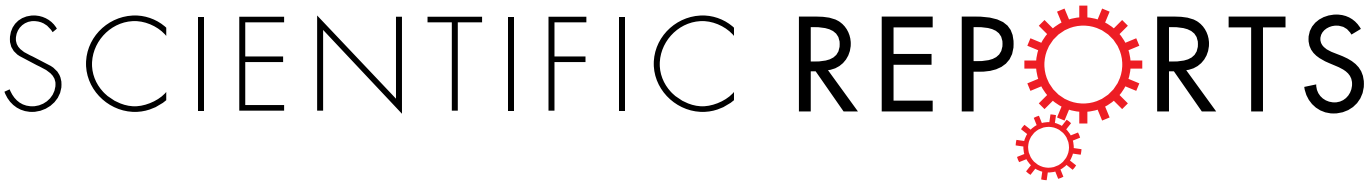

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Novel positive allosteric modulators of $GABA_A$ receptors with anesthetic activity

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$GABA_A$ receptors are the main inhibitory neurotransmitter receptors in the brain and are targets for numerous clinically important drugs such as benzodiazepines, anxiolytics and anesthetics. We previously identified novel ligands of the classical benzodiazepine binding pocket in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors using an experiment-guided virtual screening (EGVS) method. This screen also identified novel ligands for intramembrane low affinity diazepam site(s). In the current study we have further characterized compounds 31 and 132 identified with EGVS as well as 4-O-methylhonokiol. We investigated the site of action of these compounds in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes using voltage-clamp electrophysiology combined with a benzodiazepine site antagonist and transmembrane domain mutations. All three compounds act mainly through the two $\beta+/\alpha-$ subunit transmembrane interfaces of the $GABA_A$ receptors. We then used concatenated receptors to dissect the involvement of individual $\beta+/\alpha-$ interfaces. We further demonstrated that these compounds have anesthetic activity in a small aquatic animal model, *Xenopus laevis* tadpoles. The newly identified compounds may serve as scaffolds for the development of novel anesthetics.

The search for novel anesthetics has been triggered by the rising age of patients and increasing use of anesthesia outside the operating room[1,2]. A key site of action of the potent anesthetics propofol and etomidate is the major inhibitory receptor in the mammalian central nervous system, the γ-aminobutyric acid type A ($GABA_A$) receptor. These receptors are composed of five homologous subunits organized around a central Cl^- selective channel[3]. Each subunit contains a large N-terminal extracellular domain (ECD), a transmembrane domain (TMD) with four alpha-helices (TM1 to TM4), and a variable-length intracellular domain (ICD) between TM3 and TM4. 19 subunits of the $GABA_A$ receptor have been cloned (for review see: 4–6), denoting that numerous types of receptor isoforms exist[5]. The most abundant $GABA_A$ receptor in the brain comprises α_1, β_2 and γ_2 subunits[3–5,7]. The receptor possesses a $2\alpha:2\beta:1\gamma$ subunit stoichiometry[8–11], with a subunit arrangement of $\gamma\beta\alpha\beta\alpha$ anti-clockwise as seen from the synaptic cleft[10–13]. The receptor composition and arrangement influence its pharmacological properties[14,15].

Benzodiazepines modulate $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor function by binding to a high affinity site located at the $\alpha+/\gamma-$ ECD interface, homologous to the agonist binding sites at $\beta+/\alpha-$ ECD interfaces[16,17]. In addition to the high affinity binding site for benzodiazepines (site 1), there are other low affinity sites. One of these, site 2, is located at the ECD $\alpha+/\beta-$ interface[18,19]. Others, together designated as site 3, are located in the TMD, based on abolition of benzodiazepine effects by combined isoleucine substitutions at the homologous residues α_1S269, β_2N265, and γ_2S280[20].

$GABA_A$ receptors are also targets for potent intravenous anesthetics, including barbiturates, propofol and etomidate[21–25]. Interestingly, receptor sensitivity to intravenous anesthetics is affected by benzodiazepine site 3 mutations[26–30]. Diverse anesthetics not only potentiate GABA-induced Cl^- currents, but additionally at high concentrations directly activate $GABA_A$ receptors[31,32]. Photo-affinity labeling has located allosteric sites for the intravenous anesthetics etomidate and propofol to the TM1 of α and TM2, TM3 of β subunits[23,24,33].

Previously, we reported a new method to identify ligands of the high affinity benzodiazepine pocket, experimental-guided virtual screening (EGVS), integrating experimental data with homology modeling of the

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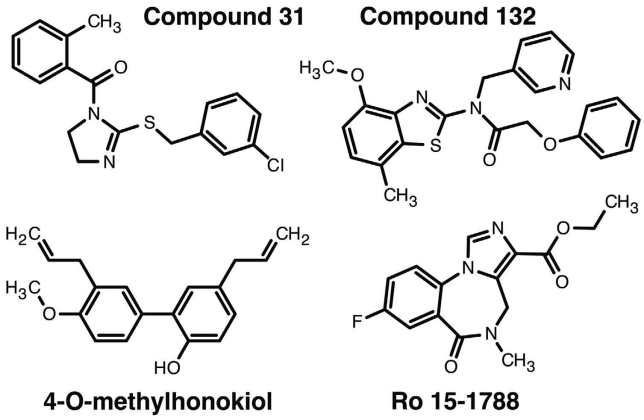

Figure 1. Chemical structure of compounds 31, 132, 4-O-methylhonokiol, and the high-affinity benzodiazepine antagonist Ro 15-1788.

$GABA_A$ receptor[34]. EGVS identified some ligands that only recognized site 1, others that recognized both site 1 and site 3[35], and another set that only recognize site 3.

Here we describe the actions of two compouds identified by EGVS, 31 and 132[34] and 4-O-methylhonokiol[36]. Using mutations and concatenated receptors we determined that the three compounds act mainly through the TMD β+/α− interfaces (site 3), and particularly the γβ+/α−β site. The anesthetic action of these drugs was explored *in vivo*, revealing potencies similar to propofol.

