃N41R and β₁R41N the variable amino acid on segment G was exchanged between these two isoforms, leading to two engineered subunits. These presumably display properties mostly derived from the parent subunit, but locally changed ligand interactions.

The binary α₁β₁R41N receptor displayed variable and often large holding currents which are indicative of spontaneous channel activity. This phenomenon has been described for several point mutations that also displayed spontaneous currents. On the other hand, the binary α₁β₁R41N receptor behaved similarly as the wild type α₁β₁ (1, 2, 3, 5) receptors. The GABA dose-response curves of both mutants are slightly left shifted (EC₅₀ ~2 µM) compared to the ones of the wild type α₁β₁ or α₁β₃ receptors (see Supplementary Fig. S9 and Supplementary Table S10). Maximum GABA currents are similar as in the wild type receptors, and the Hill coefficients are ~1.2 for both mutated receptors (see Supplementary Table S10).

Next, the modulatory effects of the compounds were examined in both mutated receptors. Interestingly, the positive modulatory effects are completely abolished for three ligands and dramatically reduced for compounds 1 and 4, whereas compound 2 is reducing GABA currents in the α₁β₁R41N receptor (see Supplementary Table S11). In contrast, the α₁β₃N41R combination displayed modulatory responses to all ligands. We observed significant changes in potency compared to the wild type (parent) α₁β₃ receptor for three ligands (see Fig. 4). For one ligand (6) potency in the wild type could not be determined due to the very low efficacy – but interestingly the loop G mutation induced β₁-like efficacy in this case (see Supplementary Fig. S12). For two additional ligands, we noted an increase in efficacy as a result of the mutant (see Fig. 4).

Interestingly, the substituent in R⁴ seems to determine how the ligand interacts with the mutated β subunit. Both compounds with a methoxy substituent in R⁴ show a left shift with a resulting potency in the mutant receptor that is intermediate between the values of the two wild type receptors α₁β₁ and α₁β₃ (see Fig. 4, Supplementary Fig. S12). For compounds bearing R⁴ = methyl we observed a strong enhancement of efficacy in the α₁β₃N41R receptor (see Fig. 4, Supplementary Fig. S12), with no significant change in potency. For the R⁴ = amino substituted compound 3, the mutation led to a marked left shift such that potency for α₁β₃N41R and for α₁β₁ are identical (complete conversion, see Fig. 4 and Supplementary Fig. S12). Overall, these observations demonstrate that both potency and efficacy of the PQ compounds are differentially determined in part by the amino acid in position 41 of the minus side segment G in α₁β₁ and α₁β₃ receptors and thus strongly support the notion that the modulatory effects are mainly elicited by the tested ligands at the extracellular α+/β− interface.

**The investigated compounds show limited α selectivity.** Each R⁴ = chloro compound was more potent in the α₁β₁ receptor compared to their respective methoxy analogues, thus, we followed up in more detail on compounds 1, 2 and 3. Compound 1 already has been investigated in 22 receptor subtypes, namely in α₅β₃ (k = 1, 2, 3, 5) and ok33-2 (k = 1–6, l = 1–3), and displayed nearly no potency differences among ok33-2 (k = 1–6, l = 1–3) or ok33 (k = 1, 2, 3, 5) receptors (see Supplementary Table S13), while displaying pronounced functional preference for α₆-containing receptors. Thus, we investigated a possible α subtype selectivity of compounds 2 and 3. For ok33-2 (k = 1–6) combinations the expression protocols are well established and all combinations express reasonably well showing consistent responses to diazepam for ok33-2 (k = 1, 2, 3, 5). In contrast, for β₁-containing combinations, this is not the case and some combinations proved to be difficult to express and characterize. Thus, in order to study the impact of the α isoforms, we utilized the β₁ subunit throughout. Table 1 shows the EC₅₀ and pEC₅₀ values obtained for the six ok33-2 (k = 1–6) subunit combinations for compounds 2 and 3.

Compound 2 modulates all six ok33-2 (k = 1–6) subtypes with EC₅₀ values in the range ~5 to ~15 µM (see Table 1), and thus without any marked potency selectivity for any of the six α isoforms. The maximum efficacies were also not indicative of any efficacy-selective effect, ranging from ~200% in the α₅-containing receptor subtype to ~600% in the α₁-containing subtype, with the exception of the ok33-2 subtype which displayed higher efficacy (>1000% modulation at 10 µM, see Supplementary Table S14).

Compound 3 exerts modulatory effects at α₁- and α₃β₃/2 receptors up to ~200% with an EC₅₀ of ~1 µM (see Supplementary Table S15). The α₃β₃/2 subtype once again was modulated with the highest efficacy in comparison. Due to the low efficacies in the ok-containing (k = 2, 4, 5) receptors, EC₅₀ values could not be determined in these receptors, but can be estimated to be in the micromolar, >10 µM range. Four of the α isoforms, namely α₁, α₂, α₃ and α₅ also produce binary ok33 receptors with robust GABA currents, while α₄ and ok33 receptors feature very small GABA currents. We compared ok33 (k = 1, 2, 3 and 5 that are diazepam insensitive) with ok33-2 (k = 1, 2, 3 and 5 that are diazepam sensitive) receptors and once
and 1.87, 1, 2, 3) combinations (see Fig. 3), we identified compound the results in the = l (l α α β γ 3 2 (see Supplementary Tables S15 and S17). effects in 3 2 for compound α in more detail. β for the extracellular 1 1 1 + − that the presence of the benzodiazepine binding sites formed by α1, α2, α3 or α5 subunits is also silent. Together with the results in the α1/β (l = 1, 2, 3) combinations (see Fig. 3), we identified compound 1 as the most potent ligand for the extracellular α1+/β1 – site and thus followed up on compound 1 in more detail.

