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Apremilast regulates acute effects of ethanol and other GABAergic drugs via protein kinase A-dependent signaling

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Abstract

Phosphodiesterase type 4 (PDE4) inhibitors prevent hydrolysis of cyclic adenosine monophosphate and increase protein kinase A (PKA)-mediated phosphorylation. PDE4 inhibitors also regulate responses to ethanol and GABAergic drugs. We investigated mechanisms by which the PDE4 inhibitor, apremilast, regulates acute effects of ethanol and GABAergic drugs in male and female mice. Apremilast prolonged the sedative-hypnotic effects of gaboxadol, zolpidem, and propofol but did not alter etomidate effects, and unexpectedly shortened the sedative-hypnotic effects of diazepam. Apremilast prolonged rotarod ataxia induced by zolpidem, propofol, and loreclezole, shortened recovery from diazepam, but had no effect on ataxia induced by gaboxadol or etomidate. The PKA inhibitor H89 blocked apremilast's ability to prolong the sedative-hypnotic effects of ethanol, gaboxadol, and propofol and to prolong ethanol- and propofol-induced ataxia. H89 also blocked apremilast's ability to shorten the sedative-hypnotic and ataxic effects of diazepam. The β 1-specific antagonist, salicylidene salicylhydrazide (SCS), produced faster recovery from ethanol- and diazepam-induced ataxia, but did not alter propofol- or etomidate-induced ataxia. SCS shortened the sedative-hypnotic effects of ethanol and diazepam but not of propofol. In *Xenopus* oocytes, a phosphomimetic (aspartate) mutation at the PKA phosphorylation site in β 1 subunits decreased the maximal GABA current in receptors containing α 1 or α 3, but not α 2 subunits. In contrast, phosphomimetic mutations at PKA sites in β 3 subunits increased the

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maximal GABA current in receptors containing $\alpha 1$ or $\alpha 2$, but not $\alpha 3$ subunits. The GABA potency and allosteric modulation by ethanol, propofol, etomidate, zolpidem, flunitrazepam, or diazepam were not altered by these mutations. We propose a model whereby apremilast increases PKA-mediated phosphorylation of $\beta 1$ - and $\beta 3$ -containing GABA_A receptors and selectively alters acute tolerance to ethanol and GABAergic drugs.

Keywords

PDE4 inhibitor apremilast; $\beta 1$ and $\beta 3$ GABA_A receptor subunits; protein kinase A; acute tolerance to alcohol and GABAergic drugs; loss of righting reflex; rotarod ataxia

1. INTRODUCTION

Phosphodiesterases (PDEs) catalyze the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate and play a key role in regulating intracellular levels of these cyclic nucleotides. Of the 11 different families of PDEs, PDE4 is the most important for controlling cAMP levels, is expressed in the brain, and is involved in alcohol and drug dependence as well as in the regulation of inflammatory and neuroimmune responses (Wen et al., 2018). Chronic alcohol intake (liquid diet model) increases neuroimmune signaling, including activation of astrocytes and microglia, and these effects are attenuated by a PDE4 inhibitor (rolipram) or genetic deletion of *Pde4b* (Avila et al., 2017). In view of the role of neuroimmune activation in regulating alcohol consumption and other behavioral effects (Erickson et al., 2019), the anti-inflammatory actions of PDE4 inhibitors may be an important part of their mechanism of action in these responses.

PDE4 inhibitors also decrease ethanol seeking and consumption in rodents (Blednov et al., 2014; Franklin et al., 2015; Hu et al., 2011; Liu et al., 2017; Wen et al., 2012). In a large genetic association study in humans, *PDE4B* was identified as a locus associated with all tobacco and alcohol use phenotypes examined (Liu et al., 2019). We recently reported that apremilast, a selective FDA-approved PDE4 inhibitor, produced stable decreases in ethanol intake in male and female mice in different drinking tests (Blednov et al., 2018b) and altered other behaviors that are correlated with ethanol consumption (Blednov et al., 2018a). For example, apremilast prolonged the acute sedative-hypnotic and ataxic effects of ethanol and decreased acute functional tolerance to ethanol. Acute functional tolerance is behavioral tolerance that occurs within an individual test session, and is distinguished from rapid tolerance which develops over 8–72 h after ethanol or drug exposure (Pietrzykowski and Treistman, 2008).

Understanding how PDE4 inhibitors decrease ethanol consumption could be beneficial for drug development to treat alcohol use disorder. We are particularly interested in apremilast because of its low side effect profile and clinical success. Apremilast, like other PDE4 inhibitors, reduces hydrolysis of cAMP leading to increased activation of protein kinase A (PKA). Current evidence indicates that PKA regulates γ -aminobutyric acid type A (GABA_A) receptor function. For example, intracerebroventricular administration of the PKA activator Sp-cAMP increases the sedative-hypnotic effects of ethanol and the GABA_A receptor agonist muscimol (Kumar et al., 2012). PKA is able to phosphorylate the large

intracellular loops of $\beta 1$ and $\beta 3$ subunits (McDonald et al., 1998). Phosphorylation of $\beta 3$ -containing receptors at S408 and S409 enhances GABA-stimulated responses, but phosphorylation of S409 alone inhibits responses, similar to effects found with $\beta 1$ subunits, which are phosphorylated solely on S409. Similar to neuronal receptors, GABA_A receptors expressed in HEK293 cells and phosphorylated by PKA on $\beta 1$ and $\beta 3$ subunits show opposing effects on GABA-stimulated currents (i.e., phosphorylation increases $\alpha 1\beta 3\gamma 2$ responses and decreases $\alpha 1\beta 1\gamma 2$ responses) (McDonald et al., 1998).

In this study, we examined mechanisms by which apremilast regulates behavioral responses to ethanol and different GABAergic drugs in mice. We found that apremilast altered recovery from the ataxic and sedative-hypnotic effects of ethanol and GABAergic drugs in a PKA-dependent manner. We also used two-electrode voltage clamp of $\alpha\beta\gamma$ GABA_A receptors expressed in *Xenopus laevis* oocytes to show that the phosphorylation states of $\beta 1$ and $\beta 3$ differentially alter receptor function depending on the type of co-expressed α subunit. Our findings suggest that apremilast-induced increases in PKA-dependent phosphorylation of $\beta 1$ - and $\beta 3$ -containing GABA_A receptors in the brain alter acute tolerance to ethanol and GABAergic drugs.

2. MATERIALS AND METHODS

2.1 Mice

Male and female C57BL/6J mice were from a colony maintained in the Animal Resources Center at The University of Texas at Austin. Original breeders were purchased and replenished every 6 months from The Jackson Laboratory (Bar Harbor, ME). Mice were group-housed by sex (4 or 5 per cage) in temperature- and humidity-controlled rooms with free access to food and water using a 12-h light/dark cycle (lights on at 7:00 a.m.). Experiments began when the mice were 2–3 months old. Mice were allowed to adapt to the testing rooms for about one week before behavioral testing. Experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and comply with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Drug Administration

Ethanol (100% stock, Aaper Alcohol and Chemical, Shelbyville, KY) solutions were prepared in 0.9% saline (20%, v/v) and injected i.p. Apremilast (Toronto Research Chemicals Inc., North York, ON, Canada) was freshly prepared as a suspension in saline with 3–4 drops of Tween-80, and 20 mg/kg p.o. was administered once daily in a volume 0.05 ml/10 g of body weight 1 h before experiments. This timeframe was chosen based on our previous working showing peak levels of apremilast in plasma, liver, and brain 1 h after administration (Blednov et al., 2018b). Gaboxadol (10 and 55 mg/kg), diazepam (6 and 50 mg/kg), and propofol (30 and 120 mg/kg) were purchased from Sigma-Aldrich (St. Louis, MO) and administered by i.p. injection (0.1 ml/10 g body weight). Gaboxadol was dissolved in saline, and diazepam and propofol were suspended in saline with 3–4 drops of Tween-80. The propofol suspension was also sonicated for 10 min. Zolpidem (5 and 60 mg/kg), loreclezole (60 mg/kg), salicylidene salicylhydrazide (SCS) (40 mg/kg), and H89 (10

mg/kg) were purchased from Tocris Bioscience (Minneapolis, MN), and etomidate (10, 15, 25, and 50 mg/kg) was purchased from Toronto Research Chemicals Inc. These drugs were freshly prepared in 0.9% saline with 3–4 drops of Tween-80 and injected at 0.1 ml/10 g of body weight for i.p. administration or at 0.05 ml/10 g of body weight for s.c. administration of H89. SCS and H89 were injected 15 min before drug treatment based on previous findings (Kumar et al., 2012) and our preliminary experiments.