Results

In an attempt to find novel ligands for the high affinity site for benzodiazepines on $GABA_A$ receptors we screened 198 compounds for displacement of the high affinity benzodiazepine site (called site 1 previously) antagonist [^{3}H]-Ro 15-1788 at receptors expressed in HEK-cells. Many high affinity ligands were identified[34]. One compound, SJM3 acted as antagonist with high affinity at site 1, but allosterically potentiated receptor activation through sites in the membrane (called sites 3 previously)[35]. Compounds 31 and 132 either did not or weakly displaced [^{3}H]-Ro 15-1788 from site 1 but potently enhanced $GABA_A$ receptor activation. 4-O-methylhonokiol shares these characteristics, potentiating $\alpha_1\beta_2\gamma_{2}$ receptors with an EC_{50} of 5.4 ± 1.8 μM, independent of the high affinity site for benzodiazepines[36]. Here, we report mechanistic and animal studies of these three compounds.

Compounds 31 and 132 are allosteric modulators of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. First, we investigated if compounds 31 and 132 were able to act as agonists. Figure 1 shows their chemical structures compounds. Both compounds at 3 and 30 μM elicited only very small currents by themselves in $\alpha_1\beta_2\gamma_{2}$ $GABA_A$ receptors expressed in *Xenopus* oocytes. These compounds elicited at the concentration of 3 μM and 30 μM currents amounting to 0.1 ± 0.06% (mean ± SD, n = 3) and 0.3 ± 0.13% (mean ± SD, n = 3), respectively of the maximal current amplitude elicited by GABA in the same oocytes. Thus neither of the compounds tested acts as an appreciable agonist on $\alpha_1\beta_2\gamma_{2}$ receptors.

Both compounds strongly enhanced currents elicited by GABA. We established the concentration response curves of this positive allosteric modulation. After two applications of GABA at a concentration eliciting 0.5–1.5% of the maximal current amplitude, the same concentration of GABA was co-applied with increasing concentrations of the tested compounds. Figure 2a,b show current traces demonstrating positive modulation by different concentrations of compounds 31 and 132, respectively. Figure 2c summarizes the results of three of such experiments. Both compounds potentiate GABA elicited currents in oocytes expressing $\alpha_1\beta_2\gamma_{2}$ receptors. For compound 31, at high concentrations apparent desensitization was observed, that could be partly due to open channel blocker effect. For both compounds no saturation at the highest concentration was obtained, because of poor solubility we could not test higher concentrations.

Potentiation by compounds 31 and 132 is not affected by Ro 15-1788. From the binding data we did not expect that the two compounds are acting through the high affinity benzodiazepine binding site 1 in $\alpha_1\beta_2\gamma_{2}$ receptors. Nevertheless, we tested whether 1 μM of the site 1 antagonist Ro 15-1788 inhibits potentiation of GABA currents by compounds 31 or 132. Either compound (3 μM) strongly potentiated currents elicited by GABA. Potentiation by either drug was not inhibited by 1 μM Ro 15-1788 (Fig. 3). Relative current amplitudes in the presence vs. absence of the antagonist were 112 ± 8% (mean ± SD, n = 4, $p > 0.05$, *t test*) for compound 31, and 115 ± 14% (mean ± SD, n = 4, $p > 0.05$, *t test*) for compound 132. This confirms that potentiation by compounds 31 and 132 does not result from action at the benzodiazepine site 1.

Compounds 31 and 132 and 4-O-methylhonokiol act at the low affinity benzodiazepine site 3 in $\alpha_1\beta_2\gamma_2$ receptors. We next investigated whether benzodiazepine site 3 TMD mutations affect potentiation by compounds 31 and 132. Combining three homologous site 3 mutations in $\alpha_1\beta_2\gamma_{2}$ receptors, α_1S269I, β_2N265I and γ_{2}S280I, eliminated the potentiation by high concentrations of diazepam[20]. We investigated the effects of these mutations individually and combined, abbreviating them as α_1M, β_2M and γ_{2}M. Recently, we described the

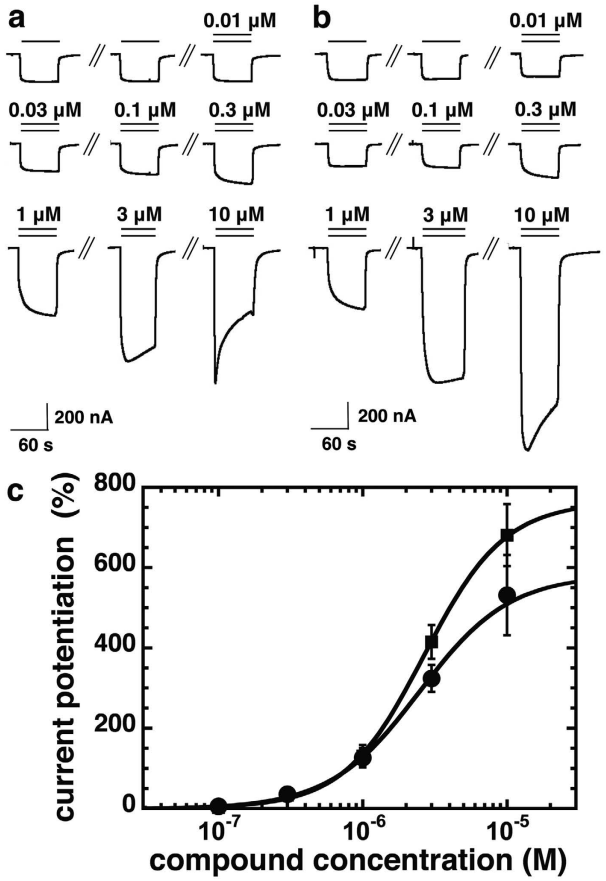

Figure 2. Compounds 31 and 132 stimulate GABA currents mediated by $\alpha 1\beta_2\gamma_{2}$ receptors in a concentration-dependent manner. $\alpha_1\beta_2\gamma_{2}$ receptors were expressed in *Xenopus* oocytes and electrophysiological experiments were performed. Original current traces of an experiment with compound 31 (**a**) and with compound 132 (**b**). Numbers indicate applied concentrations of the respective compounds. (**c**) Concentration dependence of the positive allosteric modulation by compound 31 (closed circles) and compound 132 (closed squares) in oocytes expressing $\alpha_1\beta_2\gamma_{2}$ receptors. Mean data ± SD for both compounds is shown, n = 3.

potency of GABA to activate all of these receptor subtypes expressed in *Xenopus* oocytes[37]. In order to exclude general gating effects caused by these mutations we showed that potentiation by low concentrations of diazepam and by THDOC are not affected[37]. For illustration, the localization of the mutations is shown in the crystalized homomeric β_3 receptor[38] where some of the β_3 subunits were renamed α_1, β_2 and γ_{2} (Fig. 4a).