The δ and the γ1 subunits have no impact on compound 1 potency for the α1+/β1 – site. For compound 1 the previously published data indicates that there is very little influence of the γ2 subunit on the
modulatory effect\(^22, 23\) in spite of the very high potency of this compound for the diazepam sensitive benzodiazepine sites\(^26\) of \(\alpha_k/\beta_2\) receptors (\(k = 1, 2, 3\) and \(5\)). Here we investigated the question whether the more potent interaction with the \(\alpha_1/+/\beta_1–\) site is also not influenced by the presence of a third subunit. We obtained consistent GABA responses, as well as consistent modulation by triazolam for \(\alpha_1/\beta_1–1\) receptors (triazolam sensitive, see methods and Supplementary Table S18) while the incorporation of \(\gamma_2\) seemed to be more variable. Similarly, the \(\alpha_1/\beta_16\) combination (DS2 sensitive, see methods) also proved to be well behaved (see Supplementary Table S18). Figure 5 shows that the potency and efficacy of compound 1 are not changed by the presence of either the \(\gamma_1\) or the \(\delta\) subunit.

A derivative of compound 1 that lacks affinity for the benzodiazepine binding site also modulates \(\alpha_1/\beta_1\)-containing receptors. Since many \(R^8\) and \(R^4\) substituted pyrazoloquinolinones not only interact with the \(\alpha_1/+/\beta_1–\) interfaces, but are very high affinity ligands at \(\alpha_k/+/\gamma_2–\) interfaces (i.e. benzodiazepine site ligands)\(^22, 23, 26\), we examined the affinity of our six test ligands for the \(\alpha_1/+/\gamma_2–\) site with flunitrazepam displacement assays using cerebellar membrane preparations from rat brains. The data indicate that all six ligands from the mini library are high affinity binders at the major \(\alpha_1/+/\gamma_2–\) benzodiazepine binding site (see Table 2).

We have reported previously an \(R^6\) substituted pyrazoloquinolinone with dramatically reduced benzodiazepine site affinity and robust \(\alpha_1/+/\beta_1–\) modulatory effects\(^29\). Thus, here we investigated the possibility that an analogous derivatization of compound 1 may result in similar ligand properties.

The resulting compound 7 (chloro-\(\beta_6\)-methoxy; LAU462, see Fig. 1) has indeed no affinity for the benzodiazepine binding site (see Table 2), and was thus also tested functionally in \(\alpha_1/\beta_1/\gamma_1\) and \(\alpha_1/\beta_16\) receptors. Figure 6 shows that it exerts modulatory effects quite similar to those of the parent compound 1, but with an approximately twenty-fold right shift (see Supplementary Table S19 for data tables and Supplementary Fig. S20 for a sample trace). Again, we find no impact of the third subunit (\(\gamma_1\) or \(\delta\)) on apparent potency.

In contrast to compound 1, it modulates the \(\alpha_1/\beta_16\) receptor with higher efficacy (compare Fig. 5 to 6b). Accordingly, the \(R^8\) substituent that nearly abolished the sub-nanomolar affinity for the benzodiazepine site has a comparatively weaker impact on the potency at the \(\alpha_1/+/\beta_1–\) site. Thus, compound 7 serves as proof of principle for a potential development of ligands that target this binding site exclusively, and with useful potency.

### Table 2. \(K_i\) values of compounds 1–7 determined by displacement of \([3H]\)flunitrazepam binding to rat cerebellar membranes (mean ± SEM, \(n = 3–4\)).

| \(R^8\) | \(R^4\) | \(R^6 = \text{Cl}\) | \(R^6 = \text{OCH}_3\) |
|---|---|---|---|
| H | OMe | 1 | 0.06 ± 0.02 | 4 | 0.07 ± 0.007 | 4 |
| H | Me | 2 | 0.05 ± 0.001 | 3 | 0.05 ± 0.002 | 3 |
| H | NH\(_2\) | 3 | 0.12 ± 0.03 | 6 | 1.00 ± 0.08 | 3 |
| \(\beta\)Bu | OMe | 7 | n.d. >100\(\mu\)M | — | — | — |

