Limiting Activity at β_1 -Subunit-Containing GABA_A Receptor Subtypes Reduces Ataxia^S

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ABSTRACT

GABA_A receptor (R) positive allosteric modulators that selectively modulate GABA_ARs containing β_2 - and/or β_3 - over β_1 -subunits have been reported across diverse chemotypes. Examples include loreclezole, mefenamic acid, tracazolate, and etifoxine. In general, " $\beta_{2/3}$ -selective" GABA_AR positive allosteric modulators are nonbenzodiazepines (nonBZs), do not show α -subunit isoform selectivity, yet have anxiolytic efficacy with reduced ataxic/sedative effects in animal models and humans. Here, we report on an enantiomeric pair of nonBZ GABA_AR positive allosteric modulators that demonstrate differential

β -subunit isoform selectivity. We have tested this enantiomeric pair along with a series of other $\beta_{2/3}$ -subunit selective, α -subunit isoform-selective, BZ and nonBZ GABA_A positive allosteric modulators using electrophysiological, pharmacokinetic, and behavioral assays to test the hypothesis that ataxia may be correlated with the extent of modulation at β_1 -subunit-containing GABA_ARs. Our findings provide an alternative strategy for designing anxiolytic allosteric modulators of the GABA_AR with BZ-like anxiolytic efficacy by reducing or eliminating activity at β_1 -subunit-containing GABA_ARs.

Positive allosteric modulators of the GABA_A receptor (R) such as the benzodiazepines (BZs) continue to be used to treat anxiety, despite the well-known side effect of sedation. Diverse drug discovery efforts over two decades have focused on generating "anxiolytic" (i.e., reducing anxiety without sedation) GABA_AR positive allosteric modulators. Medicinal chemistry efforts have focused primarily on modifications of the BZ template with limited success in reducing sedative liability (Whiting, 2006).

One strategy to generate anxiolytic positive allosteric modulators involves creation of positive allosteric mod-

ulators that selectively modulate individual GABA_AR subtypes involved in anxiety, while avoiding those mediating sedation. Several laboratories have focused on α -subunit isoform-selective BZ site agonists that evoke positive modulation of α_2 - and α_3 - but not α_1 -subunit-containing GABA_ARs. This " $\alpha_{2/3}$ -selective" approach is based on pharmacological and genetic data suggesting that α_2 - and α_3 -subunit-containing GABA_ARs mediate the anxiolytic actions of BZs, whereas those with α_1 -subunits, especially the $\alpha_1\beta_2\gamma_2$ subtype, are thought to mediate their sedative effects (Rudolph et al., 1999; McKernan et al., 2000). Consistent with this general theory, L-838,417 is a $\alpha_{2,3}$ -subunit-selective partial agonist BZ receptor ligand reported to be anxiolytic in animal models (McKernan et al., 2000). However, recent clinical studies designed to determine whether $\alpha_{2,3}$ -subunit selectivity imparts reduced sedative liability has resulted in equivocal results where BZ-like side effects were observed (de Haas et al., 2008, 2009). Moreover, the α_3 -subunit-selective BZ site partial agonist adiplon has potent sedative activity and was in clinical development as a sedative-hypnotic (Sprenger et al., 2007).

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ABBREVIATIONS: R, receptor; BZ, benzodiazepine; nonBZ, nonbenzodiazepine; DMSO, dimethyl sulfoxide; 5-HT, 5-hydroxytryptamine; nAChR, nicotinic acetylcholine receptor; HPLC, high-performance liquid chromatography; LD, light-dark transition; ANOVA, analysis of variance; RR, Rotarod; AD₅₀, ataxogenic half-maximal dose where half of the mice fail the RR assay; EtOH, ethanol; MED, minimal effective dose; EPM, elevated plus maze; PK, pharmacokinetic; PD, pharmacodynamic; AI, anxiolytic index; SAR, structure-activity relationship; LGIC, ligand-gated ion channel.

Selective activity at different α -subunit isoforms does not fully explain the sedative effects of all BZ site agonists. A prominent example that argues against the theory is the BZ site ligand ocinaplon. It has robust full agonist-like activity at α ₁-subunit-containing GABA_ARs in vitro, yet it has reduced sedative liability in preclinical and clinical studies (Basile et al., 2004; Lippa et al., 2005; Popik et al., 2006). Furthermore, there have been no reports of compounds with α _{2,3}-subunit selectivity achieving clinical proof-of-concept (i.e., anxiolytic), despite the passage of almost a decade since the initial proposal of the α _{2,3}-subunit selectivity hypothesis.

Apart from the intense focus on GABA_A subtype-selective BZs, other ways to elicit anxiolytic activity via the GABA_A are relatively unexplored, especially as it relates to nonBZ site positive allosteric modulators with receptor subtype selectivity (Whiting, 2006). One interesting approach focuses on GABA_A positive allosteric modulators that are selective modulators of β ₂- or β ₃- over β ₁-subunit-containing GABA_ARs. This type of selective modulation has been reported for compounds across diverse chemotypes (e.g., loreclezole, mefenamic acid, tracazolate, and etifoxine) (Halliwell et al., 1999; Thompson et al., 2002; Hamon et al., 2003; Groves et al., 2006). Empirical observations of these positive allosteric modulators in both animals and humans provide anecdotal evidence that suggests that the degree of activation of β ₁-subunit-containing GABA_ARs may contribute to their sedative/ataxic potential.

Based on this correlative evidence, we propose the hypothesis that among nonBZ site positive allosteric modulators the continuum of sedative, ataxic and hypnotic effects elicited by GABA_A activation may also depend on β -subunit isoform selectivity. Thus, limiting or eliminating activity at β ₁-subunit-containing GABA_ARs will reduce or abolish these effects, respectively. We recently identified a series of nonBZ site enaminone positive allosteric modulators with a range of potencies and efficacies, thus providing us with a template to create tools to test our hypothesis (Hogenkamp et al., 2007).

Here, we report the characterization of an enantiomeric pair of enaminones, 2-261 and 2-262, that have differential β -subunit isoform selectivity. We have tested this pair and 15 other β -subunit isoform-selective and -nonselective positive allosteric modulators for GABA_A potency/efficacy, pharmacokinetic profiles, and behavior in anxiety models. Our studies provide correlative pharmacological evidence that selective modulation of β -subunit-containing GABA_A subtypes may be another mechanism to separate anxiolytic from ataxic effects among nonBZ site GABA_A positive allosteric modulators.