Wild-type $\alpha_1\beta_2\gamma_{2}$, $\alpha_1M\beta_2\gamma_{2}$, $\alpha_1\beta_2M\gamma_{2}$, $\alpha_1\beta_2\gamma_{2}M$ and $\alpha_1M\beta_2M\gamma_{2}M$ receptors were expressed in *Xenopus* oocytes. Using electrophysiological techniques we determined the effect of these point mutations on the potentiation by compounds 31, 132 and 4-O-methylhonokiol, normalizing to the potentiation in wild type $\alpha_1\beta_2\gamma_{2}$ receptors.

Figure 4b summarizes the results obtained. The single mutations in the α_1 and in the γ_{2} subunits did not significantly alter the degree of potentiation by 3 µM of either compound 31 or 132. Relative to wild-type $\alpha_1\beta_2\gamma_{2}$ receptors, modulation in mutated $\alpha_1M\beta_2\gamma_{2}$ receptors by compound 31 amounted to 77 ± 24% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*), and by compound 132 to 118 ± 44% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*). Modulation in $\alpha_1\beta_2\gamma_{2}M$ receptors by compound 31 was 87 ± 52% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*), and by compound 132 was 91 ± 21% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*). In contrast, modulation by both compounds was strongly impaired in $\alpha_1\beta_2M\gamma_{2}$ and triply mutated $\alpha_1M\beta_2M\gamma_{2}M$ receptors. Potentiation by 3 µM compound 31 in $\alpha_1\beta_2M\gamma_{2}$ was 10.4 ± 8.5% (mean ± SD, n = 3, $p = 0.007$, *Tukey posthoc test*), and in $\alpha_1M\beta_2M\gamma_{2}M$ receptors was 2.3 ± 0.8% (mean ± SD, n = 3, $p = 0.0043$, *Tukey posthoc test*). Potentiation by compound 132 relative to that in $\alpha_1\beta_2\gamma_{2}$ was also dramatically reduced in $\alpha_1\beta_2M\gamma_{2}$ 7.3 ± 6.8% (mean ± SD, n = 3, $p = 0.003$, *Tukey posthoc test*) and $\alpha_1M\beta_2M\gamma_{2}M$ −0.4 ± 5.1% (mean ± SD, n = 3, $p = 0.0021$, *Tukey posthoc test*), respectively.

Potentiation by 1 µM 4-O-methylhonokiol was also significantly reduced only in $\alpha_1\beta_2M\gamma_{2}$ and $\alpha_1M\beta_2M\gamma_{2}M$ receptors, similar to compounds 31 and 132. Relative to wild-type $\alpha_1\beta_2\gamma_{2}$ receptors, modulation in $\alpha_1M\beta_2\gamma_{2}$ mutated amounted to 63 ± 6% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*), and in $\alpha_1\beta_2\gamma_{2}M$ receptors to 79 ± 16% (mean ± SD, n = 3, $p > 0.05$, *Tukey posthoc test*). Relative to $\alpha_1\beta_2\gamma_{2}$, residual potentiation in $\alpha_1\beta_2M\gamma_{2}$ receptors amounted to 15.6 ± 1.5% (mean ± SD, n = 3, $p = 0.0022$, *Tukey posthoc test*), and to 2.2 ± 0.2% (mean ± SD, n = 3, $p = 0.0018$, *Tukey posthoc test*) in $\alpha_1M\beta_2M\gamma_{2}M$.

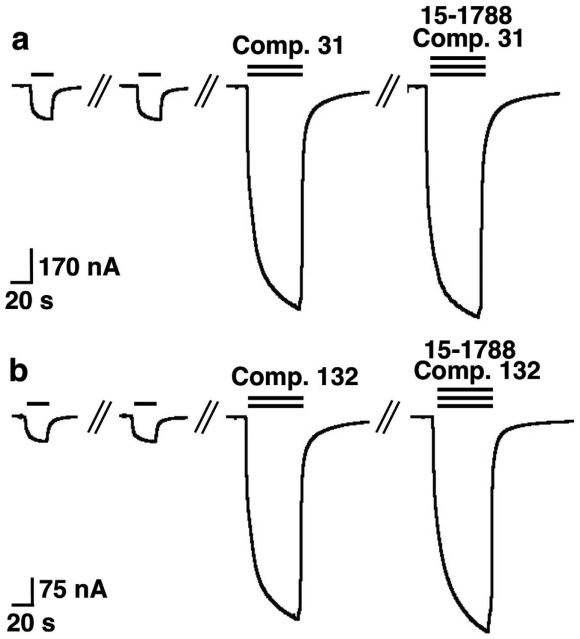

Figure 3. Compounds 31 and 132 do not act at the classical high affinity site for benzodiazepines. GABA at a concentration eliciting 0.5% of the maximal current amplitude ($EC_{0.5}$, single bars) was applied until a stable response was obtained. Subsequently, the same concentration of GABA was co-applied with 3 μM of compounds 31 (**a**) or 132 (**b**), which resulted both in a large increase of current amplitude. Co-application of Ro 15-1788 with compound and GABA did not reduce the degree of modulation in both cases. Experiments were repeated 4 times, with three different batches of oocytes, with a similar outcome.