### Figure 5. Compound 1 modulates GABA-evoked currents in \(\alpha_1/\beta_1\), \(\alpha_1/\beta_1/\gamma_1\) and \(\alpha_1/\beta_16\) receptors with similar potencies. (a) Concentration-dependent modulation of GABA \(EC_{50}\) current at \(\alpha_1/\beta_1\), \(\alpha_1/\beta_1/\gamma_1\) and \(\alpha_1/\beta_16\). Data represent means ± SEM (\(n = 3–10\)). (b) \(EC_{50}\) values were calculated for each individual experiment and are presented as mean ± SEM. One-Way ANOVA was used for multiple comparisons followed by a Tukey post hoc test and showed no significant differences between the mean \(EC_{50}\) values for each subtype.
as reflected by the rank order. Concentration-dependent modulation of GABA EC_{50} current at α1β1, α1β1γ1 and α1β1δ receptors. Based on the most potent ligand feature underlies the different potency rank orders in γ2, γ3. γ2 performs best for the α1 isoform. It is interesting to note here that the methoxy group in all cases, the degree of polarity seems more important for the pronounced potency preference for α1 selective fragrant dioxane derivatives (FDDs) 16, the β3 isoform. These are not the first β3-selective ligands that are allosteric modulators of several GABAA receptor subtypes. However, in comparison with the previously published β3 selective partial negative modulator SCS on the other hand has very high potency 17. While this compound has successfully been used in a number of interesting functional and biological assays, it so far has not been developed into a tool compound for the selective detection of β3-containing receptors. Moreover, its binding site in the TM domain is not known exactly, and it is also not yet known if it features a combined selectivity profile for certain other subunits, as it has been investigated only in a total of four receptor subtypes. We have tested the selective effects of the pyrazoloquinolinones presented here in a wider panel of receptor subtypes compared to FDDs or SCS.

The potential usefulness of highly potent PQ ligands with β3-selectivity is large. Along these lines, future efforts will be directed towards a detailed understanding of ligand features that reduce affinity for α+γ2− while retaining and improving affinity for α+β−. The long term goal is to develop ligands which can be isoform labeled and used for the specific detection and quantification of β3-containing receptors. Here, the additional activity at the benzodiazepine binding site can be overcome readily for studies in ex vivo samples by blocking this site with any unlabeled high affinity benzodiazepine site ligand 35. The described compounds offer good opportunities for isotopic labeling in the future. Compounds 1 and 4–7 contain a methoxy group, which can be used to introduce isotopic labeling in the future. Compounds 1 and 4–7 contain a methoxy group, which can be used to introduce
[11CH₃] in the last stage of the synthesis, starting from the corresponding phenols. Furthermore, Schnürch and coworkers have published a proof of principle study for the titration of nitrogen containing heterocycles⁶⁶, and this method can be applied to all described compounds. Radioligands will accelerate the testing of candidate compounds for α+/β− binding sites considerably, as the screening for new hits using functional assays is very slow.

Future applications of (suitably labelled) α+/β− specific ligands are broad. For example, it has been discussed controversially whether cerebellar Purkinje cells express β subunits: Sergeeva and colleagues found no evidence, while Kelley et al. present evidence in favor⁶⁶–⁶⁷. Tool compounds for the specific detection of α+/β− interfaces in radioligand assays or autoradiographic studies, or with which receptors that contain this interface can be manipulated selectively in acute slices or in cultured neurons, could be helpful to investigate further. The pyrazoloquinolinone scaffold is also particularly attractive for the development of tool compounds to be used in vivo, such as experimental drugs for behavioral studies, or as PET ligands, because it already has been demonstrated to possess very low toxicity and adequate bio-availability⁶⁸.

Materials and Methods

**GABA<sub>A</sub> receptor subunits and mutated subunits.** cDNAs of rat GABA<sub>A</sub> receptor subunits α1, α4, β1, β2, β3 and γ2S were cloned as described³⁹. cDNAs of the rat subunits α2, α3 and α5 were gifts from P. Malherbe, that of α6 and γ1 were gifts of P. Seeburg and that of β6 was a gift of C. Czajkowski. The mutants were constructed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) following manufacturer’s instructions. We used the wild-type rat β3-pCI vector as template and the primers GTGCGGTAGGGATCGACATCG and GCAGACTGGGGGACCCTCC resulting in a substitution of amino acid R41 (CGG) to N (AAC). The mutated rat α1-pCI vector as template and the primers CGTCGCGGATGAACTGATGTGGCC and TCCACGCGGGGCCCTTCA resulting in a substitution of amino acid R41 (CGG) to N (AAC). The mutated subunits were confirmed by sequencing.

**RNA Preparation.** In vitro transcription of mRNA was based on the cDNA expression vectors encoding for rat GABA<sub>A</sub> receptor subunits α1−6, β1−3, γ1−2, δ and the two β mutants (β1R41N and β3N41R)⁴⁰. After linearizing the cDNA vectors with appropriate restriction endonucleases, the DNA was purified and concentrated with the DNA Clean and Concentrator™ Kit (Zymo Research, Catalog No. D4005). Capped transcripts of the purified cDNA were produced using the mMESSAGE mMACHINE<sup>®</sup> T7 transcription kit (Ambion, TX, USA) and polyadenylated using the Ambion Poly A tailing kit (Ambion). After transcription and polyadenylation the RNA was purified with the MEGAclear™ Kit (Ambion, Catalog No. AM1908). The final RNA concentration was measured on NanoDrop<sup>®</sup> ND-1000 and finally diluted and stored in diethylpyrocarbonate-treated water at −80°C. For the microinjection, the RNA of α3β receptor combinations was mixed at 1:1 ratio (which leads to α3 receptors that consist of predominantly 3 beta and 2 alpha subunits⁶⁵), for α3κ3μ3 (k=1–3, l=1, m=1, n=1), receptors at 1:1:5 ratio, for α3κ3μ3 (k=4–6) and α3κ3μ (k=1, 4 and 6, l=1, 3) receptor combinations at 1:5:1 ratio. All receptor combinations had a final concentration of 36 ng/μl.