Materials and Methods

Drugs. Loreclezole (Heeres, 1985; Astleford et al., 1989), tracazolate (Bare et al., 1989), etifoxine (Putman et al., 2007), and ocinaplon (Skolnick and Epstein, 2005) were synthesized in our lab using methods reported in the literature. The enaminones were synthesized as described previously (Hogenkamp et al., 2007; Table 1). Diazepam, bretazenil, etomidate, and mefenamic acid were purchased from Sigma-Aldrich (St. Louis, MO), whereas L-838,417 (McKernan et al., 2000) was from Tocris Biosciences (Ellisville, MO). For electrophysiology experiments, drugs were first dissolved in dimethyl sulfoxide (DMSO) to 10 mM and diluted in Ringer's salt solution (0.1% total DMSO final solution). Drugs for per os administration were dissolved in polyethylene glycol-400 and administered at ≤ 2 ml/kg. Drugs administered by intraperitoneal injection were dissolved in DMSO and administered at ≤ 1 ml/kg.

Animals. Mice were male NSA (Harlan Labs, Los Angeles, CA) and weighed 24 to 28 g. Rats were male Sprague-Dawley (Harlan Labs) and weighed 250 to 350 g. Animals were housed under a 12:12-h light/dark cycle starting at 6:30 AM and tested according to University of California, Irvine Institutional Animal Care and Use Committee-approved protocols. Oocytes were obtained from *Xenopus laevis* frogs using procedures approved and monitored by the Institutional Animal Care and Use Committee.

Two-Electrode Voltage-Clamp Oocyte Electrophysiology. cDNA clones were provided as kind gifts from the following sources: human α 7, α 4, and β 2 nicotinic acetylcholine receptors (nAChRs) were

TABLE 1

Potency and maximal efficacy of test compounds at α ₁ β ₁ γ ₂ or α ₁ β ₂ γ ₂ GABA_ARs in rank order of ascending efficacy at α ₁ β ₁ γ ₂

The mouse RR AD₅₀ value is provided for each compound if determinable over the range of doses tested. The peak brain levels associated with the AD₅₀ or the highest doses tested, when an AD₅₀ could not be attained, are expressed in micromolar. In vitro potency is expressed as the EC₅₀ (micromolar) followed by its 95% confidence interval in parentheses, and maximal efficacy is expressed as the percentage of modulation of an EC₁₀ GABA effect. Brain levels are expressed as the mean $\pm$ S.E.M. ($n = 3-9$). All test compounds were administered by intraperitoneal injection except for 2-261, 2-262, and mefenamic acid, which were administered orally.

Compound	Maximal Efficacy at α ₁ β ₁ γ ₂	EC ₅₀ at α ₁ β ₂ γ ₂	Maximal Efficacy at α ₁ β ₂ γ ₂	EC ₅₀ at α ₁ β ₂ γ ₂	RR AD ₅₀	Brain Level
	%	μ M	%	μ M	mg/kg	μ M
L-838,417 ^a	0	N.D.	0	N.D.	30	8 $\pm$ 1
2-325	16	N.D.	1330	0.22 (0.1–4.6)	>30	2.1 $\pm$ 0.4
Mefenamic acid	18	N.D.	315	3.6 (2.5–5.1)	>500	3.1 $\pm$ 0.7
2-313	33	N.D.	727	0.14 (0.09–0.2)	>50	1.2 $\pm$ 0.1
2-261	34	N.D.	1101	0.3 (0.1–0.6)	>100	3.7 $\pm$ 0.5
2-301	47	N.D.	686	0.22 (0.1–0.7)	>100	4.0 $\pm$ 1.5
Ocinaplon	81	2.8 (0.7–11)	72	2.1 (0.3–19)	54	12.5 $\pm$ 1.5
Bretazenil	95	0.05 (0.01–0.42)	58	0.004 (0.001–0.008)	25	11.7 $\pm$ 3.0
Diazepam	109	0.11 (0.03–0.5)	166	0.05 (0.02–0.1)	2	0.64 $\pm$ 0.3
2-128	113	2.4 (1.2–4.9)	552	0.9 (0.6–1.3)	>120 (40% failure at 120)	5.8 $\pm$ 2.0
Loreclezole	125	40 (31–52)	498	4.8 (3.1–7.5)	83	11.7 $\pm$ 3.2
2-262	181	1.4 (0.7–2.7)	1102	0.04 (0.02–0.09)	43	0.8 $\pm$ 0.3
2-249	249	3.6 (1.4–9.5)	905	0.03 (0.01–0.1)	3	0.6 $\pm$ 0.1
2-148	369	6.9 (3–12)	817	0.2 (0.1–0.4)	5	1.1 $\pm$ 0.1
Tracazolate	375	4.7 (2–12)	821	0.6 (0.3–1.3)	42	7.6 $\pm$ 1.7
2-314	655	0.2 (0.1–0.4)	814	0.11 (0.03–0.4)	2.5	0.30 $\pm$ 0.06
Etomidate	819	18 (12–27)	1307	2 (1–8)	N.D.	N.D.

N.D., not determined.

^a L-838,417 is inactive at both α ₁-containing subtypes because of selectivity for α _{2/3} over α ₁-subunits.

from Dr. Jon Lindstrom (The University of Pennsylvania, Philadelphia, PA); human GABA_A receptor subunits (α_1 , α_2 , α_3 , β_1 , β_2 , β_3 , γ_2 , and δ) were from CoCensys Inc. (Irvine, CA); and rat δ and ϵ nAChRs were from Dr. James Boulter (University of California, Los Angeles, CA). 5-HT_{3A} was purchased from the American Type Culture Collection (Manassas, VA).

Preparation, microinjection, and maintenance of oocytes was as described previously (Ng et al., 2007). Individual oocytes were injected with 0.005 to 50 ng of each subunit mRNA as follows (ratio of subunits in parentheses): GABA_A receptor subunit combinations ($\alpha_{1,2}$, or 3; $\beta_{1,2}$, or 3 γ_{2L} ; or δ): (5:1:1). Stage IV to V oocytes were plucked from ovary membranes and defolliculized with collagenase type IA (Worthington Biochemicals, Freehold, NJ) for 45

min, rinsed 10 times with Ringer's salt solution, and then cRNA was injected at 50 nl. Oocytes were tested 3 to 28 days after injection ($n = 3-7$ /compound) in Ringer's salt solution by linear drug application method using electrodes with 1- to 2- ω tip resistance. Changes in membrane current were passed through a pre-amplifier and then through a T200 patch amplifier (Axon Instruments, Sunnyvale, CA), with a bandpass filter of 2 kHz. pCLAMP software (Molecular Devices) was used to monitor, record, and analyze data. All compounds were tested with a 30-s pretreatment before coapplication, with EC₁₀ (concentration of GABA that evokes 10% of the maximal response) GABA for the control response. The GABA EC₁₀ was determined in each individual oocyte expressing the receptor subtype of interest. For example, the EC₁₀

Fig. 1. Structural diversity of GABA_AR allosteric modulators in the present study.

value ranges from 3×10^{-7} to 10^{-6} M at $\alpha_1\beta_1\gamma_2$ and from 3×10^{-6} to 10^{-5} M at $\alpha_1\beta_2\gamma_2$ isoforms. The EC_{100} value was 10^{-3} M. Responses in presence of test compound were calculated as percentage of modulation above control. Concentration-response curves were fit to nonlinear regression analysis on Prism 4.0 (GraphPad Software Inc., San Diego, CA) for percentage of maximal stimulation, EC_{50} values, and their 95% confidence limits. In cases where 0% modulation was not defined, the bottom of the concentration-response curve was constrained to zero. However, when several concentrations tested resulted in a well defined 0% response then constraining to zero was unnecessary. Percentage of stimulation corresponding to brain levels of the compounds tested was extrapolated from these concentration-response curves.