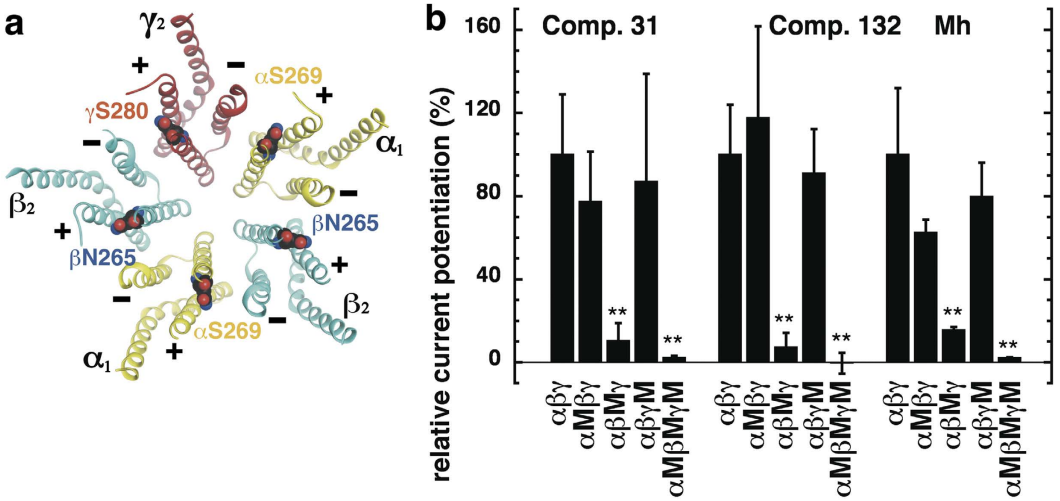

Figure 4. (**a**) Model structure of the $GABA_A$ receptor transmembrane domain. The major isoform of the $GABA_A$ receptor is composed of two α_1, two β_2, and one γ_2 subunits. The model structure depicts the crystalized homomeric β_3 $GABA_A$ receptor (PDB structure 4COF)[38]. In this figure, some of the β_3 subunits were renamed α_1 (yellow), β_2 (blue) and γ_2 (red); structures are shown in ribbon representation. The mutated residues α_1S269, β_2N265, and γ_2S280 are located at the interfaces between subunits. (**b**) Potentiation of the GABA response by compound 31 (3 μM), compound 132 (3 μM), and 4-O-methylhonokiol (1 μM, abbreviated Mh) in wild-type $\alpha_1\beta_2\gamma_2$, single mutant (α_1M, β_2M, γ_2M), and triple mutant receptors expressed in *Xenopus* oocytes. The bars indicate mean ± SD, n = 3.

The above data suggests that the modulatory site(s) for the three compounds we studied is located in one or both of the β+/α− TMD subunit interfaces on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors.

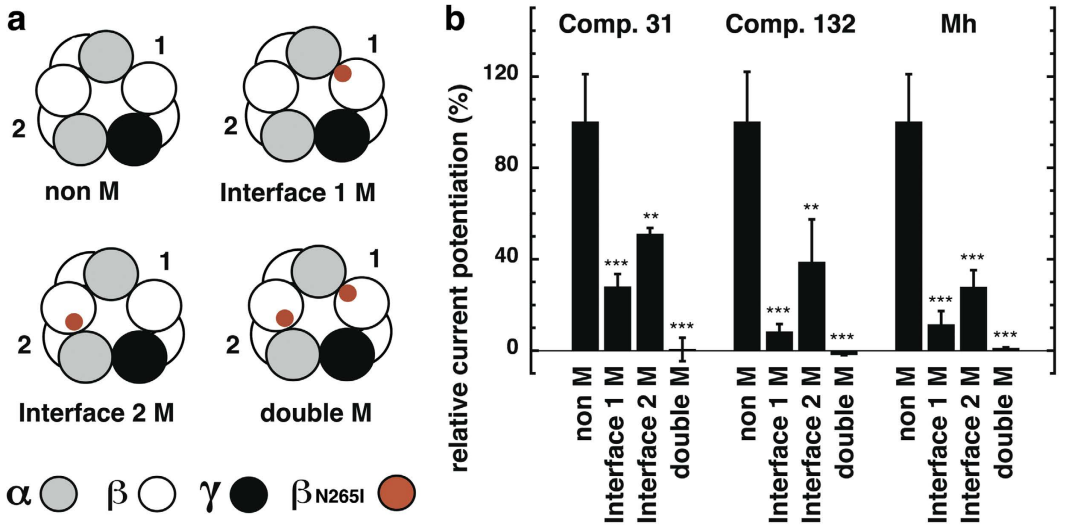

Figure 5. Individual roles of the two β+/α− interfaces in channel modulation by compounds 31, 132 and 4-O-methylhonokiol. (**a**) Scheme showing the four concatenated wild-type and mutant receptors. 1 and 2 refer to the two different β+/α− subunit interfaces, interface 1 and interface 2. The location of the β_2N265I mutations is indicated in red color. Concatenated receptors were prepared containing no mutation (α_1-β_2-α_1/γ_{l2}-β_2, non M), a mutation at interface 1 (α_1-β_2-α_1/γ_{l2}-β_2M, interface 1 M), a mutation at interface 2 (α_1-β_2M-α_1/γ_{l2}-β_2, interface 2 M), or mutations in both sites (α_1-β_2M-α_1/γ_{l2}-β_2M, double M). Interface 2 harbors a binding site for GABA with higher apparent affinity for channel gating than the one positioned at the interface 1[54]. (**b**) Potentiation by compound 31 (3μM), compound 132 (3μM), and 4-O-methylhonokiol (1μM), using an $EC_{0.5-1.5}$ concentration of GABA for each concatenated receptor subtype. Bars indicate mean ± SD, n = 3.

Role of the individual β+/α− subunit interfaces in channel modulation by compounds 31, 132 and 4-O-methylhonokiol. Each $GABA_A$ receptor contains two β+/α− subunit interfaces. Combined mutation at these interfaces greatly reduces the modulatory effects of compounds 31, 132 and 4-O-methylhonokiol. Using α_1-β_2-α_1 and γ_{l2}-β_2 subunit concatemers, we studied the effects of individual mutated interfaces. We designated receptors containing the mutant in the γ_{l2}-β_2 construct interface 1 M, and the mutation in the α_1-β_2-α_1 construct interface 2 M (Fig. 5a). The α_1-β_2M-α_1 and γ_{l2}-β_2M constructs were built, and were co-expressed with non-mutated dual or triple subunit constructs forming α_1-β_2-α_1/γ_{l2}-β_2M and α_1-β_2M-α_1/γ_{l2}-β_2 receptors. Both constructs were expressed together to form the double mutant receptor α_1-β_2M-α_1/γ_{l2}-β_2M.