**Two electrode voltage clamp (TEV) in Xenopus laevis oocytes.** Mature female Xenopus laevis (Nasco, WI) were anesthetized in a bath of ice-cold 0.17% Tricain (Ethyl-m-amino-benzoate, Sigma, MO) before decapitation in full accordance with all rules of the Austrian animal protection law (see http://www.ris.bka.gv.at/Dokumente/BgbIAuth/BGBLA_2012_1_114/BGBLA_2012_1_114.pdf) and the Austrian animal experiment by-laws (see https://www.ris.bka.gv.at/Dokumente/BgbIAuth/BGBLA_2012_II_522/BGBLA_2012_II_522.pdf) which implement the European Directive 2010/63/EU (see http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF) into the Austrian law (all information accessed on July 27, 2016). The frog’s ovaries were transferred to ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES; pH 7.5). Stage 5–6 oocytes with the follicle cell layer around them were roughly dissected with forceps into packages of 10–15 cells and washed in Ca<sup>2+</sup>-free ND96 medium. Cells were then digested with collagenase (type IA, Sigma, NO, 1 mg/mL ND96) at 18°C shaking at 30 rpm for 30–60 minutes and gently demembranated with the aid of a glass pipette with appropriate tip diameter and a platinum loop. Demembranated cells were stored at 18°C for at least 6 hours in ND96 solution containing penicillin G (10 000 IU/100 mL) and streptomycin (10 mg/100 mL) in 6 hours to preselect and exclude damaged cells from further treatment. Healthy demembranated oocytes were injected with an aqueous solution of mRNA. A total of 4.5 ng of mRNA per oocyte was injected with a Nanoject II (Drummond). After injection of mRNA, oocytes were incubated at 18°C (ND96 + antibiotic) for 2–3 days for α3 receptors and for 3–4 days for α3γ2 or α3β2 receptors before recording. When cells were measured at later time points, oocytes were stored at +4°C instead of 18°C.

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of Ca<sup>2+</sup>-containing NDE solution medium [96 mM NaCl, 5 mM HEPES–NaOH (pH 7.5), 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂]. For current measurements the oocytes were impaled with two microelectrodes (1–3 MΩ resistance) filled with 2 M KCl. The oocytes were constantly washed by a flow of 6 mL/min NDE that could be switched to NDE containing GABA and/or drugs. The EC<sub>50</sub> was determined at the beginning of each experiment. Drugs were diluted into NDE from DMSO-solutions resulting in a final concentration of 0.1% (by volume) perfusing the oocytes. Compounds were co-applied with GABA until a peak response was observed. Between two applications, oocytes were washed in NDE for up to 15 min to ensure full recovery from desensitization. Maximum currents measured in mRNA injected oocytes were in the microampere range for all subtypes of GABA<sub>A</sub> receptors. To test for modulation of GABA induced currents by drugs a concentration of GABA that was titrated to trigger 3–5% of the respective maximum GABA-elicted current of the individual oocyte (EC<sub>50</sub>) was applied to the cell with increasing concentrations of compounds. In order to monitor receptor composition, diazepam (~200% modulation at 1 μM) was used to investigate the incorporation of the γ2<sup>25</sup> subunit, DS2 (>800% modulation at 1 μM) for the incorporation
of the δ subunit and triazolam (>200% modulation at 10 μM) for the γ1 incorporation\(^{41}\). Enhancement of the chloride current was defined as \((I_{\text{GABA}} - \text{Comp})/I_{\text{GABA}}\) = 1, where \(I_{\text{GABA}} - \text{Comp}\) is the current response in the presence of a given compound and \(I_{\text{GABA}}\) is the control GABA current. All recordings were performed at room temperature at a holding potential of 60 mV using a Dagan TEV-200A two-electrode voltage clamp (Dagan Corporation, Minneapolis, MN). Data were digitized, recorded and measured using an Axon Digidata- 1500 low-noise data acquisition system (Axon Instruments, Union City, CA). Data acquisition was done using pCLAMP v.10.5 (Molecular Devices™, Sunnyvale, CA).

Data were analysed using GraphPad Prism v.6 and plotted as concentration-response curves. These curves were normalized and fitted by non-linear regression analysis to the equation \(Y = \text{Bottom} + (\text{Top-bottom})/1 + 10^\left(\log(KD \cdot X)\right)nM\), where \(EC_{50}\) is the concentration of the compound that increases the amplitude of the GABA-evoked current by 50%, and \(nH\) is the Hill coefficient. Data are given as mean ± SEM from at least three oocytes of two and more oocyte batches. Statistical significance was calculated using an extra sum of squares \(F\)-Test (see Figs 3 and 4). P-values of <0.05 were accepted as statistically significant.