Pharmacokinetic Studies. Blood was removed at various time points after drug administration via cardiac puncture under halothane anesthesia and centrifuged at 1000g for 6 min to separate the plasma. After euthanization, brains were perfused with saline and removed, stored at -20°C until processed for extraction and high-performance liquid chromatography (HPLC) analysis. Plasma and brain extracts were run through HPLC in a 30 to 70% acetonitrile/phosphate buffer mobile phase through a C18 column, detecting the maximal absorption wavelength via UV-spectrometry as described previously (Ng et al., 2007). Extraction methods were repeated for liquid chromatography/mass spectrometry determination. Approximately 0.5 g (brain) or 250 μl (plasma) containing the analyte was precipitated with acetonitrile, and the supernatant was evaporated and reconstituted in acetonitrile. The samples were analyzed by reversed phase high-performance liquid chromatography in a 2790 HPLC (Waters, Milford, MA)

using a Nova-Pak C18 column (Waters) and were detected using a QuatroUltima triple quad mass spectrometer (Waters) by collision-induced dissociation/tandem mass spectrometry.

Mouse Light-Dark Transition. Naive mice were acclimated (1 h) to a darkened room before administration of test compounds. Testing occurred during peak brain levels of compounds, in automated light-dark transition (LD) boxes (Coulbourn Instruments, Allentown, PA), tracked by infrared beam-breaking collar and TruScan software (Coulbourn Instruments). Light bulbs were placed 60 cm above the floor of the test box where light intensity was 400 lux and centered on the lit half of the box. The time spent in the dark was recorded. Data were analyzed with Prism 4.0 (GraphPad Software Inc.) for statistical significance by one-way ANOVA with Dunnett's multiple comparison post-hoc test.

Rat Elevated Plus Maze. Rats were group housed and handled daily for 3 days before testing in the elevated plus maze (EPM; Coulbourn Instruments). Testing was conducted in a dimly lit (2-lux) room, with two 60-W bulbs pointed at the ceiling near the open arms (122 cm above the maze, 400 lux at the surface of the maze). The maze was cleaned between each run. All compounds were tested at times that correspond to peak brain concentrations. Automated counting of time spent in the open arms of the maze was achieved by using the MedPC-IV program (MED Associates, St. Albans, VT). Data were analyzed with Prism 4.0 (GraphPad Software Inc.) for statistical significance by one-way ANOVA with Dunnett's multiple comparison post-hoc test.

Mouse Rotarod. Naive mice were trained on a Rotarod (RR; Columbus Instruments, Columbus, OH) in four sessions (6–15 rpm) over 2 days to successfully complete the 2-min trial before final testing (6 rpm). On day 3, the mice were administered

Fig. 2. Modulation of submaximal (EC_{10}) GABA-evoked currents by 2-261 and 2-262. Top, representative current tracings from two-electrode voltage-clamp recordings in *X. laevis* oocytes expressing human $\alpha_1\beta_2\gamma_2$ (A) or $\alpha_1\beta_1\gamma_2$ (B) GABA_ARs. Substitution of the β_2 - with the β_3 -subunit results in a similar response. Control currents for modulation were 10% of maximal current (EC_{100}). Drug exposures are indicated by the bars. Bottom, concentration-response relationship for modulation of EC_{10} currents by 2-261 and 2-262. Data represents mean $\pm$ S.E.M. ($n = 3-8$).

compound and tested over a period of 360 min at various intervals. The percentage of animals remaining on the RR throughout each 2-min trial was recorded. The results that coincided with the time of peak effect were analyzed by the method of Litchfield and Wilcoxon (1949) to determine the ataxogenic half-maximal dose where half of the mice fail the RR assay (AD_{50}).

Effect of 2-261 versus Diazepam on Ethanol-Induced RR Deficit. Naive mice were trained on an RR (Columbus Instruments) in four sessions (6–15 rpm) over 2 days to successfully complete the 2-min trial before final testing (6 rpm). On the third day, the mice (12 mice/condition) were administered an AD_{25} dose (1 g/kg i.p.) of EtOH followed 10 min later by 2-261 (30 mg/kg p.o.), diazepam (1 mg/kg i.p.), or test drug vehicle, and then they were

tested for RR performance at various time intervals. The percentage of animals remaining on the RR throughout the 2-min trial was recorded and analyzed. Among the EtOH-treated animals, the statistical difference between test drug- and vehicle-treated animals was determined by the Fischer-Yates exact probability test.

Results

2-261 and 2-262 Evoke β - but Not α -Subunit Isoform-Selective Positive Allosteric Modulation of GABA_ARs in *X. laevis* Oocytes. We previously synthesized a series of nonBZ site GABA_AR positive allosteric modulators, which

Fig. 3. α -Subunit selectivity of 2-261 and 2-262. Concentration-response curves for 2-261 and 2-262 across α_1 -, α_2 -, or α_3 -subunit-containing GABA_AR subtypes. The curves depicted (left) show the effect of 2-261 on $\alpha_1\beta_1\gamma_2$ versus $\alpha_1\beta_2\gamma_2$ (A), $\alpha_2\beta_1\gamma_2$ versus $\alpha_2\beta_2\gamma_2$ (B), and $\alpha_3\beta_1\gamma_2$ versus $\alpha_3\beta_2\gamma_2$ (C) GABA_ARs, with the filled and open symbols denoting the presence of β_2 - or β_1 -subunits, respectively. 2-261 elicits high-efficacy modulation GABA currents at $\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_2\gamma_2$, and $\alpha_3\beta_2\gamma_2$ but not at $\alpha_1\beta_1\gamma_2$, $\alpha_2\beta_1\gamma_2$, or $\alpha_3\beta_1\gamma_2$. 2-262 (right) elicits a similar pattern of responses at $\alpha_1\beta_1\gamma_2$ versus $\alpha_1\beta_2\gamma_2$ (D), $\alpha_2\beta_1\gamma_2$ versus $\alpha_2\beta_2\gamma_2$ (E), and $\alpha_3\beta_1\gamma_2$ versus $\alpha_3\beta_2\gamma_2$ (F), except that greater responses are observed at β_1 -subunit-containing GABA_ARs when concentrations exceed 10^{-7} M.