Wild type concatenated receptors α_1-β_2-α_1/γ_{l2}-β_2 were also expressed. This receptor has an EC_{50} for GABA of approximately 120 μM[12]. Results are standardized to the potentiation observed in α_1-β_2-α_1/γ_{l2}-β_2 concatenated receptors. As shown in Fig. 5b, in the double mutant concatemeric receptors (α_1-β_2M-α_1/γ_{l2}-β_2M) potentiation for all three compounds was abolished. Relative to α_1-β_2-α_1/γ_{l2}-β_2 concatenated receptors, in the double mutant α_1-β_2M-α_1/γ_{l2}-β_2M receptor modulation by compound 31 was reduced to 1 ± 5% (mean ± SD, n = 3, $p < 0.0005$, *Tukey posthoc test*), by compound 132 to −2 ± 0.4% (mean ± SD, n = 3, p = 0.0001, *Tukey posthoc test*), and by 4-O-methylhonokilol to 1 ± 0.6% (mean ± SD, n = 3, p = 0.0001, *Tukey posthoc test*). In receptors containing only one mutation, either interface 1 or interface 2, modulation by compound 31 was reduced significantly compared to concatenated wild type receptors α_1-β_2-α_1/γ_{l2}-β_2. With interface 1 M, residual relative potentiation was 28 ± 6% (mean ± SD, n = 3, p = 0.0005, *Tukey posthoc test*) and with interface 2 M it was 51 ± 3% (mean ± SD, n = 3, p = 0.0059, *Tukey posthoc test*) of wild-type. Potentiation of interface 1 M and interface 2 M receptors differed significantly (p = 0.0035, *t test*). Therefore, both TMD β+/α− sites seem to contribute differently to modulation by compound 31, interface 1 being more efficacious.

Likewise, modulation by compound 132 was sensitive to the mutations at both sites. In interface 1 M receptors relative potentiation was reduced to 8 ± 4% (mean ± SD, n = 3, p = 0.0002, *Tukey posthoc test*), and in interface 2 M receptors to 39 ± 19% (mean ± SD, n = 3, p = 0.0035, *Tukey posthoc test*). Again, interface 1 M produced a larger impact than 2 M, although the difference was at the statistical limit (p = 0.0510, *t test*). Modulation by 4-O-methylhonokiol in interface 1 M and 2 M receptors was reduced to 11 ± 6% (mean ± SD, n = 3, $p < 0.0001$, *Tukey posthoc test*) and 28 ± 8% (mean ± SD, n = 3, p = 0.0004, *Tukey posthoc test*), respectively. The two mutations produced significantly different effects (p = 0.043, *t test*), with the interface 1 M effect again larger.

Therefore, potentiation by all three compounds displayed similar sensitivity patterns with homologous mutations in distinct β+/α− TMD sites of α_1-β_2-α_1/γ_{l2}-β_2 receptors. Both sites are necessary for full modulation, while interface 1 produces a larger impact than interface 2.

Effect of the β_2N265S mutation. The β_2N265 residue is important for allosteric modulation of $GABA_A$ receptors by many compounds acting through the TMD. This residue was initially described as a determinant for the modulatory action of loreclezole, where the β_2N265S mutation created a receptor unresponsive to this compound[39]. Mutations in this residue also abolish potentiation by the anesthetics etomidate and propofol[28,40].

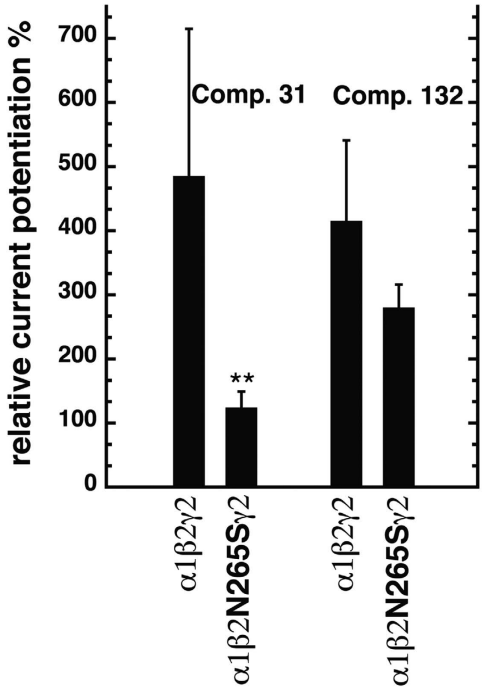

Figure 6. Effect of the β_2N265S mutation on modulation by compounds 31 and 132. Wild-type $\alpha_1\beta_2\gamma_{l2}$ and mutated $\alpha_1\beta_2$N265Sγ_{l2} receptors were expressed in *Xenopus* oocytes and studied. Potentiation of GABA currents was determined using 3 μM of compound 31 or 132. Bars indicate mean ± SD, n = 4–11.

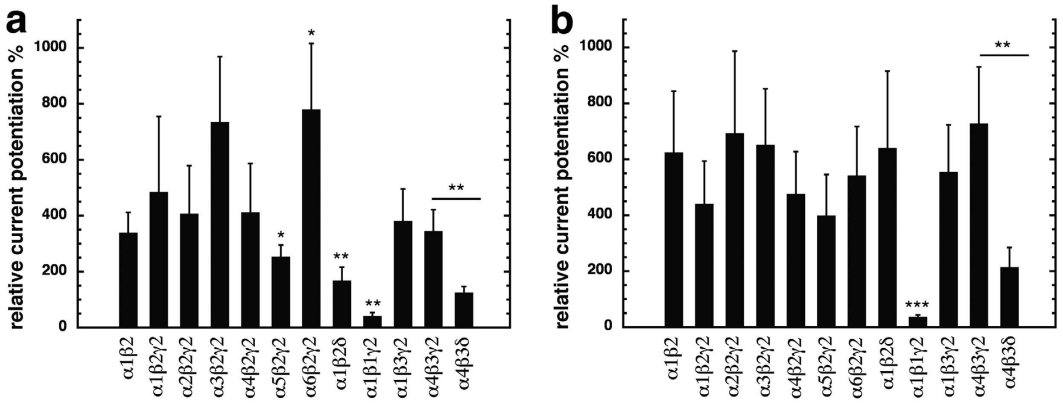

Figure 7. Subunit specificity of current potentiation in different $GABA_A$ receptors. Different subunit combinations were expressed in *Xenopus* oocytes. Potentiation of GABA currents at a GABA concentration eliciting 0.5–1.5% of the maximal current amplitude was determined using 3 μM of compound 31 (**a**) or 132 (**b**). Bars indicate mean ± SD, n = 4–11.