2.3 Loss of the Righting Reflex

Responses to sedative-hypnotic doses of ethanol and other drugs were measured as the duration of the loss of righting reflex (LORR). When mice became ataxic, they were placed in the supine position in V-shaped plastic troughs until they were able to right themselves three times within 30 s. The duration of the LORR was defined as the time elapsed between being placed in the supine position until recovering the righting reflex. Saline or apremilast (20 mg/kg, p.o.) was injected once 1 h before i.p. injection of gaboxadol (55 mg/kg), zolpidem (60 mg/kg), propofol (120 mg/kg), etomidate (25 and 50 mg/kg), or diazepam (50 mg/kg). To study the role of PKA on apremilast responses, mice were treated with apremilast (20 mg/kg, p.o.) 1 h before testing and then treated with H89 (10 mg/kg, s.c.) 15 min before i.p. injection of ethanol (3.6 g/kg), gaboxadol (55 mg/kg), propofol (120 mg/kg), or diazepam (50 mg/kg). To study the role of β 1 subunits, mice were pretreated with SCS (40 mg/kg, i.p.) 15 min before i.p. injection of ethanol (3.6 g/kg), propofol (120 mg/kg), or diazepam (50 mg/kg).

2.4 Rotarod Ataxia

Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments, Columbus, OH) at 10 rpm, and training was considered complete when mice were able to remain on the rotarod for 60 s. Every 15 min after drug injection, each mouse was placed on the rotarod and latency to fall was measured until the mouse was able to remain on the rotarod for 60 s. Saline or apremilast (20 mg/kg, p.o.) was injected once 1 h before i.p. injection of gaboxadol (10 mg/kg), diazepam (6 mg/kg), zolpidem (5 mg/kg), propofol (30 mg/kg), loreclezole (60 mg/kg), or etomidate (10 mg/kg). To study effects of PKA inhibition, mice were treated with apremilast (20 mg/kg, p.o.) or saline (p.o.) 1 h before testing and then treated with saline or the PKA inhibitor H89 (10 mg/kg, s.c.) 15 min before injection of ethanol (2 g/kg), diazepam (6 mg/kg), or propofol (30 mg/kg). To investigate the role of β 1-containing GABA_A receptors, saline or the β 1-specific antagonist SCS (40 mg/kg, i.p.) was injected 15 min before i.p. injection of ethanol (2 g/kg), diazepam (6 mg/kg), propofol (30 mg/kg), or etomidate (15 mg/kg).

2.5 Electrophysiology

Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI). Experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin and comply with the ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The complementary DNAs encoding the rat GABA_A subunits α 1, β 1, β 3, and γ 2 were provided by Dr. M. H. Akabas (Albert Einstein College of Medicine); human α 2 (provided by Dr. Neil Harrison, Columbia University) was subcloned into pGEMHE, and rat α 3 was optimized and synthesized by GenScript

(Piscataway, NJ). Mutations in the β cDNAs were made through site-directed mutagenesis using QuikChange (Agilent Technologies, Santa Clara, CA). The *in vitro* transcription of GABA_A subunits was performed using mMessage mMachine (Life Technologies, Grand Island, NY). Manually isolated *Xenopus laevis* oocytes were injected with 50 nl capped complementary RNAs encoding wild-type or mutant subunits in different ratios, depending on the subunits: $\alpha\beta3\gamma2$, 0.5:0.3:0.5 ng/oocyte and $\alpha\beta1\gamma2$, 0.5:0.5:0.5 ng/oocyte. The injected oocytes were incubated at 15°C in sterilized Modified Barth's solution for 1–4 days before recording.

The responses of GABA_A receptors expressed in oocytes were studied using two-electrode voltage clamp. Oocytes were discarded if the maximal current was over 30 μ A or if the baseline was unstable or drifted to positive values. Final drug dilutions were freshly prepared each day. GABA concentration-response curves were determined using increasing concentrations of GABA (0.1–3000 μ M) applied for 20–30 s followed by a 5–15 min washout. The oocyte's response to each concentration was expressed as the percentage of the maximal current produced by that oocyte. To verify the presence of the $\gamma2$ subunit in the expressed receptors, responses to GABA were evaluated in the presence of Zn⁺⁺ (10 μ M). Flunitrazepam, zolpidem, etomidate, and propofol stocks were prepared in DMSO. The maximal GABA concentration was applied for 20 s, and after a 15-min washout, the GABA concentration that produced 5% of the maximal response was applied. If the resulting current was not between 3 and 7% of the maximal response, the GABA concentration was adjusted accordingly until the response was within those parameters. This was defined as the nominal EC₅ GABA. After two consecutive applications of EC₅ GABA, the modulators were coapplied with EC₅ GABA in between EC₅ GABA alone applications. Ethanol or zinc were reapplied alone for 60 s immediately before their co-application with EC₅ GABA.

2.6 Statistical Analysis

Statistical analyses were performed using Prism 8 (GraphPad Software, Inc., La Jolla, CA) software. Data are reported as mean \pm S.E.M values (number of mice and oocytes used are reported in the figure legends). For behavioral tests, sex as a factor was not significant so we combined the data from male and female mice (with the exception of data in Figures 3D and 6 which were collected only in male mice). Data were analyzed by one- or two-way ANOVA and Tukey's *post hoc* tests. For electrophysiology, GABA concentration-response curves were determined using non-linear fitting of a Hill equation with variable slope. Current values elicited by a maximal GABA concentration were analyzed over three consecutive days using two-way ANOVA (multiple comparisons with Sidak's correction). Drug responses in the presence of EC₅ GABA were quantified as the percent change in current from the average of the EC₅ GABA alone responses obtained immediately before and after the drug. One-way ANOVA testing was used to detect significant differences in drug modulation between mutant receptors.

3. RESULTS

3.1 Apremilast prolongs duration of the LORR induced by gaboxadol, zolpidem, and propofol

We previously showed that apremilast (20 mg/kg, p.o.) prolonged the sedative-hypnotic effects of ethanol in male and female C57BL/6J mice (Blednov et al., 2018a). Here we measured the duration of the LORR following injection of different GABAergic sedative-hypnotic drugs in male and female C57BL/6J mice after pretreatment with saline or apremilast (20 mg/kg, p.o.). Apremilast significantly prolonged the duration of LORR induced by 55 mg/kg of gaboxadol [$t(13) = 29.80$, $p < 0.0001$], 60 mg/kg of zolpidem [$t(15) = 6.39$, $p < 0.0001$], and 120 mg/kg of propofol [$t(13) = 8.48$, $p < 0.0001$] (Figure 1A–C) but did not alter LORR induced by etomidate (Figure 1D). However, apremilast significantly shortened the duration of LORR induced by 50 mg/kg of diazepam [$t(16) = 9.36$, $p < 0.0001$] (Figure 1E).