included the racemic enaminone designated 2-247 (Hogenkamp et al., 2007). The individual enantiomers of 2-247 (2-261 and 2-262) differ only in the chirality of the *sec*-butyl amide side chain (Fig. 1). We chose to test 2-247, 2-261, 2-262, and subsequently other enaminones from our compound library for subunit-selective modulation of GABA-evoked currents in oocytes expressing different α - and β -subunit isoform combinations. At GABA_ARs containing β_2 - and β_3 -subunits, the enantiomers 2-261 and 2-262 both evoked high-efficacy positive modulation of GABA-evoked control currents (control currents were "EC₁₀" or 10% of maximal current), with maximal modulation to $\sim$ 1000% at $\alpha_1\beta_2\gamma_2$ (Fig. 2A) and $\alpha_1\beta_3\gamma_2$ (data not shown) GABA_ARs for both compounds. This efficacy far exceeds those of the typical full agonist BZ ($\sim$ 166% maximum for diazepam; Table 1) tested at $\alpha_1\beta_2\gamma_2$ in the same assay. GABA-dependent modulation by 2-261 and 2-262 at β_2 - and β_3 -subunit-containing GABA_ARs did not appear to be strongly dependent on α -subunits, with equivalent maximal modulation observed at α_1 -, α_2 -, and α_3 -subunits (Fig. 3).

In contrast, maximal modulation by 2-261 and 2-262 at β_1 -subunit-containing GABA_ARs was significantly lower than observed at those with β_2 - and β_3 -subunits. For example, at $\alpha_1\beta_1\gamma_2$, maximal modulation was 181% for 2-262 and only 34% for 2-261 (Fig. 2B), consistent with β -subunit isoform selectivity. The ratio of maximal modulation at $\alpha_1\beta_2\gamma_2$ versus $\alpha_1\beta_1\gamma_2$ is 32-fold for 2-261 and 6-fold for 2-262.

To further characterize the β -subunit selectivity of 2-261 and 2-262, we obtained GABA concentration-response data in the presence and absence of 2-261 and 2-262 at $\alpha_1\beta_1\gamma_2$ and $\alpha_1\beta_2\gamma_2$ subtypes. Both enantiomers induced a significant leftward shift of the GABA concentration-response curve at $\alpha_1\beta_2\gamma_2$, with little or no shift at $\alpha_1\beta_1\gamma_2$ (Fig. 4).

Positive Allosteric Modulation by 2-261 and 2-262 Occurs at the Same Site. The interaction of the two enantiomers was examined at $\alpha_1\beta_1\gamma_2$ GABA_ARs to establish whether they share the same site. We hypothesized that the low-efficacy modulator (2-261) should block the effects of the high-efficacy modulator (2-262) if these compounds have a common site of action. Modulation evoked by 2-262 was inhibited by 2-261, and this blockade was overcome by increasing concentrations of 2-262 consistent with a shared site of action (Fig. 5).

Anxiolytic Activity of 2-261 and 2-262 in Mice and Rats. In the mouse LD paradigm, both enantiomers reduced the time spent in the dark chamber. The minimal effective dose (MED) for 2-261 in this assay was 1 mg/kg p.o., with activity observed up to 30 mg/kg p.o. (Fig. 6A). 2-262 was more potent, with an MED value of 0.1 mg/kg p.o. and activity up to 1 mg/kg p.o. (Fig. 6B). For 2-262, anxiolytic activity was lost at 3 mg/kg p.o., possibly due to the onset of apparent sedative/ataxic effects of 2-262 (see below). The BZ receptor agonist alprazolam (0.1 mg/kg i.p.) was also active in this anxiety model.

Fig. 4. Electrophysiological shift of GABA by 2-261 and 2-262. GABA concentration-response curves derived from $\alpha_1\beta_2\gamma_2$ (A) versus $\alpha_1\beta_1\gamma_2$ (B) GABA_ARs $\pm$ 0.7 μ M 2-261. Likewise, the effect of GABA on $\alpha_1\beta_2\gamma_2$ (C) versus $\alpha_1\beta_1\gamma_2$ (D) GABA_ARs $\pm$ 3 μ M 2-262 is depicted in the bottom panels. GABA responses are expressed as $I/I_{\max}$, with each data point representing the mean $\pm$ S.E.M. ($n = 3-4$). The dashed line represents the GABA_{max} current with GABA only.

Fig. 5. Electrophysiological evidence that 2-261 blocks modulation by 2-262. **A**, representative voltage-clamp recording illustrating surmountable blockade of 2-262-induced modulation by 2-261 in oocytes expressing human GABA_A $\alpha_1\beta_1\gamma_2$ GABA_ARs. Drugs were applied as indicated by the bars. Dashed line indicates EC₁₀ control current magnitude used for calculating percentage of modulation. **B**, quantitative analysis of percentage of modulation (mean $\pm$ S.E.M.; $n = 3$).

We measured brain levels of 2-261 and 2-262 in a parallel group to correlate brain concentrations of drug at anxiolytic doses with GABA_AR modulation in vitro. 2-261 and 2-262 were administered at their MEDs as determined in the LD paradigm described above. At their anxiolytic MEDs, the peak brain levels of 2-261 and 2-262 were 4.0 ± 0.6 and 2.7 ± 0.4 nM, respectively. These levels correspond to an ~ 19 and $\sim 80\%$ modulation of EC₁₀ GABA-induced currents (in oocytes) at the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype by 2-261 and 2-262, respectively (Fig. 2A). Using similar analyses, these levels would have no measurable activity at $\alpha_1\beta_1\gamma_2$ GABA_ARs. Similar anxiolytic activity was observed in the rat EPM paradigm (Fig. 7).

Effects of 2-261 and 2-262 in the RR Assay and the Pharmacokinetic Profile of Both Enantiomers at Ataxic Doses. We tested 2-261 and 2-262 in the RR assay in mice to obtain AD₅₀ values to quantitate ataxia. No RR failures were observed over a period of 360 min in 2-261-treated mice at doses up to 60 mg/kg p.o. (Fig. 8A). Only 10% of animals failed the RR assay at 120 mg/kg p.o. so that the AD₅₀ value could not be determined. In contrast, 2-262 has an AD₅₀ value of 43 mg/kg p.o. (Fig. 8B).