As shown in Fig. 6, the β_2N265S mutation in $\alpha_1\beta_2\gamma_{l2}$ receptors significantly reduced potentiation by 3 μM compound 31, from 485 ± 230% in wild-type receptors (mean ± SD, n = 11), to 125 ± 25% in the mutated receptor (mean ± SD, n = 4, p = 0.009, *Tukey posthoc test*). In contrast, this mutation did not significantly reduce potentiation by 3 μM compound 132: 415 ± 126% in wild-type receptors (mean ± SD n = 6) versus 280 ± 36% in the mutated receptor (mean ± SD, n = 4, p > 0.05, *Tukey posthoc test*). We have shown earlier that potentiation by 4-O-methylhonokiol in receptors carrying the β_2N265S mutation was greatly reduced to about 40%[36].

Subunit specificity of compounds 31 and 132. From the above experiments we inferred that the β+/α− TMD subunit interfaces mediate potentiation of compounds 31 and 132. We also wanted to know if potentiation by these compounds depends on subunit isoforms. First we replaced the α_1 subunit by different α subunit isoforms: $\alpha_1\beta_2\gamma_{l2}$, $\alpha_2\beta_2\gamma_{l2}$, $\alpha_3\beta_2\gamma_{l2}$, $\alpha_4\beta_2\gamma_{l2}$, $\alpha_5\beta_2\gamma_{l2}$ and $\alpha_6\beta_2\gamma_{l2}$ (Fig. 7). Compound 31 displayed a similar degree of potentiation in $\alpha_1\beta_2\gamma_{l2}$ receptors 485 ± 230% (mean ± SD, n = 11), $\alpha_2\beta_2\gamma_{l2}$ receptors, 407 ± 172% (mean ± SD, n = 4, p > 0.05, *Tukey posthoc test*), $\alpha_3\beta_2\gamma_{l2}$ receptors, 735 ± 234%, (mean ± SD, n = 5, p > 0.05, *Tukey posthoc test*),

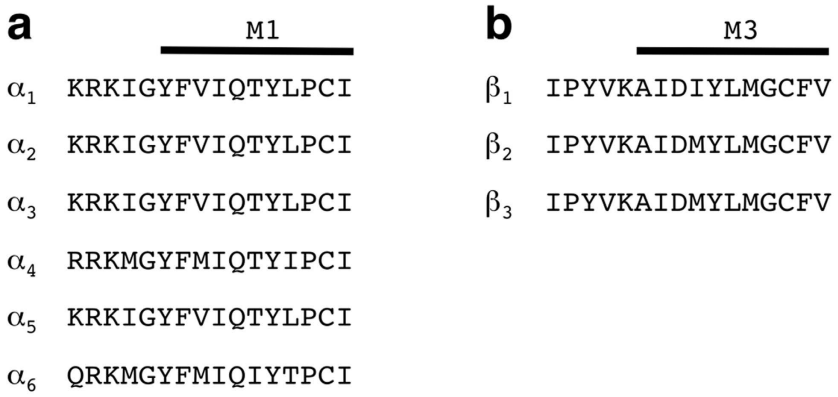

Figure 8. Alignment of the rat amino acid residue sequences of different α (a) and β (b) subunit isoforms. (**a**) Sequences preceding M1 and the first part of M1 in α subunits are shown. (**b**) Sequences preceding M3 and the first part of M3 in β subunits are shown.

and $\alpha_4\beta_2\gamma_2$ receptors, 412 ± 175% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*). The $\alpha_5\beta_2\gamma_2$ receptor showed a significant decrease in potentiation compared to $\alpha_1\beta_2\gamma_2$ receptors to 253 ± 92% (mean ± SD, n = 8; $p = 0.023$, *Tukey posthoc test*), and the $\alpha_6\beta_2\gamma_2$ receptor an increase in potentiation, amounting to 780 ± 236% (mean ± SD, n = 4; $p = 0.048$, *Tukey posthoc test*). This discrepancy in modulation of $\alpha_5\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ receptors maybe explained by the fact that 3 of the first 7 residues of M1 located at the minus side of the α subunit are different (Fig. 8a). Compound 132 produced similar potentiation in receptors with all α subunits tested. Amounting to 415 ± 126% (mean ± SD, n = 6) in $\alpha_1\beta_2\gamma_2$ receptors, 694 ± 293% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) in $\alpha_2\beta_2\gamma_2$ receptors, 652 ± 215% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) in $\alpha_3\beta_2\gamma_2$ receptors, 476 ± 152% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) in $\alpha_4\beta_2\gamma_2$ receptors, 399 ± 147% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) in $\alpha_5\beta_2\gamma_2$ receptors, and 542 ± 175% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) in $\alpha_2\beta_2\gamma_2$ receptor type. These results indicate that although the type of α subunit has differential effects on potentiation between compounds 31 and 132, these compounds modulate all receptor subtypes studied.

Next, we examined the role of the β subunit, replacing the β_2 by β_1 or β_3. For compounds 31 and 132, $\alpha_1\beta_3\gamma_2$ receptors showed a similar potentiation as $\alpha_1\beta_2\gamma_2$ receptors. Amounting to 381 ± 115% for compound 31 and 555 ± 169% for compound 132 (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*). In the case of $\alpha_1\beta_1\gamma_2$ receptors, potentiation by both compounds was significantly reduced compared to that in $\alpha_1\beta_2\gamma_2$, 41 ± 13% for compound 31 and 37 ± 7% for compound 132 (n = 4; $p = 0.0023$, *Tukey posthoc test* for compound 31; n = 4; $p = 0.0004$, *Tukey posthoc test,* for compound 132). These results indicate that the type of β subunit is important for the potentiation by both compounds. It is interesting to note in this context that β_1 and β_2/β_3 differ not only in the residue 265, but also in the fourth residue of M3 predicted to be close to the latter residue (Fig. 8b).

When the γ_2 subunit was omitted, no statistical difference was observed for either compound tested. Compound 31 displayed a similar degree of potentiation between $\alpha_1\beta_2\gamma_2$ receptors and $\alpha_1\beta_2$ receptors, 339 ± 73% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*). Compound 132 also showed a similar potentiation between both receptors, potentiation in $\alpha_1\beta_2$ receptors amounting to 625 ± 219% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*). When the γ_2 subunit was replaced by a δ subunit, in the case of $\alpha_1\beta_2\delta$ receptors, potentiation was affected only in the case of compound 31, where a significant reduction was observed relative to $\alpha_1\beta_2\gamma_2$ receptors. Where potentiation amounted to 168 ± 48% (mean ± SD, n = 4, $p = 0.0026$, *Tukey posthoc test*) for compound 31, and 640 ± 275% (mean ± SD, n = 4, $p > 0.05$, *Tukey posthoc test*) for compound 132. In $\alpha_4\beta_3\delta$ receptors, potentiation by both compounds was statistically reduced relative to $\alpha_4\beta_3\gamma_2$ receptors. For compound 31 potentiation amounted to 345 ± 77% (mean ± SD, n = 4) in $\alpha_4\beta_3\gamma_2$ receptors, and was reduced to 125 ± 22% (mean ± SD, n = 4, $p = 0.0015$, *Tukey posthoc test*) in $\alpha_4\beta_3\delta$ receptors. Potentiation of compound 132 in $\alpha_4\beta_3\gamma_2$ receptors was 728 ± 203% (mean ± SD, n = 4), and was reduced to 214 ± 71% (mean ± SD, n = 4, $p = 0.0031$ *Tukey posthoc test*, for compound 132) in $\alpha_4\beta_3\delta$ receptors.