3.2 Apremilast prolongs ataxia induced by zolpidem, loreclezole, and propofol

We previously showed that apremilast (20 mg/kg, p.o.) prolongs recovery from the acute ataxic effect of ethanol (Blednov et al., 2018a). Here, we investigated whether this effect occurs with other GABAergic drugs using lower doses that are selective for certain GABA_A receptor subtypes. Apremilast (20 mg/kg, p.o.) did not alter recovery from rotarod ataxia induced by gaboxadol (10 mg/kg, i.p.), which is a selective agonist for receptors containing $\alpha 4$ and δ subunits (Figure 2A). However, it significantly prolonged recovery from the motor impairing effects of zolpidem (5 mg/kg) ($F_{1,30} = 13.7$, $p < 0.001$, effect of pretreatment; $F_{7,210} = 144$, $p < 0.0001$, effect of time; $F_{7,210} = 9.5$, $p < 0.0001$, pretreatment x time interaction), which is a positive allosteric modulator of receptors containing $\alpha 1$ and $\gamma 2$ subunits (Figure 2B). Apremilast also prolonged recovery from loreclezole (60 mg/kg) ($F_{1,20} = 101$, $p < 0.0001$, effect of pretreatment; $F_{9,180} = 68.6$, $p < 0.0001$, effect of time; $F_{9,180} = 23.8$, $p < 0.0001$, pretreatment x time interaction) (Figure 2D), which is a positive allosteric modulator of receptors that contain $\beta 2$ or $\beta 3$ subunits. Additionally, apremilast prolonged recovery from propofol (30 mg/kg) ($F_{1,29} = 11.9$, $p < 0.01$, effect of pretreatment; $F_{6,174} = 91.5$, $p < 0.0001$, effect of time; $F_{6,174} = 3.9$, $p < 0.01$, pretreatment x time interaction) (Figure 2E), which is not selective for β subunits (Rudolph and Antkowiak, 2004). In contrast, pretreatment with apremilast produced faster recovery from the motor impairing effects of diazepam (6 mg/kg, Figure 2C) ($F_{1,28} = 19.6$, $p < 0.001$, effect of pretreatment; $F_{8,224} = 136$, $p < 0.0001$, effect of time; $F_{8,224} = 7.8$, $p < 0.0001$, pretreatment x time interaction), which is a positive allosteric modulator of receptors containing $\gamma 2$ with $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits. Apremilast did not alter recovery from ataxia induced by etomidate (10 mg/kg) (Figure 2F). At low doses, etomidate is a positive allosteric modulator of receptors that contain $\beta 2$ or $\beta 3$ subunits (Sieghart and Savic, 2018).

3.3 A PKA inhibitor prevents apremilast modulation of LORR induced by ethanol, gaboxadol, or propofol

Since apremilast is a PDE4 inhibitor and increases activation of PKA, we next used the kinase inhibitor H89 (Hidaka et al., 1984) to evaluate the role of PKA on apremilast-induced increases in LORR duration in male and female C57BL/6J mice. Pretreatment with H89 (10

mg/kg, s.c.) did not alter the duration of the LORR induced by ethanol (3.6 g/kg, i.p.), gaboxadol (55 mg/kg, i.p.), or propofol (120 mg/kg, i.p.), but it completely blocked the ability of apremilast (20 mg/kg, p.o.) to prolong the sedative-hypnotic effect of these drugs [effect of pretreatment on LORR induced by ethanol ($F_{1,34} = 118.0$, $p < 0.0001$), gaboxadol ($F_{1,34} = 479.5$, $p < 0.0001$), and propofol ($F_{1,34} = 140.1$, $p < 0.0001$)] (Figure 3A–C). Pretreatment with H89 also did not alter duration of the LORR induced by diazepam (50 mg/kg), but it blocked the ability of apremilast to shorten diazepam-induced LORR ($F_{1,20} = 161.7$, $p < 0.0001$) (Figure 3D).

3.4 Blockade of PKA prevents apremilast modulation of rotarod ataxia induced by ethanol, diazepam, or propofol

We next examined potential PKA-dependent effects of apremilast on ataxia induced by ethanol and GABAergic drugs. In male and female C57BL/6J mice, H89 (10 mg/kg, s.c.) reversed the ability of apremilast (20 mg/kg, p.o.) to prolong recovery from the ataxic effects of 2 g/kg of ethanol ($F_{1,22} = 32.1$, $p < 0.0001$, effect of pretreatment; $F_{9,198} = 181$, $p < 0.0001$, effect of time; $F_{9,198} = 10.7$, $p < 0.0001$, pretreatment x time interaction) (Figure 4A) and 30 mg/kg of propofol ($F_{1,21} = 38.1$, $p < 0.0001$, effect of pretreatment; $F_{6,126} = 152$, $p < 0.0001$, effect of time; $F_{6,126} = 15.7$, $p < 0.0001$, pretreatment x time interaction) (Figure 4B). H89 also reversed the ability of apremilast to speed recovery from ataxia induced by 6 mg/kg of diazepam ($F_{1,21} = 97.9$, $p < 0.0001$, effect of pretreatment; $F_{8,168} = 447$, $p < 0.0001$, effect of time; $F_{8,168} = 35.9$, $p < 0.0001$, pretreatment x time interaction) (Figure 4C).

3.5 A GABA_A receptor $\beta 1$ subunit antagonist accelerates recovery from ataxia induced by ethanol or diazepam

Because PKA can regulate GABA_A receptor function through phosphorylation of $\beta 1$ and $\beta 3$ subunits (McDonald et al., 1998), we first investigated the importance of $\beta 1$ -containing receptors using SCS, a $\beta 1$ -specific antagonist (Thompson et al., 2004). Pretreatment of C57BL/6J male mice with SCS (40 mg/kg, i.p.) induced faster recovery from the motor impairing effects of ethanol ($F_{1,10} = 25.6$, $p < 0.001$, effect of treatment; $F_{7,70} = 191$, $p < 0.0001$, effect of time; $F_{7,70} = 8.5$, $p < 0.0001$, treatment x time interaction) (Figure 5A) and diazepam ($F_{1,10} = 22.6$, $p < 0.001$, effect of treatment; $F_{8,80} = 194$, $p < 0.0001$, effect of time; $F_{8,80} = 10.2$, $p < 0.0001$, treatment x time interaction) (Figure 5B). As predicted, SCS did not change recovery from ataxia induced by propofol ($F_{5,45} = 337$, $p < 0.0001$, effect of time) or etomidate ($F_{7,70} = 41$, $p < 0.0001$, effect of time) (Figure 5C and D).

3.6 A GABA_A receptor $\beta 1$ subunit antagonist shortens the duration of LORR induced by ethanol or diazepam, but not by propofol

We next examined the effect of SCS (40 mg/kg, i.p.) pretreatment on the sedative-hypnotic effects of ethanol, diazepam, and propofol in male C57BL/6J mice. SCS significantly shortened the duration of LORR induced by 3.6 g/kg of ethanol [$t(10) = 12.9$, $p < 0.0001$] or 50 mg/kg of diazepam [$t(10) = 12.3$, $p < 0.0001$], but did not alter the sedative-hypnotic effect of 120 mg/kg of propofol (Figure 6A–C). A summary of results from our behavioral tests are shown in Table 1.