**Radioligand displacement assays.** Rat cerebellar membranes were prepared and radioligand binding assays were described as described previously\(^{42}\). In brief, membrane pellets were incubated for 90 min at 4°C in a total of 500 μL of a solution containing 50 mM Tris/citrate buffer, pH = 7.1, 150 mM NaCl and 2 mM \(^{3}H\)luminaltrazepam in the absence or presence of either 5μM diazepam (to determine unspecific binding) or various concentrations of receptor ligands (dissolved in DMSO, final DMSO-concentration 0.5%). Membranes were filtered through Whatman GF/B filters and washed 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. Nonlinear regression analysis of the displacement curves used the equation: log(inhibitor) vs. response - variable slope with Top = 100% and Bottom = 0% Y = \(100/(1 + \text{IC}_{50} \cdot X/\text{KD} + X)\) and an equilibrium binding constant KD for rat cerebellum was determined (SD ± SEM n = 3 independent experiments): 4.8 ± 0.3 nM

\(IC_{50}\) values were converted to Ki using the Cheng-Prusoff relationship\(^{39}\) Ki = IC\(_{50}*(1 + (S/KD))\) with S being the concentration of the radioligand (2 nM) and the KD value described above (4.8 nM).

All analyses were performed using GraphPad Prism version 7 for PC, GraphPad Software, La Jolla California USA, www.graphpad.com.

**Investigated compounds.** We tested pyrazoloquinolinolines with combined substituents at the position R\(^{8}\) and R\(^{9}\) on ring A (Cl, OMe and tBu) and \(R^{4}\) on position D (methoxy, methyl, amino). The following compounds were used: Compound 1 (POZ 028): C\(_{17}H_{15}ClN\(_{3}\)O\(_{2}\): 8-Chloro-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3(5H)-one [\(\text{Cpd 9}\)]\(^{23}\); Compound 2 (LAU156): C\(_{17}H_{15}ClN\(_{3}\)O\(_{2}\): 8-Chloro-2-(4-methylphenyl)-2H-pyrazolo[4,3-H]-quinolin-3(5H)-one [\(\text{Cpd 10}\)]\(^{23}\); Compound 3 (LAU206): C\(_{18}H_{15}ClN\(_{3}\)O\(_{2}\): 8-Chloro-2-(4-aminophenyl)-2H-pyrazolo[4,3-H]-quinolin-3(5H)-one [\(\text{Cpd 11}\)]\(^{23}\); Compound 4 (LAU176): C\(_{18}H_{15}N\(_{3}\)O\(_{2}\): 8-Methoxy-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; Compound 5 (DCBS76): C\(_{18}H_{15}N\(_{3}\)O\(_{2}\): 8-Methoxy-2-(4-methylphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; Compound 6 (DCBS96): C\(_{18}H_{15}N\(_{3}\)O\(_{2}\): 2-(4-Aminophenyl)-8-methoxy-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; Compound 7 (LAU462): C\(_{18}H_{15}ClN\(_{3}\)O\(_{2}\): 6-(tert-Butyl)-8-chloro-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; Compound 8 (Diazepam) (Sigma-Aldrich, St. Louis, MO, USA); Compound 9 (Triaizolan) (Sigma-Aldrich, MO, USA); Compound 10 (DS2) (R&D Systems, MN, USA).

**Compounds synthesis.** Commercially available reagents were used without further purification. Reactions were monitored by thin layer chromatography with silica gel 60 F\(_{254}\) plates (E. Merck, Darmstadt, Germany). HPLC chromatography was carried out with the Autopurification system by Waters using fluoro-phenyl columns. \(^{1}H\) and \(^{13}C\) NMR spectra were recorded on Bruker AC 200 (\(^{1}H\): 200 MHz, \(^{13}C\): 50 MHz), Bruker Avance UltraShield 400 (\(^{1}H\): 400 MHz, \(^{13}C\): 101 MHz) or Bruker Avance IIHD 600 spectrometer equipped with a Prodigy BBO cryo probe (\(^{1}H\): 600 MHz, \(^{13}C\): 151 MHz). Chemical shifts are reported in parts per million (ppm) and were calibrated using DMSO-\(d_{6}\) as internal standard. Multiplicities are denoted by s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet) and m (multiplet). Melting points were determined with a Büchi Melting Point B-545 apparatus. HR-MS was measured on an Agilent 6230 LC TOFMS mass spectrometer equipped with an Agilent Dual AJS ESI-Source.

Compounds 1 (POZ 028), 2 (LAU156), 3 (LAU206) and 4 (LAU176) were synthesized and published previously\(^{23}\). Synthesis of 5 (DCBS76) was conducted in analogy to previously outlined synthetic routs\(^{23,44,45}\). The synthesis of 6 (DCBS96) was improved as described. Compound 7 (LAU462) was synthesized according to reported protocols\(^{23,46,47}\).