Previous work[36] showed that potentiation by 4-O-methylhonokiol was dependent on the α subunit in a similar fashion as compound 31, since modulation was reduced by receptors containing α_5 and α_6 subunits. The type of β subunit was also important, as the presence of the β_1 subunit strongly reduced potentiation by this compound. On the contrary, the presence of a γ or a δ subunit did not affect potentiation.

Anesthetic activity in tadpoles. The anesthetic activity for compounds 31, 132 and 4-O-methylhonokiol was determined as loss of righting reflex (LoRR) in *Xenopus* tadpoles, Fig. 9 shows the concentration dependence curve for each. Compound 31 yielded an EC_{50} of 2.7 μM (95% confidence interval = 2.0 to 3.7 μM), while the EC_{50} compound 132 was 1.2 μM (95% confidence interval = 0.73 to 2.0 μM), and 4-O-methylhonokiol EC_{50} = 1.0 μM (95% confidence interval = 0.46 to 2.2 μM). For comparison, the EC_{50} for propofol-induced LoRR in tadpoles is 1.3 μM[41]. Anesthesia was fully reversible for compound 31; for animals tested with compound 132, recovery was minimal at concentrations above 3 μM. For 4-O-methylhonokiol, animals tested at a concentration of 10 μM did not recover, whereas recovery was complete at 3 μM and lower concentrations.

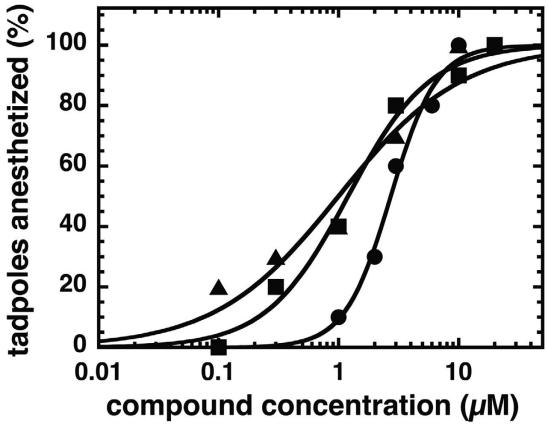

Figure 9. Concentration-response curves for loss of righting reflexes (LORR) in tadpoles for compounds 31 (closed circle), 132 (closed square), and 4-O-methylhonokiol (closed triangle). The percent of animals anesthetized is plotted against aqueous anesthetic concentration, overlaid with logistic fits. Each point represents data from ten animals. Data were fitted to a Hill equation.

Discussion

Here we functionally characterized compounds 31, 132 and further investigated the properties of 4-O-methylhonokiol. All three compounds are potent allosteric potentiators of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors that do not act at site 1. We suspected that they instead act through the low affinity TMD site(s) for benzodiazepines (site 3). Indeed, potentiation by compounds 31, 132 and 4-O-methylhonokiol was abolished in the triple mutant receptor α_1S269Iβ_2N265Iγ_2S280I as well as by the single mutation β_2N265I, but unaffected by the homologous mutations α_1S269I and γ_2S280I. Assuming that these TM2 mutations alter drug actions through local steric effects in adjacent TMD interfacial sites, our results indicate that of the five such sites, only the two $\beta+/\alpha-$ interfaces 1 and 2 mediate the potentiating effects of these three compounds.

We further dissected the contribution of the individual $\beta+/\alpha$- subunit interfaces using concatenated subunit assemblies. The $\gamma\beta+/\alpha-\beta$ interface (interface 1) and $\alpha\beta+/\alpha-\gamma$ interface (2) participated differently in modulation by the three compounds studied. For all compounds the contribution of the interface 1 to drug modulation is apparently greater than that of the interface 2.

We and others have shown that the intravenous anesthetics etomidate, propofol and pentobarbital also act via TMD interfacial sites[26–29,37]. In $\alpha_1\beta_2\gamma_2$ receptors, β_2N265I reduced potentiation by all compounds, α_1S269I reduced potentiation exclusively by pentobarbital, and the γ_2S280I mutation increased potentiation by etomidate, while reducing potentiation by propofol and pentobarbital[37]. Different sets of residues located at subunit interfaces have been photo-labeled by etomidate, barbiturate, and propofol analogs, revealing that some anesthetics selectively bind within different TMD interfaces[23,24,33,42,43]. Additionally, mutations of residues at the $\beta+/\alpha-$ subunit interface affecting anesthetic action have been shown to affect modulation by valerenic acid. This suggests that the binding pocket for this compound is also at or near the anesthetics binding site[44]. Other subunit interfaces were not investigated.

Work by our group using receptor concatenation determined that both $\beta+/\alpha-$ subunit interfaces participated equally in modulation by propofol. In contrast, modulation by etomidate was found to be more affected by the $\gamma\beta+/\alpha-\beta$ interface site (interface 1) than the $\alpha\beta+/\alpha-\gamma$ site (interface 2)[37]. Interestingly, studies using another mutation (β_2M286W) and different concatenated subunit assemblies suggest that etomidate interactions are equivalent in the two $\beta+/\alpha-$ sites of $\alpha_1\beta_2\gamma_2$ receptors[45].

The homologs of β_2N265 in β_1 and β_3 are serine and asparagine, respectively, and this single residue dramatically influences sensitivity to loreclezole[39], etomidate and propofol[26,28,29,39,40]. Potentiation by compound 31 was strongly affected by this mutation while that by compound 132 was affected less. On the other hand, potentiation by 132 was severely reduced in $\alpha_1\beta_1\gamma_2$ receptors, compared to $\alpha_1\beta_2\gamma_2$ receptors. Potentiation by 4-O-methylhonokiol is also reduced by the β_2N265S mutation or substitution of β_1 for β_2[36].