We measured the peak brain and plasma concentrations of 2-261 at the maximal dose tested (120 mg/kg p.o.) and 2-262 at its AD₅₀ (43 mg/kg p.o.) to determine whether limited brain bioavailability could account for the limited ability of 2-261 to induce RR deficit. The maximal brain levels observed for 2-261 and 2-262 were 3.7 ± 0.5 and 0.8 ± 0.3 μM, respectively. At 10 mg/kg p.o., 2-261 and 2-262 had plasma half-lives of 1.5 and 1.6 h and peak brain levels of 0.7 ± 0.2 and 0.26 ± 0.11 μM, respectively (Fig. 9). Therefore, the differences in RR effects cannot be ascribed to limitations in bioavailability of 2-261 relative to 2-262.

Estimation of Anxioreactivity by Dose and Anxioreactive Index. To estimate the dose separation between anxiolysis and ataxia, we compared the RR AD₅₀ value with the MED value for anxiolytic activity. For 2-262, the separation is $430\times$ (RR AD₅₀ $\div$ LD MED). For 2-261, RR failures never exceeded 10%; thus, the separation could only be estimated as $>120\times$.

To examine the influence of bioavailability on anxioreactivity, we compared brain levels at ataxic and anxiolytic doses. The ratio of peak brain concentration at the ataxic AD₅₀ value divided by the peak brain concentration at the anxiolytic MED value yields an anxioreactive index (AI) for comparisons between compounds. For 2-262 in the LD paradigm, the AI corresponding to the MED (0.1 mg/kg p.o.) is ~ 296 (800 nM at AD₅₀ $\div$ 2.7 nM at MED). In contrast, for 2-261, the AI corresponding to the MED (1 mg/kg p.o.) is >925 (3700 nM at 120 mg/kg $\div$ 4 nM at MED), because the AD₅₀ value could not be calculated for 2-261. Based on the PK/pharmacodynamic (PD) relationship, 2-261 has a significantly greater AI than 2-262.

Does the Extent of Modulation at β_1 -Subunit-Containing GABA_ARs Determine the AI? To address this question, we tested additional enaminones and other reference positive allosteric modulators for 1) β -subunit isoform selectivity in vitro to determine the β -subunit isoform selectivity SAR in the enaminone series, 2) the ability to cause RR deficit, and 3) the PK profile to determine whether a PK/PD correlation exists. The data from the enaminones tested on $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_1\gamma_2$ GABA_ARs are summarized in Table 1 (SAR details can be found in Supplemental Table 1). These enaminones potentiated EC₁₀ GABA-evoked currents with varying degrees of β -subunit isoform selectivity and efficacy. As observed for 2-261 and

Fig. 6. Anxiolytic activity of 2-261 and 2-262 in mice. Dose-dependent effects of 2-261 (A) and 2-262 (B) versus alprazolam (0.1 mg/kg i.p.) on time (seconds) spent in the dark in the mouse LD paradigm after per os administration of each drug. The effects are compared with vehicle control. Each bar represents the mean $\pm$ S.E.M. time spent in the dark during a 5-min interval ($n = 5-42$ animals). Statistically significant differences from vehicle at *, $P < 0.05$ and **, $P < 0.01$ after ANOVA and post-hoc Dunnett's test.

2-262, the enaminones do not appear to be α -subunit isoform-selective (Hogenkamp et al., 2007). Moreover, these enaminones had a generally small effect when tested for receptor selectivity at selected Cys-loop LGICs (i.e., α_7 , $\alpha_4\beta_2$, and $\alpha_1\beta_1\delta\epsilon$ nicotinic acetylcholine; 5-HT_{3A}; and *N*-methyl-D-aspartate receptors; Table 2).

Like 2-261, 2-313 showed β -subunit isoform selectivity, with maximal modulation of 727% at β_2 -subunit-containing GABA_ARs and only 33% at those containing β_1 -subunits. The isopropyl amide 2-249 was found to resemble 2-262, with maximal modulation of β_1 - and β_2 -subunit-containing GABA_ARs in vitro of 249 and 905%, respectively. 2-301 was highly selective for β_2 -subunit-containing GABA_ARs, but the maximal efficacy was reduced compared with 2-261 (maximal modulation of $\beta_2 = 686\%$). 2-325 has almost absolute selectivity, with a maximal modulation of β_1 -subunit-containing GABA_ARs of 16%, while modulating β_2 -subunit-containing GABA_ARs to over the GABA_{max} current ($>1300\%$).

Fig. 7. Anxiolytic activity of 2-261 and 2-262 in rats. Dose-dependent effects of 2-261 (A) versus 2-262 (B) on time spent in the open arms in the rat EPM paradigm measured 30 min after intraperitoneal administration of each enantiomer. The effects are compared with vehicle control and diazepam (1.0 mg/kg i.p.). Each bar represents the mean $\pm$ S.E.M. of time spent in the open arms during a 5-min interval ($n = 8-35$ animals). Statistically significant differences from vehicle control at *, $P < 0.05$ and **, $P < 0.01$ after ANOVA and post-hoc Dunnett's test.

Correlation between Activity at β_1 -Subunit-Containing GABA_ARs and RR Deficit. Selected enaminones and nonenaminone reference compounds (Fig. 1) were tested in RR assays to determine AD₅₀ values. In parallel, PK assays were performed to determine brain concentrations corresponding to RR deficit to allow determination of whether a PK/PD correlation exists among peak brain concentrations, RR response, and activation of β_1 -subunit-containing GABA_ARs.

Fig. 8. Effect on Rotarod performance by 2-261 and 2-262 in mice. Time course of RR performance in mice after per os administration of various doses of 2-261 (A) versus 2-262 (B). The AD_{50} values for 2-261 and 2-262 at the time of peak effect were >120 and 43 mg/kg, respectively, as calculated by the method of Litchfield and Wilcoxon (1949) ($n = 8-10$ mice/time point).

An apparent threshold effect is observed when comparing activity at β_1 -containing GABA_ARs and RR failure (Fig. 10A). In contrast, there is no clear correlation between activity at β_2 -containing GABA_ARs and RR failure (Fig. 10B). Specifically, compounds with high efficacy at β_1 -containing GABA_ARs induce RR failures, whereas those with low activity at β_1 -containing GABA_ARs do not. This same general phenomenon was observed for the enantiomeric pair 2-261 and 2-262 described above.

Compound 2-325 has almost no efficacy ($\sim 16\%$) at the $\alpha_1\beta_1\gamma_2$ receptor subtype when tested at $10 \mu\text{M}$. It fails to induce RR deficit at 30 mg/kg i.p., despite a maximal efficacy of >1300% at the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype. The brain level of

2-325 at 30 mg/kg i.p. is $2.1 \mu\text{M}$, which is $\sim 9.5\times$ its EC_{50} value at $\alpha_1\beta_2\gamma_2$ GABA_ARs. The AD_{50} value for 2-301 is >100 mg/kg i.p. At this dose, the peak brain 2-301 level is $4.0 \mu\text{M}$, which is $\sim 18\times$ the EC_{50} value ($0.22 \mu\text{M}$) for stimulation of the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype; yet, no RR deficit is observed, despite brain levels that correspond to maximal stimulation (maximum, 686%) in the oocyte electrophysiological assays.