8-Methoxy-2-(4-methylphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one 5 (DCBS76). Compound 5 was synthesized according to the literature\(^{23,44,45}\). In 74% yield (yellow solid, 85 mg, 0.28 mmol). \(^{1}H\) NMR (400 MHz, DMSO-\(d_{6}\)) \(\delta\) 2.32 (s, 3H), 3.93 (s, 3H), 7.22–7.27 (m, 2H), 7.29 (dd, \(J = 9.1, 2.9\) Hz, 1H), 7.58 (d, \(J = 2.8\) Hz, 1H), 7.67 (d, \(J = 9.0\) Hz, 1H), 8.08–8.16 (m, 2H), 8.65 (s, 1H), 12.79 (br s, 1H). \(^{13}C\) NMR (101 MHz, DMSO-\(d_{6}\)) \(\delta\) 55.7, 102.5, 105.3, 118.7 (2 C), 119.6, 120.0, 121.2, 129.0 (2 C), 129.7, 132.9, 137.8, 137.9, 142.7, 157.5, 161.4. HR-MS: calculated [C\(_{18}H_{16}N_{3}O_{2}\)]\(^{+}\): 306.1237; found [C\(_{18}H_{16}N_{3}O_{2}\)]\(^{+}\): 306.1230 (diff: 2.23 ppm). TLC (10% MeOH in CH\(_{2}\)Cl\(_{2}\)): R\(_{f}\) = 0.54. M.p.: decomposes > 300°C.
2-(4-Aminophenyl)-8-methoxy-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one 6 (DCBS96). 8-Methoxy-2-(4-nitrophenyl)-1,2-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one (20 mg, 0.06 mmol) was dissolved in 2.5 mL MeOH, Pd/C (10 wt-%) was added and the reaction mixture was stirred at room temperature under hydrogen atmosphere. After 18 h the reaction mixture was passed through a bed of silica and the solvent was removed under reduced pressure. The residue was purified by HPLC and neutralized with 1 mL satd. NaHCO3. The precipitate was washed with water (2 × 2 mL) and dried in vacuo to give 2-(4-aminophenyl)-8-methoxy-2,5-dihydro-3H-pyrazolo[4,3-c]quinolin-3-one as yellow solid (16 mg, 0.052 mmol, 87%). 1H NMR (600 MHz, DMSO-<em>d</em><sub>6</sub>) δ 3.90 (s, 3H), 4.99 (br s, 2H), 6.58–6.64 (m, 2H), 7.21 (dd, <em>J</em> = 9.0, 2.9 Hz, 1H), 7.52 (d, <em>J</em> = 2.9 Hz, 1H), 7.63 (d, <em>J</em> = 9.0 Hz, 1H), 7.78–7.83 (m, 2H), 8.54 (s, 1H). 13C NMR (151 MHz, DMSO-<em>d</em><sub>6</sub>) δ 55.6, 102.3, 105.4, 113.6 (2C), 119.2, 120.9 (2C), 121.5, 129.9, 130.0, 137.6, 142.0, 145.6, 157.3, 160.6. HR-MS: calculated [C<sub>17</sub>H<sub>15</sub>N<sub>4</sub>O<sub>2</sub>+2H]: 307.1190; found [C<sub>17</sub>H<sub>15</sub>N<sub>4</sub>O<sub>2</sub>: 307.1196 (diff.: -2.21 ppm). TLC (5% EtOAc in CH<sub>2</sub>Cl<sub>2</sub>): <em>R</em><sub>f</sub> = 0.25. M.p.: decomposes >300°C.

**Data Availability.** The datasets generated during and/or analysed during the current study are available from the corresponding author upon request.