In subunit specificity studies, compounds 31 and 132 potentiated receptors containing the α_4 and α_6 subunits, contrasting with benzodiazepine site 1 agonists. Both compounds also potentiated receptors carrying the δ subunit, although to different degrees. Thus, these compounds not only acted at receptors shown to be located synaptically as $\alpha_1\beta_{2-3}\gamma_2$, $\alpha_2\beta_2\gamma_2$ and $\alpha_3\beta_2\gamma_2$[46], but also at $\alpha_5\beta_2\gamma_2$ receptors and receptors containing the δ subunit, which are all located extra-synaptically[5,46,47]. Similarly, SJM-3 modulates both synaptic and extrasynaptic receptors[35].

The similarities between the three compounds we studied and the clinical anesthetics propofol, etomidate and pentobarbital suggested their possible use as sedative-hypnotics in animals. Indeed, all three compounds induced reversible loss of righting reflexes (LoRR) in *Xenopus laevis* tadpoles with EC_{50}s comparable to the anesthetics propofol[41], and etomidate[48]. However, LoRR was not reversible with high concentrations of compound 132 and 4-O-methylhonokiol.

In summary, the newly identified compounds 31 and 132 modulate both synaptic and extrasynaptic $GABA_A$ receptors at molecular sites different from the classical benzodiazepine pocket. These compounds, together with 4-O-methylhonokiol, act through $\beta+/\alpha-$ TMD interfaces, with strongest effects through the interface 1. These

compounds potently produced LoRR in aquatic animals and thus may be useful lead compounds in the search for novel anesthetic, sedative-hypnotic or anxiolytic drugs.

Methods

Construction of mutated receptor subunits. The point mutations α_1S269I, β_2N265I, β_2N265S and γ_2S280I were prepared using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

Construction of concatenated subunits. Construction of tandem and triple subunit cDNAs. The tandem construct γ_2-β_2, and triple construct α_1-β_2-α_1 has been described previously[12]. Site-directed mutagenesis of β_2N265 to I was done in the tandem construct and the triple construct using the QuikChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

Expression of $GABA_A$ receptors in Xenopus oocytes. Capped cRNAs were synthesized (Ambion, Austin, TX, USA) from the linearized plasmids with a cytomegalovirus promotor (pCMV vectors) containing the different subunits, respectively. A poly-A tail of about 400 residues was added to each transcript using yeast poly-A polymerase (United States Biologicals, Cleveland, OH, USA). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad) for visualization of the RNA. Known concentrations of RNA ladder (Invitrogen) were loaded as standard on the same gel. cRNAs were precipitated in ethanol/isoamylalcohol 19:1, the dried pellet dissolved in water and stored at −80 °C. cRNA mixtures were prepared from these stock solutions and stored at −80 °C.

Animal experiments were carried out in strict accordance to the Swiss ethical guidelines, and have been approved by the local committee of the Canton Bern Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15). Surgery was done under anesthesia, and all efforts were made to diminish animal suffering. Xenopus laevis oocytes were prepared, injected and defolliculated as described previously[49,50]. Oocytes were injected with 50 nL of the cRNA solution containing wild type or mutated rat α_1, β_2 and γ_2 subunits of the $GABA_A$ receptors at a concentration of 10 nM:10 nM:50 nM[51]. For concatenated tandem and triple constructs, cRNA combinations ratios of 25: 25 nM were used. Injected oocytes were incubated in modified Barth's solution at 18 °C for at least 24 h before the measurements.

Functional characterization of $GABA_A$ receptors. Currents were measured using a modified two-electrode voltage clamp amplifier Oocyte clamp OC-725 (Warner Instruments) in combination with a XY-recorder (90% response time 0.1 s) or digitized at 100 Hz using a PowerLab 2/20 (AD Instruments) using the computer programs Chart (ADInstruments GmbH, Spechbach, Germany). Tests with a model oocyte were performed to ensure linearity in the larger current range. The response was linear up to 15 μA. The holding potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Na-HEPES (pH 7.4). Concentration response curves for the compounds were fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of the compound, EC_{50} the concentration eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration-response curves. For all receptors studied, modulation was measured at a GABA concentration eliciting 0.5–1.5% of the maximal GABA current amplitude. GABA was applied twice alone for 20–60 s, and then in combination with the different compounds for 45 s or 1 min. The duration of washout periods was 4 min in between agonist or agonist/drug aplications to prevent receptor desentization. At the beginning of the experiments, GABA applications were repeated when the elicited current amplitude altered by >5%. Potentiation was calculated by the following equation: $(I_{Modulator + GABA}/I_{GABA} - 1) * 100\%$. The perfusion solution was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.4 mm from the surface of the oocyte. This allowed fast changes in agonist concentration around the oocyte. The rate of change was estimated 70% in less than 0.5 s[14]. The perfusion system was cleaned between drug applications by washing with DMSO to avoid contamination. All media contained a final concentration of 0.5% DMSO (v/v) to ensure drug solubility.

All data are from at least two different batches of oocytes. Data represent mean ± SD. An unpaired *t test* was used to compare two means. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by a *Tukey post hoc* test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Loss of righting reflex assay in Xenopus tadpole. Animals were used and experiments were carried out with approval and according to the guidelines of the MGH Institutional Animal Care and Use Committee. General anesthetic potency was assessed in *Xenopus* laevis tadpoles as previously described[41,52,53]. In brief, groups of 10 tadpoles were placed in aqueous solutions containing compound 31, 132, or 4-O-methylhonokiol, and tested every five minutes for loss of righting reflexes (LoRR). Each animal was assigned a score of either awake or LoRR, and the percent of animals anesthetized was plotted against the concentration of the compound tested. Concentration-response data was fitted by non-linear least squares to logistic functions of the form $Y = 1/(1 + 10\text{^}((LogEC_{50} - Log[Drug])*HillSlope))$ using Graphpad Prism 6. Results are reported as EC_{50s} and 95% confidence intervals.

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Author Contributions

M.C.M. and R.B. performed electrophysiological experiments. D.P. and A.N. performed LoRR experiments. M.C.M., S.A.F. and E.S. designed the experiments, analysed the data, and wrote the manuscript.

Additional Information

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