Mefenamic acid is a clinically used nonsteroidal anti-inflammatory drug that is structurally different from the enaminones. It has no appreciable activity at the $\alpha_1\beta_1\gamma_2$ receptor subtype and is also incapable of eliciting RR deficit, despite brain concentrations equal to the EC_{50} value for activity at the $\alpha_1\beta_2\gamma_2$ receptor subtype (Table 1). A similar lack of RR deficit is observed with other compounds (e.g., 2-313), despite brain levels in excess of levels required for maximal stimulation of β_2 -subunit-containing GABA_ARs.

The anxiolytic BZ receptor ligand ocinaplon has maximal stimulation of $\alpha_1\beta_1\gamma_2$ GABA_ARs subtypes of $\sim 81\%$. The AD_{50} value for ocinaplon is 54 mg/kg i.p., which corresponds to a brain level ($12.5 \mu\text{M}$) that is $\sim 4.5\times$ the EC_{50} value for stimulation of the $\alpha_1\beta_1\gamma_2$ GABA_AR subtype and induces 76% stimulation. This is consistent with the observation that when compounds reach brain levels associated with activity exceeding the 47% stimulation of the $\alpha_1\beta_1\gamma_2$ receptor subtype observed with 2-301, RR deficit will occur regardless of compound potency as reflected by their AD_{50} values. Based on these studies, a PK/PD correlation emerges where activity exceeding a threshold efficacy at the $\alpha_1\beta_1\gamma_2$ GABA_AR subtype is required for eliciting RR deficit.

A threshold level of activation is required to observe RR deficit, and it lies in between the efficacy for 2-301 and ocinaplon (Fig. 10A). In contrast, a threshold could not be identified when a similar bar graph analysis was performed based on efficacy at the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype (Fig. 10B) or at α_1 -, α_2 -, or α_3 -subunit (data not shown)-containing GABA_ARs. There also was no correlation with γ - or δ -subunit-containing receptors (data not shown). This β -subunit isoform selectivity does not appear to be predictive of anxiolytic activity as compounds with great variations in efficacy, such as 2-148 and 2-313, can still show robust anxiolytic activity (Fig. 11).

Efficacy Required for Achieving an AD_{50} in the RR Assay. The degree of stimulation of $\alpha_1\beta_1\gamma_2$ GABA_ARs necessary to achieve an RR AD_{50} can be deduced by measuring the peak brain levels of each of the compounds at the AD_{50} dose and extrapolating the degree of stimulation by this concentration of compound observed in the oocyte assays. Ten compounds of diverse chemotypes reveal that a range of 52 to 270% potentiation at $\alpha_1\beta_1\gamma_2$ GABA_ARs is necessary to observe an AD_{50} for RR deficit (Table 3). In contrast, the range is 58 to 1060% for $\alpha_1\beta_2\gamma_2$ GABA_ARs. A similar analysis using activity at α_1 -, α_2 -, or α_3 -subunit-containing GABA_AR subtypes does not result in a comparably narrow range of stimulation observed with the $\alpha_1\beta_1\gamma_2$ GABA_AR. Despite robust efficacy at the $\alpha_1\beta_2\gamma_2$ subtype, compounds such as 2-261 do not show potentiation of ethanol-induced RR failure commonly displayed by BZs (Fig. 12).

Discussion

We provide clear evidence in support of the hypothesis that a correlation exists between the efficacy of compounds at

Fig. 9. Pharmacokinetics of 2-261 and 2-262 in mice. The pharmacokinetic profile of 2-261 (A) and 2-262 (B) in mice where plasma and brain levels (micromolar) are shown at various time (minutes) points after 10 mg/kg p.o. Each data point represents the mean $\pm$ S.E.M. levels ($n = 4$ animals/time point).

TABLE 2

Effect of 2-261, 2-262, and other GABA_AR active enaminones on related LGICs

Compound	% Modulation of Selected LGICs				
	α_7 nAChR	$\alpha_4\beta_2$ nAChR	$\alpha_1\beta_1\delta\epsilon$ nAChR	5-HT _{3A}	N-Methyl-D-aspartate
2-128	272 $\pm$ 81	9.2 $\pm$ 8	0	0	0
2-148	0	0	0	-37 $\pm$ 8	0
2-261	166 $\pm$ 46	23 $\pm$ 8	0	0	33 $\pm$ 23
2-262	175 $\pm$ 116	26 $\pm$ 15	0	0	0
2-313	535 $\pm$ 55	17 $\pm$ 12	13 $\pm$ 7	0	16 $\pm$ 4
2-314	111 $\pm$ 52	0	0	0	21 $\pm$ 12
2-325	0	0	0	0	-19 $\pm$ 1

The effect is expressed as percentage of modulation $\pm$ S.E.M. ($n = 3-4$) of an EC₅₀ concentration of agonist at the respective LGICs in the presence of 10 μ M test compound.

β 1-subunit-containing GABA_ARs and ataxia. 2-261 and 2-262, a pair of enantiomers that share a common site of action, have differential ataxic liability that is related to the degree of $\alpha_1\beta_1\gamma_2$ GABA_AR subtype activation. Strong evidence for this relationship results from the use of a chemically diverse set of positive allosteric modulators as tools. This correlation becomes evident when PK/PD studies on these GABA_AR positive allosteric modulators revealed that a threshold of activity at β 1-subunit-containing GABA_ARs must be exceeded to observe performance deficit in the RR assay. We have unequivocally demonstrated that RR deficit can be reliably predicted based on activity at β 1-subunit-containing GABA_ARs. No such correlation could be shown for the other major subunits (i.e., β_2 , β_3 , α_1 , α_2 , or α_3). There also was no dependence upon γ - or δ -subunit-containing receptors. Moreover, limited ataxia due to reduced potency and/or efficacy are not confounds because many of our compounds meet or greatly exceed the potency/efficacy of the commonly used full agonist BZs. It is interesting to note that the existence of an association between β 1-subunit-containing GABA_ARs and sedation/ataxia can be deduced from the literature. Collectively, these reports are prescient in view of our current hypothesis.