**References**

1. Sieghart, W. Allosteric modulation of GABA<sub>α</sub> receptors via multiple drug-binding sites. *Adv Pharmacol* 72, 53–96 (2015).
2. Andersson, J. D. & Halldin, C. PET radioligands targeting the brain GABA<sub>α</sub>/benzodiazepine receptor complex. *Labelled Comp Rad* 56, 196–206 (2013).
3. Ernst, M. & Sieghart, W. GABA<sub>α</sub> receptor subtypes: structural variety raises hope for new therapy concepts. *eNeuroforum* 6, 97–103 (2015).
4. Alkini, M. K. & Schofield, P. Widespread expression of GABA<sub>α</sub> receptor subunits in peripheral tissues. *Neurosci Res* 35, 145–153 (1999).
5. Olsen, R. W. & Sieghart, W. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. *Pharmacov* 60, 243–260 (2008).
6. Tretter, V., Ehya, N., Fuchs, K. & Sieghart, W. Stoichiometry and assembly of a recombinant GABA<sub>α</sub> receptor subtype. *J Neurosci* 17, 2728–2737 (1997).
7. Galz, J.-L. & Changeux, J.-P. Neurotransmitter-gated ion channels as unconventional allosteric proteins. *Curr Opin Struct Biol* 4, 554–565 (1994).
8. Puthenkal, R. et al. Structural Studies of GABA<sub>α</sub> receptor binding sites: Which experimental structure tells us what? *Front Mol Neurosci* 9 (2016).
9. Curtis, D. R., Duggan, A. W. & Johnston, G. A. Glycine, strychnine, picrotoxin and spinal inhibition. *Brain Res* 14, 759–762 (1969).
10. Tibbs, R. E. & Gouaux, E. Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* 474, 54–60 (2011).
11. Johnston, G. A. Advantages of an antagonist: bicuculline and other GABA antagonists. *Brit J Pharmacol* 169, 328–336 (2013).
12. Yagle, M. A. et al. [3H]Ethynylbicycloorthobenzoate ([3H]EBOB) binding in recombinant GABA<sub>α</sub> receptors. *Neurotoxicology* 24, 817–824 (2003).
13. Sigel, E. Mapping of the benzodiazepine recognition site on GABA<sub>α</sub> receptors. *Curr Top Med Chem* 2, 833–839 (2002).
14. Maeda, J. et al. Visualization of alpha5 subunit of GABA<sub>α</sub>/benzodiazepine receptor by 11C Ro15-4513 using positron emission tomography. *Synapse* 47, 200–208 (2003).
15. Lingford-Hughes, A. et al. Imaging the GABA benzodiazepine receptor subtype containing the alpha5-subunit in vivo with [11C] Ro15 4513 positron emission tomography. *J Cereb Blood F Met* 22, 878–889 (2002).
16. Sergeeva, O. A. et al. Fragrant dioxane derivatives identify beta1-subunit-containing GABA<sub>α</sub> receptors. *J Biol Chem* 285, 23985–23993 (2010).
17. Thompson, S. A. et al. Salicylidene salicylhydrazide, a selective inhibitor of 31-containing GABA<sub>α</sub> receptors. *Brit J Pharmacol* 142, 97–106 (2004).
18. Sanna, E. et al. Direct activation of GABA<sub>α</sub> receptors by loreclezole, an anticonvulsant drug with selectivity for the beta-subunit. *Neuropharmacology* 35, 1753–1760 (1996).
19. Hill-Venning, C., Belelli, D., Peters, J. A. & Lambert, J. J. Subunit-dependent interaction of the general anesthetic etomidate with the gamma-aminobutyric acid type A receptor. *Brit J Pharmacol* 120, 749–756 (1997).
20. Khom, S. et al. Valeric acid potentiates and inhibits GABA<sub>α</sub> receptors: molecular mechanism and subunit specificity. *Neuropharmacology* 53, 178–187 (2007).
21. Jurd, R. et al. General anesthetic actions in vivo strongly attenuated by a point mutation in the GABA<sub>α</sub> receptor beta3 subunit. *FASEB J* 17, 250–252 (2003).
22. Varagic, Z. et al. Subtype selectivity of alpha+ beta- site ligands of GABA<sub>α</sub> receptors: identification of the first highly specific positive modulators at alpha6beta2/3gamma2 receptors. *Brit J Pharmacol* 169, 384–399 (2013).
23. Varagic, Z. et al. Identification of novel positive allosteric modulators and null modulators at the GABA<sub>α</sub> receptor alpha+ beta-interface. *Brit J Pharmacol* 169, 371–383 (2013).
24. Minihedyari, P. et al. Unexpected Properties of delta-Containing GABA<sub>α</sub> Receptors in Response to Ligands Interacting with the alpha+ beta- Site. *Neurochem Res* 39, 1057–1067 (2014).
25. Ramerstorfer, J. et al. The GABA<sub>α</sub> Receptor α+ β− Interface: A Novel Target for Subtype Selective Drugs. *J Neurosci* 31, 870–877 (2011).
26. He, X. et al. Studies of molecular pharmacophore/receptor models for GABA<sub>α</sub>/BZa subtypes: binding affinities of symmetrically substituted pyrazolo[4,3-c]quinolin-3-ones at recombinant alpha x beta 3 gamma 2 subtypes and quantitative structure-activity relationship studies via a comparative molecular field analysis. *Drug Deliv* 16, 77–91 (1999).
27. Che Has, A. T. et al. Zolpidem is a potent stoichiometry-selective modulator of α1/3 GABA<sub>α</sub> receptors: evidence of a novel benzodiazepine site in the α1-0.1 interface. *Sci Rep* 6, 28874 (2016).
28. Dillon, G. H. et al. U-93631 causes rapid decay of gamma-amino- butyric acid-induced chloride currents in recombinant rat gamma- amino- butyric acid type A receptors. *Mol Pharmacol* **44**, 860–865 (1993).