3.7 Mutation of phosphorylation sites on $\beta 1$ or $\beta 3$ subunits alters the maximal response to GABA in heterologously expressed GABA_A receptors

The direct application of apremilast (1–10 μ M) to $\alpha 1\beta 2\gamma 2$ or $\alpha 1\beta 3\gamma 2$ GABA_A receptors heterologously expressed in *Xenopus* oocytes did not alter receptor function (Figure S1).

In order to study a homogeneous population of receptors possessing a known phosphorylation state, we expressed mutated $\beta 1$ and $\beta 3$ subunits in combination with $\alpha 1$, $\alpha 2$, or $\alpha 3$, along with $\gamma 2$ subunits. In these β subunits, the relevant serines were replaced by phosphomimetic or non-phosphorylatable residues. The GABA sensitivity of each subunit combination and the GABA-induced maximal current were determined over three consecutive days to control for changes in levels of receptor expression. The analysis of GABA-mediated maximal current yielded the same significant differences, whether the data were analyzed dayby-day by two-way ANOVA, or pooled and analyzed by one-way ANOVA.

The relevant serine residue in the intracellular loop of the $\beta 1$ subunits (S409) was replaced by either an aspartate (phosphomimetic) or alanine (non-phosphorylatable) residue. When assessing the maximal GABA concentration-induced currents mediated by $\alpha 1\beta 1\gamma 2$ combinations, the receptors containing the non-phosphorylatable subunit [$\beta 1(A)$] showed larger currents than the phosphomimetic subunit [$\beta 1(D)$] ($F_{2,102} = 21.93$, $p < 0.0001$, effect of days after injection; $F_{2,102} = 17.54$, $p < 0.0001$, effect of phosphorylation state; $F_{4,102} = 3.35$, $p < 0.05$, days after injection \times phosphorylation state interaction) (Figure 7A). The same result was observed for $\alpha 3\beta 1\gamma 2$ combinations, except that the difference was not yet significant on day 2 after injection ($F_{2,107} = 40.24$, $p < 0.0001$, effect of days after injection; $F_{2,107} = 28.38$, $p < 0.0001$, effect of phosphorylation state; $F_{4,107} = 3.53$, $p < 0.001$, days after injection \times phosphorylation state interaction) (Figure 7E). Currents mediated by receptors containing the wild-type subunit [$\beta 1(S)$] were not consistent compared with the phosphomimetic and non-phosphorylatable subunits, likely reflecting a variable endogenous phosphorylation state. When $\beta 1$ was expressed with $\alpha 2$ and $\gamma 2$ subunits, there were no differences in the maximal currents in $\beta 1$ -containing receptors with differing phosphorylation states (Figure 7C). The sensitivity to GABA was not affected by the phosphorylation state of the $\beta 1$ 409 residue in any subunit combination (Figure 7B, D and F, and Table 2).

For $\alpha 1\beta 3\gamma 2$ combinations, both relevant serines in the intracellular loop of $\beta 3$ (408 and 409) were replaced by either aspartates or alanines. The phosphomimetic [$\beta 3(DD)$] subunit showed a larger maximal GABA-induced current than the non-phosphorylatable [$\beta 3(AA)$] and wild-type [$\beta 3(SS)$] subunits, except on day 1, when the differences were not yet significant ($F_{2,153} = 71.0$, $p < 0.0001$, effect of days after injection; $F_{2,153} = 13.9$, $p < 0.0001$, effect of phosphorylation state; $F_{4,153} = 1.34$, $p > 0.05$, days after injection \times phosphorylation state interaction) (Figure 8A). The $\alpha 2\beta 3\gamma 2$ receptors showed a similar trend on days 1 and 2, but the only significant difference was on day 2 between the phosphomimetic [$\beta 3(DD)$] and non-phosphorylatable [$\beta 3(AA)$] subunits ($F_{2,138} = 30.35$, $p < 0.0001$, effect of days after injection; $F_{2,138} = 6.07$, $p < 0.01$, effect of phosphorylation state; $F_{4,138} = 0.42$, $p > 0.05$, days after injection \times phosphorylation state interaction) (Figure 8C). We observed no differences in the maximal currents in $\alpha 3\beta 3\gamma 2$ combinations

on any day (Figure 8E). In $\beta 3$ -containing receptors, the sensitivity to GABA was not affected by the phosphorylation state of the $\beta 3$ 408 and 409 residues in $\alpha 1$ and $\alpha 3$ combinations (Figure 8B and F, and Table 2). Small differences in the GABA EC_{50} values were observed in $\alpha 2\beta 3\gamma 2$ receptors (Figure 8D and Table 2).

In order to determine if the phosphorylation state of β subunits affects GABA_A receptor function by different allosteric modulators, we co-applied a submaximal GABA concentration (EC_5) with ethanol (200 mM), propofol (2 μ M), etomidate (1 μ M) (Figure 9), zolpidem (0.1 μ M), flunitrazepam (0.1 μ M), or diazepam (0.1 and 3 μ M) to oocytes expressing $\alpha 1\beta 3\gamma 2$ receptors (Figure 10). We also tested diazepam modulation of $\alpha 1\beta 1\gamma 2$ receptors (Figure 10). We corroborated expression of $\gamma 2$ along with α and β subunits by measuring the effect of an endogenous modulator, zinc (10 μ M), which inhibits $\alpha\beta$ and $\alpha\beta\gamma$ receptors with different potencies (Figure 9). None of the allosteric modulators showed a differential effect that depended on the phosphorylation state of β subunits.

4. DISCUSSION

Apremilast profoundly altered the behavioral effects of ethanol and GABA_A receptor-specific drugs in male and female mice. Apremilast prolonged the duration of the LORR induced by ethanol, gaboxadol, zolpidem, and propofol and also prolonged ataxia induced by ethanol, zolpidem, propofol, and loreclezole. Surprisingly however, apremilast shortened the duration of the LORR and of ataxia induced by diazepam. The PKA inhibitor H89 blocked apremilast modulation of behavior by ethanol, propofol, gaboxadol, and diazepam, suggesting that apremilast alters acute tolerance to ethanol and other GABAergic drugs via PKA-mediated phosphorylation of GABA_A receptors. Our results with apremilast are consistent with work showing that increasing PKA activity intracerebroventricularly increases the sedative-hypnotic effects of ethanol or the GABA_A receptor agonist muscimol (Kumar et al., 2012). Conversely, other work has shown that inhibiting PKA decreases ethanol's sedative-hypnotic effects (Thiele et al., 2000).

Our behavioral studies suggest that α , $\beta 1$, and $\beta 3$ subunits are important for apremilast modulation of GABAergic drugs. Therefore, we studied how the phosphorylation state of $\beta 1$ or $\beta 3$ expressed with different α subunits altered GABA_A receptor function and modulation. In *Xenopus* oocytes, apremilast did not produce any direct changes in GABA_A receptor function, suggesting a low level of PDE4 activity as previously reported (Stahl et al., 2015). To study β phosphorylation, we used mutated receptors expressed in oocytes. Decreased maximal GABA-induced currents were observed in phosphomimetic $\beta 1$ -containing subunits expressed with $\alpha 1$ or $\alpha 3$, but not $\alpha 2$ subunits. In contrast, increased maximal GABA-induced currents were observed in phosphomimetic $\beta 3$ -containing subunits expressed with $\alpha 1$ and $\alpha 2$. Our findings in oocytes agree with those in HEK293 cells (McDonald et al., 1998), showing that $\beta 1$ phosphorylation decreases and $\beta 3$ phosphorylation increases GABA_A responses. These same β phosphorylation effects were observed in $\alpha 1$ -containing receptors in oocytes, and thus apremilast prolongation of zolpidem (an $\alpha 1$ -selective modulator) responses *in vivo* suggests that it acts by increasing phosphorylation of $\alpha 1\beta 3$ -containing receptors. Furthermore, the differential GABA_A responses of $\alpha 2$ and $\alpha 3$ subunits in combination with phosphomimetic $\beta 1$ - or $\beta 3$ -containing receptors suggest that these α

subunits can modulate ethanol- and diazepam-induced ataxia. Additional support for this comes from our previous study showing that deletion of $\alpha 2$ shortened recovery from ethanol and flurazepam, whereas deletion of $\alpha 3$ prolonged ataxia by both drugs (Blednov et al., 2013).