Clinically Tested/Used Compounds Support the β 1-Subunit Hypothesis. The most compelling anecdotal evidence relating receptor subtype selectivity and sedation is based on clinically used compounds. For example, mefenamic acid, a nonsteroidal anti-inflammatory drug that has been used for several decades, is a selective modulator of β_2 -subunit-containing GABA_ARs that has virtually no activity at β 1-subunit-containing GABA_ARs (Halliwell et al., 1999). This drug has no sedative activity at clinically relevant (i.e., in-

flammation) serum levels (~ 83 μ M) that would supersaturate $\alpha_1\beta_2\gamma_2$ GABA_ARs (Cryer and Feldman, 1998). We have shown that the maximal stimulation of β_2 -subunit-containing GABA_ARs by mefenamic acid exceeds that of diazepam. Therefore, a lack of agonist BZ-like efficacy is not the explanation for lack of ataxia. Likewise, the anticonvulsant loreclezole has $\beta_{2/3}$ -subunit selectivity, diazepam-like efficacy, and anxiolytic/anticonvulsant activity in animal models with reduced sedative effects (Smith et al., 2004; Groves et al., 2006) and is a non-sedating antiepileptic in human clinical trials (Fisher and Blum, 1995). Etifoxine, with $\beta_{2/3}$ -subunit selectivity, also has diazepam-like efficacy at GABA_ARs (Smith et al., 2004) and is used clinically as a non-sedating anxiolytic (Nguyen et al., 2006). We firmly believe that these clinical observations along with our own data provide a cogent argument that designing compounds (see Supplemental Data for SAR on β -subunit isoform selectivity) with reduced activity at β 1-subunit-containing GABA_ARs will ultimately result in anxiolytic drugs.

Neuroanatomical Correlates of β -Subunit Selectivity Support the β 1-Subunit Hypothesis. The neuroanatomical localization of the β 1-subunit explains, in part, why limited efficacy at β 1-subunit-containing GABA_ARs may reduce sedative/ataxic liability. The β 1-subunits are reported to be associated with extrasynaptic GABA_ARs and are expressed in brainstem and arousal-related areas of the rat brain (Pirker et al., 2000; Sun et al., 2004; Harrison, 2007). Several regions in the brain responsible for either the induction or maintenance of sleep have β 1-subunit-containing GABA_AR subtypes. For example, an important area for modulating sleep and facilitating EEG synchronization is the reticular thalamic nucleus that has primarily β 1-subunit-

Fig. 10. Activity at $\alpha_1\beta_1\gamma_2$ GABA_ARs is predictive of ataxia. Rank order of apparent maximal percentage of modulation of EC₁₀ GABA-evoked currents by various test compounds in oocytes expressing the $\alpha_1\beta_1\gamma_2$ (A) or $\alpha_1\beta_2\gamma_2$ (B) GABA_AR subtypes. Maximal modulation was defined by that calculated from the concentration-response curves generated for each compound in the oocyte assays or that observed at ≥ 10 μ M when limited by compound solubility. All compounds with efficacy below the threshold (dashed line) at the $\alpha_1\beta_1\gamma_2$ -subtype receptor pass the RR test, whereas those with efficacy above the threshold fail the RR test as defined by a calculable AD₅₀. Filled columns, passing RR test; open columns, failing RR test. *, L-838,417 causes RR failure, with AD₅₀ = 30 mg/kg i.p. but has no activity at $\alpha_1\beta_1\gamma_2$ or $\alpha_1\beta_2\gamma_2$ GABA_AR subtypes.

Fig. 11. Anxiolytic activity of 2-313 and 2-148 in mice. Effects of 2-313 (A) and 2-148 (B) versus alprazolam (0.1 mg/kg) on time (seconds) spent dark in the mouse LD paradigm after intraperitoneal administration of each compound. The effects of the test compounds are compared with vehicle control. Each bar represents the mean $\pm$ S.E.M. time spent in the dark chamber during a 5-min interval (n = 6–30 animals). Significantly different from vehicle control at *, P < 0.05 after ANOVA and post-hoc Dunnett's test.

containing GABA_ARs and receives input from the basal forebrain, substantia nigra, and globus pallidus and generally enhances arousal/attention (Paré et al., 1990; Hazrati and Parent, 1991; Asanuma, 1994). The reticular thalamic nucleus projects to the thalamic relay nuclei to promote sleep, whereas the intralaminar thalamic nuclei receive innervation from the ascending reticular activating system and facilitate awareness (McCormick and Bal, 1997; Hartings et al., 2000). The positive modulation of β_1 -subunit-containing GABA_ARs in these nuclei normally serves to induce sleep such as with agonist BZs; thus, compounds devoid of activity at β_1 -subunit-containing GABA_ARs would be expected have reduced sedative potential (Pirker et al., 2000; Van der Werf

et al., 2002; Huntsman and Huguenard, 2006). The distribution of the β_1 -subunit in human brain has not been fully characterized. It is assumed that its distribution in rodent brain is representative of that in humans. Nevertheless, human clinical evidence on the sedative potential of β_1 -subunit-selective compounds (e.g., etifoxine, loreclezole, and mefenamic acid) is consistent with this brain structure-function relationship.

A PK/PD Correlation Provides Support of an Activity Threshold for Ataxia. The strongest argument that ataxic liability depends on β_1 -subunit-containing GABA_ARs is provided by the correlative evidence derived from the PK/PD relationship of our test compounds. The ability to identify a threshold of activity at $\alpha_1\beta_1\gamma_2$ GABA_ARs, which

TABLE 3

Degree of $\alpha_1\beta_1\gamma_2$ or $\alpha_1\beta_2\gamma_2$ GABA_AR subtype stimulation by the peak brain levels attained at the AD₅₀ dose of various test compounds

Brain levels and AD₅₀ values of compound are from Table 1. The degree of activation associated with these levels of compound is derived from the concentration-response curves for each compound and defined as a percentage of stimulation of EC₁₀ GABA in oocytes expressing the $\alpha_1\beta_1\gamma_2$ or $\alpha_1\beta_2\gamma_2$ GABA_AR subtypes. All test compounds were administered by intraperitoneal injection except for 2-262 which was administered orally.

Compound	AD ₅₀	Brain Levels at AD ₅₀	Stimulation at $\alpha_1\beta_1\gamma_2$	Stimulation at $\alpha_1\beta_2\gamma_2$
	mg/kg	μ M		%
Ocinaplon	54	12.5	76	58
Bretazenil	25	11.7	65	69
Diazepam	2	0.64	90	130
2-128 ^a	>120	5.8	76	518
Loreclezole	83	11.7	99	382
2-262	43	0.8	71	1060
2-249	3	0.6	52	857
2-148	5	1.1	71	739
Tracazolate	42	7.6	270	803
2-314	2.5	0.3	230	583

^a 2-128 has poor solubility and bioavailability so that an accurate AD₅₀ could not be determined; therefore, the brain levels of the compound were determined at the highest dose (120 mg/kg) tested where 40% of the animals failed the RR test.