29. Maldifassi, M. C., Baur, R. & Sigel, E. Molecular mode of action of CGS 9895 at alpha1 beta2 gamma2 GABA_A receptors. *J Neurochem* **138**, 722–730 (2016).
30. Miller, P. S. & Aricescu, A. R. Crystal structure of a human GABA_A receptor. *Nature* **512**, 270–275 (2014).
31. Kücke, A. M., Teißen, J. A., Seifling-Clark, J., Wagner, D. A. & Czajkowski, C. Structural Requirements for Imidazobenzodiazepine Binding to GABA_A Receptors. *Mol Pharmacol* **63**, 289–296 (2003).
32. Anstre, Q. M. et al. Mutations in the Gabr1 gene promote alcohol consumption through increased tonic inhibition. *Nat Commun* **4**, 2816 (2013).
33. Ueno, S., Wick, M. J., Ye, Q., Harrison, N. L. & Harris, R. A. Subunit mutations affect ethanol actions on GABA_A receptors expressed in Xenopus oocytes. *Brit J Pharmacol* **127**, 377–382 (1999).
34. Mortensen, M., Patel, R. & Smart, T. G. GABA Potency at GABA_A Receptors Found in Synaptic and Extrasynaptic Zones. *Front Cell Neurosci* **6**, 1 (2012).
35. Korpi, E. R. et al. Cerebellar GABA_A receptors in two rat lines selected for high and low sensitivity to moderate alcohol doses: pharmacological and genetic studies. *Alcohol* **9**, 225–231 (1992).
36. Groll, B., Schnurch, M. & Mihovilovic, M. D. Selective Ru(0)-catalyzed deuteration of electron-rich and electron-poor nitrogen-containing heterocycles. *J Org Chem* **77**, 4432–4437 (2012).
37. Kelley, M. H. et al. Alterations in Purkinje cell GABA_A receptor pharmacology following oxygen and glucose deprivation and cerebral ischemia reveal novel contribution of (3(1))-subunit-containing receptors. *Eur J Neurosci* **37**, 555–563 (2013).
38. Grins, Y. & Prusoff, W. H. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (IS0) of an enzymatic reaction. *Biochem Pharmacol* **22**, 3099–3108 (1973).
39. Fryer, R. L. et al. Structure-activity relationship studies at benzodiazepine receptor (BZDR): a comparison of the substituent effects of pyrazolquinolinone analogs. *J Med Chem* **36**, 1669–1673 (1993).
40. Savini, L. et al. High affinity central benzodiazepine receptor ligands. Part 2: quantitative structure-activity relationships and comparative molecular field analysis of pyrazolo[4,3-c]quinolin-3-ones. *Biorg Med Chem* **9**, 431–444 (2001).
41. Neumann, J. J., Rakshit, S., Droge, T. & Glorius, F. Palladium-catalyzed amidation of unactivated C(sp3)-H bonds: from amines to indolines. *Angew Chem Int Edit* **48**, 6892–6895 (2009).
42. Sieghart, W. & Schuster, A. Affinity of various ligands for benzodiazepine receptors in rat cerebellum and hippocampus. *Biochem Pharmacol* **33**, 4033–4038 (1984).
43. Cheng, Y. & Prusoff, W. H. Relationship between the inhibition constant (K(I)) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**, 3099–3108 (1973).
44. Ebert, V., Scholze, P. & Sieghart, W. Extensive heterogeneity of recombinant gamma-aminobutyric acid A receptors expressed in alpha 4 beta 3 gamma 2-transfected human embryonic kidney 293 cells. *Neuropharmacology* **35**, 1323–1330 (1996).
45. Pak, F., Wu, B., Shi & Cu-catalyzed, Z.-J. intromolecular amidation of unactivated C(sp3)-H bonds to synthesize N-substituted indolines. *J Org Chem* **77**, 4432–4437 (2012).
46. Ebert, V., Scholze, P. & Sieghart, W. Extensive heterogeneity of recombinant gamma-aminobutyric acid A receptors expressed in alpha 4 beta 3 gamma 2-transfected human embryonic kidney 293 cells. *Neuropharmacology* **35**, 1323–1330 (1996).
47. Pak, F., Wu, B., Shi & Cu-catalyzed, Z.-J. intromolecular amidation of unactivated C(sp3)-H bonds to synthesize N-substituted indolines. *J Org Chem* **77**, 4432–4437 (2012).
48. Hörlein, G., Kübel, B., Studeneer, A. & Salbeck, G. Heterocyclen durch Anellierung an 4-Pyridinole, II Thieno-[3,2-c]pyridin-3-ole. *Chem Eur J* **22**, 6487–6490 (2016).
49. Hörlein, G., Kübel, B., Studeneer, A. & Salbeck, G. Heterocyclen durch Anellierung an 4-Pyridinole, II Thieno[3,2-c]pyridin-3-ole. *Liebigs Ann Chem* **1979**, 387–391 (1979).

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Author Contributions
X.S. and D.C.B.S. contributed equally to the work. X.S., D.C.B.S. and M.E. conceived the study. D.C.B.S., M.S. and M.D.M. planned chemical synthesis, D.C.B.S. performed chemical synthesis and computational modelling. X.S. planned and supervised electrophysiological experiments. X.S., J.P., Z.V., K.B., M.T. and S.R. and R.H. performed electrophysiological measurements and data analysis. X.S. and K.B. performed statistical analysis. P.S. and F.S. performed binding studies and generated the mutated constructs. X.S. and D.C.B.S. wrote the manuscript. D.C.B.S., X.S., M.T., K.B. and M.E. prepared figures. All authors reviewed the final manuscript and provided important input.

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