We also studied modulation of $\alpha 1\beta\gamma 2$ GABA_A receptors in different phosphorylation states by the GABAergic drugs used in the behavioral tests. We found that the allosteric effects of ethanol and other GABAergic drugs did not depend on β subunit phosphorylation. Thus, we propose that the behavioral effects of apremilast result from PKA-mediated alterations in GABA_A receptor responses to GABA rather than to changes in allosteric modulation by these drugs.

We were particularly interested in the effects of apremilast on ataxia because the lower drug doses used for this behavior would be expected to have more GABA_A receptor specificity than the higher doses required to induce LORR. Apremilast did not alter ataxia induced by gaboxadol, which in low doses selectively targets GABA_A receptors containing $\alpha 4$ and δ subunits (Chandra et al., 2006). However, apremilast prolonged recovery from the ataxic effects of zolpidem, which has a relative preference for receptors containing $\alpha 1$ and $\gamma 2$ subunits at low doses, but can also potentiate $\alpha 2/3\beta\gamma 2$ receptors at higher concentrations (Sieghart and Savic, 2018). In contrast, we found that apremilast shortened recovery from LORR and from ataxia induced by diazepam, which acts at receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits in combination with $\gamma 2$ subunits (efficacy $\alpha 3 > \alpha 2 > \alpha 1 \sim \alpha 5$) (Sieghart and Savic, 2018). These differential effects suggest that α subunits may influence apremilast modulation of certain GABAergic drugs.

It is not known which α subunits mediate the ataxic effect of diazepam since diazepam-induced rotarod ataxia is not altered in $\alpha 1$, $\alpha 2$, or $\alpha 3$ knock-in mice carrying a mutation that prevents benzodiazepine binding to the respective α subunit (Low et al., 2000; Rudolph et al., 1999). Instead it appears that benzodiazepine binding to any two of these subunits is sufficient for diazepam to produce ataxia. None of the knock-in mice have undergone LORR testing in response to GABAergic drugs.

The findings in α subunit knock-in mice raised the possibility that differential modulation of receptors containing $\alpha 1$ and $\alpha 3$ subunits may account for contrasting effects of apremilast on behavioral responses to zolpidem and diazepam. Based on our electrophysiological recordings, the α -selectivity shown by diazepam and zolpidem in heterologous systems (Sieghart and Savic, 2018), and the most common combinations of GABA_A subunits apparently present in brain (Benke et al., 1994), we propose the following: PKA-mediated phosphorylation reduced the function of $\alpha 3\beta 1$ -containing GABA_A receptors resulting in reduced diazepam-induced ataxia, while PKA-mediated phosphorylation of $\alpha 1\beta 3$ -containing receptors increased their function and drove the increase in zolpidem-induced ataxia. Other factors in play could be the selective expression of α subunits in different neuronal circuits (Pirker et al., 2000), or a differential selectivity for the pharmacologically active metabolites derived from diazepam (Nikas et al., 2015). Alternatively, in the case of diazepam, PKA modulation by apremilast may involve another target such as a protein that interacts with GABA_A receptors rather than a specific GABA_A receptor subunit.

Apremilast also prolonged ataxia induced by propofol (which shows no selectivity towards β subunits in trimeric receptors) (Rudolph and Antkowiak, 2004) and loreclezole, (which preferentially modulates GABA_A receptors containing β 2 or β 3 subunits) (Sieghart and Savic, 2018). PKA activation increases phosphorylation of β 1 and β 3, but not β 2 subunits (McDonald et al., 1998), suggesting that β 3 phosphorylation is responsible for apremilast modulation of propofol and loreclezole responses *in vivo*. As demonstrated in HEK293 cells (McDonald et al., 1998) and here in oocytes, phosphorylation of β 3 increases inhibitory GABA_A receptor-induced currents, which would be expected to increase the intoxicating motor effects and decrease acute tolerance to these drugs. Our finding that apremilast prolongs ataxia induced by ethanol and GABAergic drugs that can potentiate β 3-containing receptors (propofol and loreclezole) is consistent with phosphorylation of β 3 subunits being important for development of acute tolerance to ethanol.

Apremilast did not alter ataxia by etomidate, which also acts on β 2/ β 3 subunits (Sieghart and Savic, 2018), but only β 2 subunits are critical for the ataxic effects of etomidate (Reynolds et al., 2003). Because β 2 subunits are not a target for PKA-mediated phosphorylation, the etomidate ataxic effect was not modified after apremilast administration. This stands in contrast with the increase in the propofol ataxic effect. Although etomidate and propofol have many similarities, including sharing GABA_A receptors as main pharmacological targets, they also have clear differences in their molecular pharmacology and behavioral effects that could be responsible for this divergence (Drexler et al., 2009; Rudolph and Antkowiak, 2004).

Our findings that apremilast accelerated recovery from diazepam-induced ataxia and shortened duration of diazepam-induced LORR are consistent with a role for β 1 subunits. The function of β 1 subunits in GABA_A receptor responses has not been well characterized, and there is also limited information about their role in behavioral responses to ethanol. To determine if apremilast modulation could be mediated by β 1 subunits, we used the β 1-specific antagonist SCS (Thompson et al., 2004). SCS produced faster recovery from diazepam-induced ataxia and LORR, mimicking the effect of apremilast. These findings suggest that PKA-induced phosphorylation of β 1-containing receptors, which would be expected to decrease neuronal GABA_A responses (McDonald et al., 1998), is involved in the ability of apremilast to accelerate recovery from the behavioral effects of diazepam. Our results agree with work showing that allosteric GABA_A modulators with limited activity at β 1-containing GABA_A receptors have reduced ability to cause ataxia (Gee et al., 2010).

When β 1-containing receptors were blocked with SCS, the recovery from etomidate-induced ataxia was not affected given that this drug acts through β 2-containing receptors to impair motor responses on the rotarod (Reynolds et al., 2003). Propofol-mediated ataxia was also unaffected, providing the first evidence that this propofol effect is not mediated by β 1-containing receptors. Propofol-induced LORR was also not modified by blocking β 1-containing receptors. The role of β 3-containing receptors in both etomidate and propofol-induced LORR has already been shown (Jurd et al., 2003), and while the increase in GABA-mediated currents through β 3-containing receptors after apremilast administration explains the increase in propofol-induced LORR, the absence of change in etomidate-induced LORR

seems to indicate a more complex mechanism of action. Perhaps apremilast modifies one or more of the other pharmacological targets of etomidate (Rudolph and Antkowiak, 2004).

Limiting β 1-mediated responses using SCS also reduced the ataxic and sedative-hypnotic effects of ethanol, but unlike diazepam responses, SCS did not mimic the effect of apremilast. While these findings indicate a contributory role for β 1 subunits in ethanol-induced ataxia, under conditions of enhanced PKA activation by apremilast, the increased ataxic effects of ethanol appear to be mediated primarily by β 3-containing receptors.