Fig. 12. Lack of ethanol potentiation of RR failure by 2-261. The effect of 2-261 (30 mg/kg p.o.) versus diazepam (1 mg/kg i.p.) on EtOH (1 g/kg i.p.)-induced RR deficit in mice. The effect is expressed as a percentage of mice passing the RR assay, with 12 mice tested under each condition. Significantly different from diazepam vehicle + EtOH at *, $P < 0.01$ by the Fischer-Yates exact probability test.

must be exceeded to observe RR deficit (Fig. 10A), that is not observed with the other subunits evaluated strongly supports our hypothesis. Furthermore, this β -subunit isoform selectivity does not appear to be predictive of anxiolytic activity as compounds with extreme differences in efficacy, such as 2-148 and 2-313, still show robust anxiolytic activity.

Based on the compounds evaluated, the empirically derived threshold for ataxia lies in between 47% (2-301) and 81% (ocinaplon) stimulation of EC₁₀ GABA at the $\alpha_1\beta_1\gamma_2$ GABA_AR subtype. Yet, upon cursory analysis, arguments can be made that inconsistencies with our β_1 -subunit hypothesis occur. For example, etomidate, which is $\beta_{2/3}$ -subunit-selective,

can cause sedation and anesthesia (Hill-Venning et al., 1997). However, this anomaly can be readily explained by a scenario where increasing doses of etomidate will result in brain levels of drug that are associated with activity that exceed the threshold at β_1 -subunit-containing GABA_ARs necessary for sedation. Etomidate also has direct channel-activating properties that can account for its anesthetic action. In contrast, 2-325 neither causes ataxia nor anesthesia; yet, it has a similar maximal efficacy at the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype as etomidate. The most parsimonious explanation for this difference is that 2-325, unlike etomidate, has virtually no activity at $\alpha_1\beta_1\gamma_2$ GABA_ARs or direct channel effects.

A Range of Efficacy at β_1 -Subunit-Containing GABA_ARs Can Be Defined for RR Activity. A well-defined range (52–270%) of activity at β_1 -subunit-containing GABA_ARs appears necessary to achieve an RR AD₅₀. It is noteworthy that among the compounds tested, none with measurable AD₅₀ values are associated with brain levels that are below the threshold level required for RR deficit. This is extraordinary when there is the possibility of confounding variables such as off-target effects or active metabolites that can cause RR deficit.

The $\sim 5\times$ difference (52–270%) in the maximal and minimal efficacy measured may be a result of two compounds. First, the steep concentration-response curve of 2-314 is a potential confound. A single log unit change in the concentration (10^{-7} – 10^{-6} M) results in a just-detectable (<15%) response to maximal modulation of $\sim 635\%$ (i.e., Hill slope >2) at $\alpha_1\beta_1\gamma_2$ GABA_ARs, resulting in the magnification of the experimental error (Supplemental Fig. 1). Second, tracazolate levels at its AD₅₀ value are associated with 270% enhancement of $\alpha_1\beta_1\gamma_2$ GABA_ARs, which may result from its opposing action mediated by adenosine A₁ and A₂ receptors (Daly et al., 1988). Tracazolate is an antagonist of adenosine A₁ and A₂ receptors, with micromolar potency where it is predicted to promote arousal and wakefulness (Van Dort et al., 2009). Therefore, greater stimulation of $\alpha_1\beta_1\gamma_2$ GABA_ARs by tracazolate would be required for physiological antagonism of its actions at these adenosine receptor subtypes. It is interesting to note that when tracazolate and 2-314 are excluded from determining the range of activity necessary to achieve an AD₅₀, it results in a much narrower $\sim 1.9\times$ range (52–99%) of modulation. In contrast, the activity at the $\alpha_1\beta_2\gamma_2$ GABA_AR subtype is much broader, even upon exclusion of tracazolate and 2-314, with an $\sim 18\times$ range (58–1060%) of efficacy associated with RR deficit and is thus unlikely to be the key receptor subtype in mediating ataxia. Regardless, the existence of a narrow range of activity that is chemotype-independent and discernible, despite the influence of multiple confounds lends strong support for the broad applicability of our hypothesis.

The β_1 -Subunit Hypothesis also Explains the Ataxic Actions of BZ Site Agonists. The ataxic actions of the BZ site agonists can be explained by our hypothesis. Both diazepam and bretazenil have efficacies that exceed the threshold activity required at $\alpha_1\beta_1\gamma_2$ GABA_ARs for sedation, whereas L-838,417 does not. The observation of an AD₅₀ value (30 mg/kg i.p.) for L-838,417 at a brain level of 8 μ M is probably the result of an off-target(s)-mediated effect because it has <1 nM affinity for its site of action (McKernan et al., 2000). In contrast, the anxiolytic profile of our nonBZ site positive allosteric modulators cannot be explained by the $\alpha_{2/3}$ -subunit isoform selectivity hypothesis alone. For ex-

ample, 2-325, 2-313, and 2-301 have maximal efficacies mediated by the $\alpha_1\beta_2\gamma_2$ subtype that are 4- to 8-fold greater than that of diazepam. Yet, they do not cause RR deficit at brain levels expected to saturate the $\alpha_1\beta_2\gamma_2$ GABA_AR, a subtype that according to the $\alpha_{2/3}$ -subunit selectivity hypothesis is thought to mediate the sedative/ataxic effects of the agonist BZs. The two hypotheses are not mutually exclusive but both may be necessary to provide a full explanation for the anxioreactivity of certain GABA_AR positive allosteric modulators.

Summary. The reduction of activity at β_1 -subunit-containing GABA_ARs reduces ataxia among the chemically diverse compounds that we have tested. It is highly unlikely that this correlation between activity at β_1 -subunit-containing GABA_AR subtypes and ataxia as measured by RR deficit is purely coincidental. The doses of the agonist BZs that induce RR deficit and a reduction in spontaneous locomotor activity are overlapping (Bourin et al., 1992; Crabbe et al., 1998). Because the latter measure is believed to be a reflection of sedative activity and the former measure indicates motor ataxia, it renders the ability to discriminate the two adverse effects by dose-separation difficult, if not impossible. If the β_1 -subunit-selective enaminones in the present study are like the agonist BZs in this regard, it is conceivable that the lack of Rotarod deficit may reflect their inability to induce both effects. Nevertheless further testing for the robustness of this general hypothesis is still necessary. For example, phenotype characterization of β_1 -subunit “knockout” or “knockdown” mice would be corroborative where full agonist BZ receptor ligands such as diazepam would be predicted to have reduced sedative/ataxic activity. The characterization of a pair of enantiomers and related enaminones has enabled us to design GABA_AR positive allosteric modulators with reduced activity at β_1 -subunit-containing GABA_ARs. These compounds have predictably reduced ataxic effects but retain robust anxiolytic activity. Our approach of enhancing activity at β_2/β_3 -subunit and reducing/eliminating activity at β_1 -subunit-containing GABA_ARs may be an effective strategy to create anxioreactive positive allosteric modulators if its validity is confirmed by clinical testing.

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