Other phosphorylation-dependent mechanisms that regulate GABA_A receptors in neurons were not captured by our heterologous expression system. For example, PKC also phosphorylates β 3 S408/S409 in cultured cortical neurons (Brandon et al., 2000), which modifies interaction with proteins like AP2 in the intracellular loop, ultimately interfering with the receptor's clathrin-mediated endocytosis (Nakamura et al., 2015). Thus, the phosphorylation state of β subunits in neurons may be determined by additional factors that regulate GABA_A receptor function and trafficking that are absent in the heterologous system. Furthermore, cAMP elevation and subsequent PKA activation have been shown to mediate the increase of GABA release from presynaptic terminals (Diao et al., 2017; Kelm et al., 2008; Lachamp et al., 2009), adding another possible apremilast mechanism for influencing GABAergic transmission. Despite the limitations of our oocyte studies, our behavioral results indicate that PKA-mediated phosphorylation of β 1 or β 3 subunits is important for the effects of apremilast on responses to GABAergic drugs.

In our hypothetical model shown in Figure 11, apremilast activates PKA-mediated phosphorylation of β 1- and β 3-containing GABA_A receptors, producing differential regulation of ethanol and other GABAergic positive allosteric modulators. We propose that apremilast reduces the ataxic and sedative-hypnotic effects of diazepam via phosphorylation of β 1 receptors, while its opposing effects on β 3-containing receptors decrease acute tolerance to ethanol and other GABAergic drugs.

PDE4 is present throughout the brain and because apremilast acts as a nonselective inhibitor of all PDE4 subclasses, it can produce widespread effects that would depend on GABA_A receptor composition and distribution. Phosphorylation of β 3 subunits is consistent with the ability of apremilast to prolong ethanol-induced ataxia and decrease acute functional tolerance, as we observed in male and female mice (Blednov et al., 2018a), and is also consistent with the increased response to β 3-acting drugs (propofol and loreclezole) by apremilast observed here. Given that β 3 subunits are widely expressed in brain compared to the more discrete localization of β 1 subunits (Hortnagl et al., 2013), and that expression levels of β 1 are decreased in C57BL/6J mice compared with other strains (Mulligan et al., 2019), the net *in vivo* effects of apremilast on ethanol responses may be explained by actions on β 3-containing GABA_A receptors. Effects of the α 1-selective modulator zolpidem on α 1 β 3-containing receptors would also be consistent with its modulation by apremilast *in vivo*.

In summary, we propose that apremilast-induced phosphorylation of β 3-containing GABA_A subunits increases synaptic inhibition and decreases acute tolerance to ethanol and

GABAergic drugs, which could contribute to the reduced alcohol drinking and related behaviors observed in mice (Blednov et al., 2018a; 2018b). Development of tolerance is one of the criteria for diagnosing alcohol dependence in humans, and our findings show that apremilast may be a promising candidate to reduce acute tolerance (and alcohol drinking) through PKA modulation of GABAergic signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
EC₅	effective concentration 5
GABA_A receptor	γ-aminobutyric acid type A receptor
i.p.	intraperitoneal
LORR	loss of righting reflex
PDE4	phosphodiesterase type 4
PKA	protein kinase A
p.o.	per os
SCS	salicylidene salicylhydrazide
s.c.	subcutaneous

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HIGHLIGHTS

- Apremilast regulates alcohol and GABAergic drug responses *in vivo*
- Apremilast regulation occurs in a protein kinase A (PKA)-dependent manner
- Phosphorylation of $\beta 1$ and $\beta 3$ subunits differentially alters GABA_A receptor function
- Apremilast acts via PKA to alter acute tolerance to alcohol and GABAergic drugs

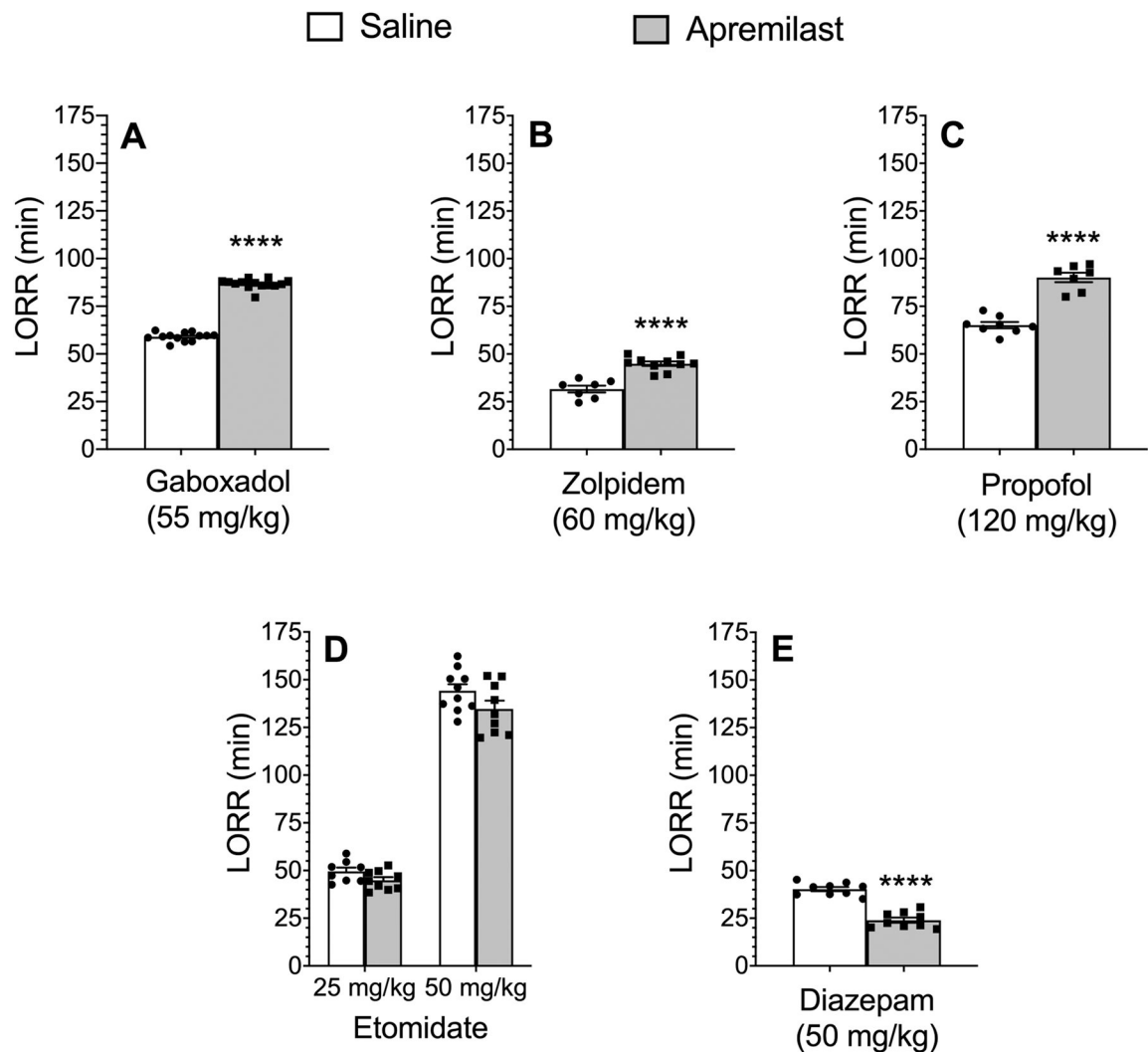
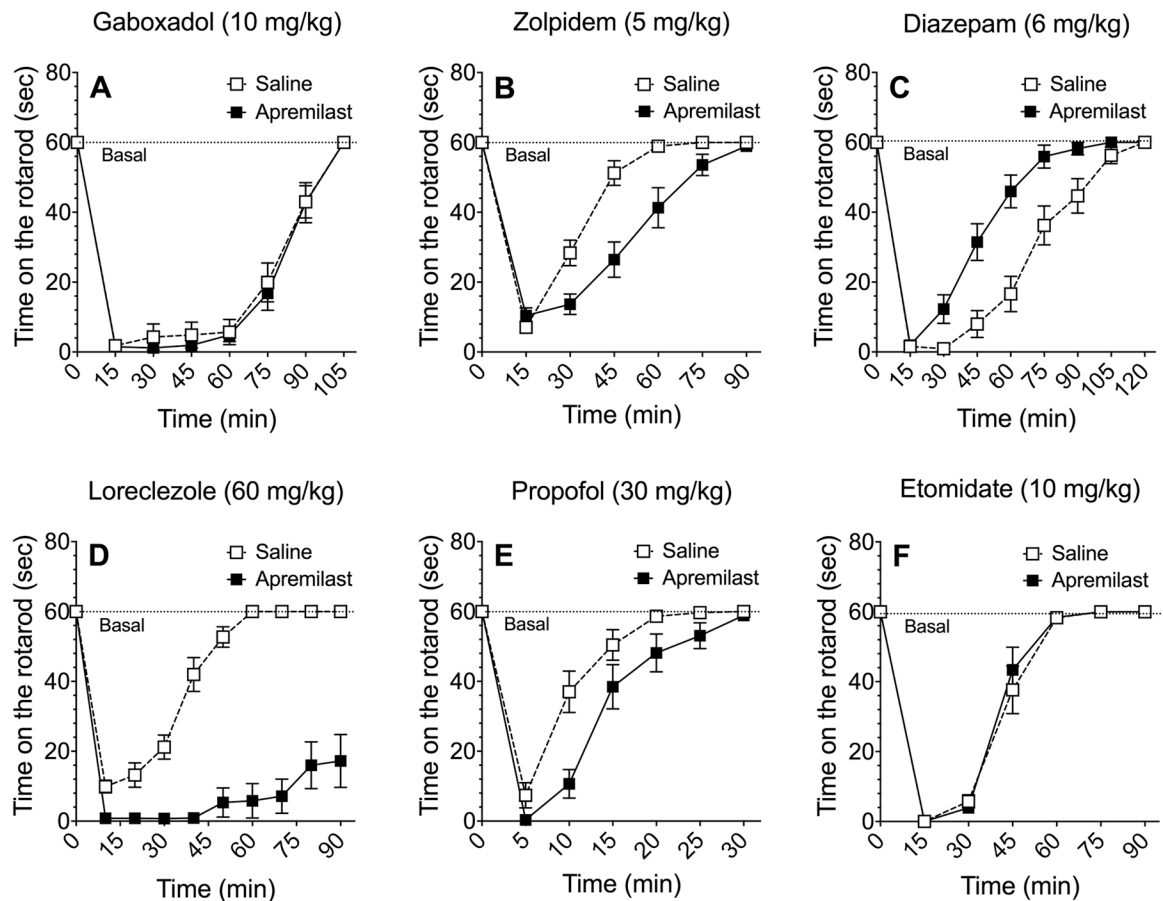


Figure 1.

Effect of apremilast on the loss of righting reflex (LORR) induced by different sedative-hypnotics. Duration of LORR in saline- vs. apremilast (20 mg/kg)-pretreated male and female C57BL/6J mice after i.p. injection of (A) gaboxadol (n = 13–14), (B) zolpidem (n = 7–10), (C) propofol (n = 7–8), (D) etomidate (n = 8–10), or (E) diazepam (n = 9). Data from male and female mice were combined. ****p < 0.0001 compared with the saline-treated group, two-tailed t-test.

**Figure 2.**

Effect of apremilast on recovery from ataxia induced by GABAergic drugs. Time on the rotarod in saline- vs. apremilast (20 mg/kg)-pretreated male and female C57BL/6J mice after i.p. injection of (A) gaboxadol (n = 16), (B) zolpidem (n = 16), (C) diazepam (n = 15), (D) loreclezole (n = 10–12), (E) propofol (n = 15–16), or (F) etomidate (n = 11–12). Data from male and female mice were combined and analyzed by two-way repeated measures ANOVA.

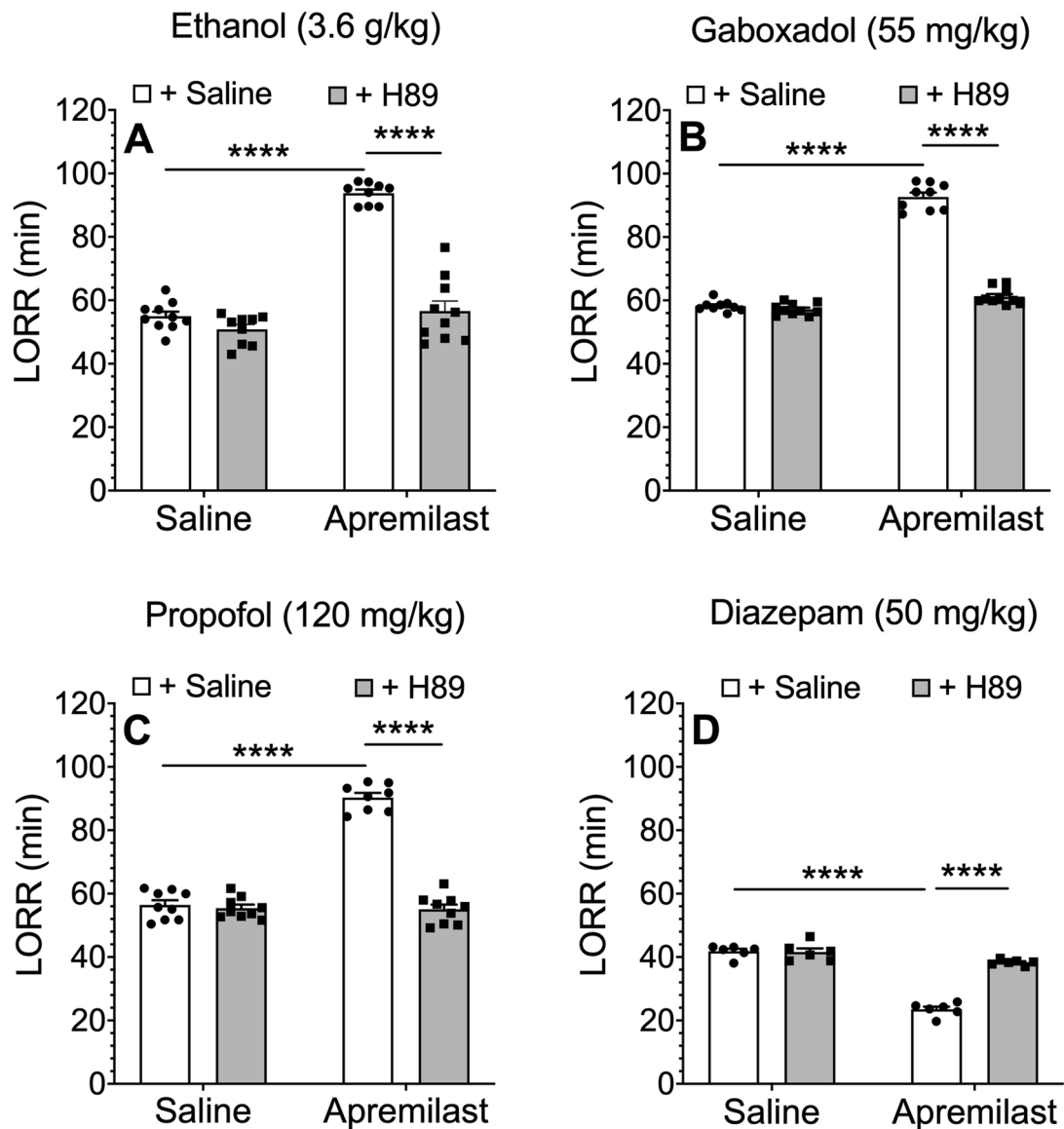


Figure 3.

The PKA inhibitor H89 prevents apremilast-induced changes in duration of the loss of righting reflex (LORR) by ethanol, gaboxadol, propofol, and diazepam. Duration of LORR induced by i.p. injection of (A) ethanol ($n = 9-10$), (B) gaboxadol ($n = 9-10$), (C) propofol ($n = 8-9$), or (D) diazepam ($n = 6$) in C57BL/6J mice pretreated with saline (p.o.) or apremilast (20 mg/kg, p.o.) 1 h before LORR assay then saline (s.c.) or H89 (10 mg/kg, s.c.) was given 15 min before sedative-hypnotic drug. Data from male mice (D) or males and females combined (A-C) were analyzed by two-way ANOVA and Tukey's *post hoc* tests, **** $p < 0.0001$.

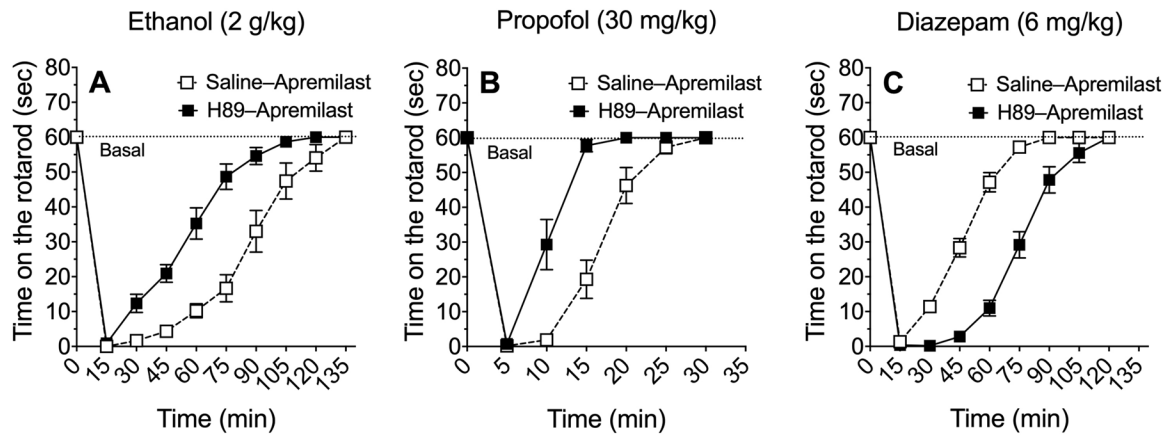


Figure 4.

The PKA inhibitor H89 prevents apremilast modulation of rotarod ataxia induced by ethanol, diazepam, or propofol. Time on the rotarod after i.p. injection of (A) ethanol ($n = 12$), (B) propofol ($n = 11-12$), and (C) diazepam ($n = 11-12$) in male and female C57BL/6J mice pretreated with saline (s.c.) + apremilast (20 mg/kg, p.o.) or H89 (10 mg/kg, s.c.) + apremilast (20 mg/kg, p.o.). Data from male and female mice were combined and analyzed by two-way repeated measures ANOVA.

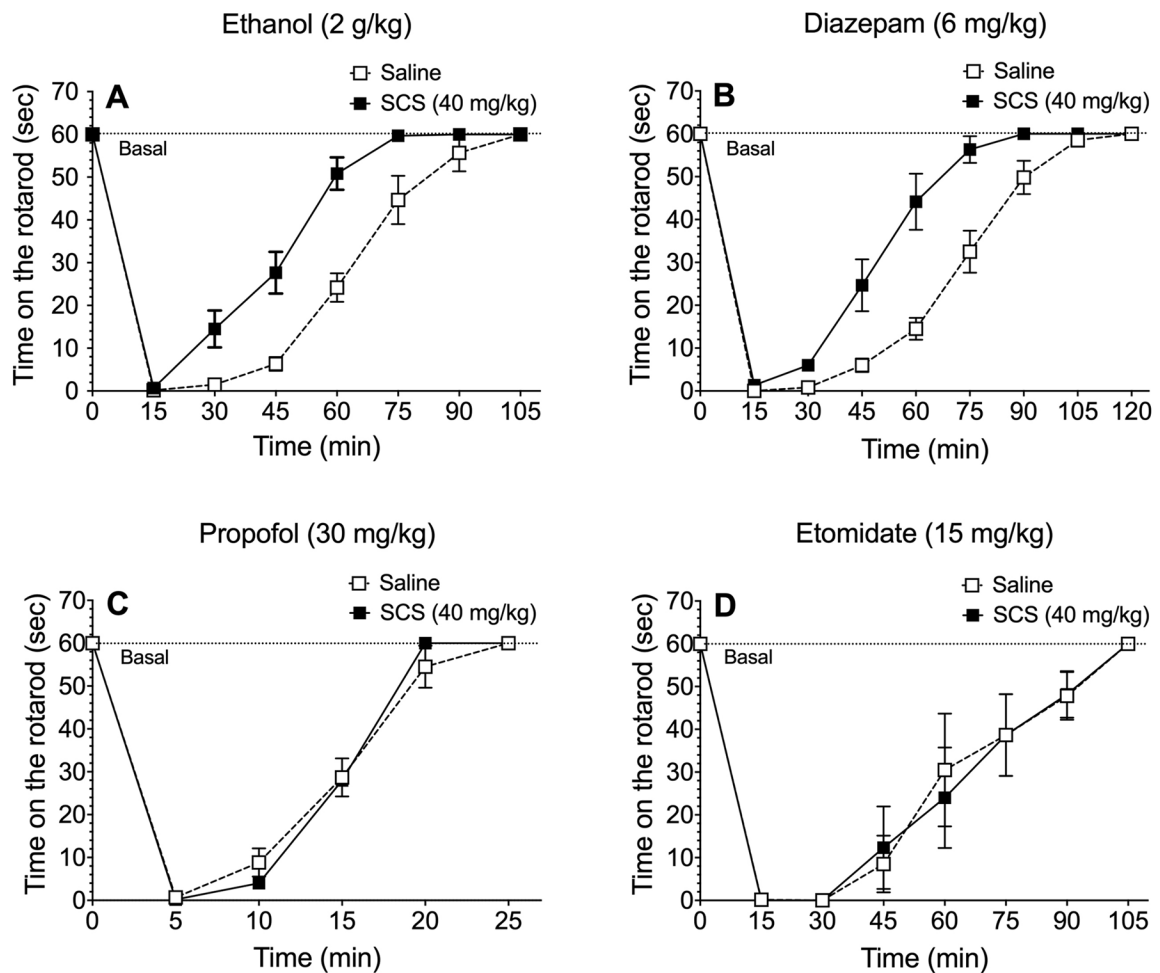


Figure 5.

A GABA_A receptor β 1 subunit antagonist accelerates recovery from ataxia induced by ethanol or diazepam. Time on the rotarod after i.p. injection of (A) ethanol (n = 6), (B) diazepam (n = 6), (C) propofol (n = 5–6), and (D) etomidate (n = 6) in male C57BL/6J mice pretreated with saline (i.p.) or salicylidene salicylhydrazide (SCS, 40 mg/kg, i.p.). Data were analyzed by two-way repeated measures ANOVA.

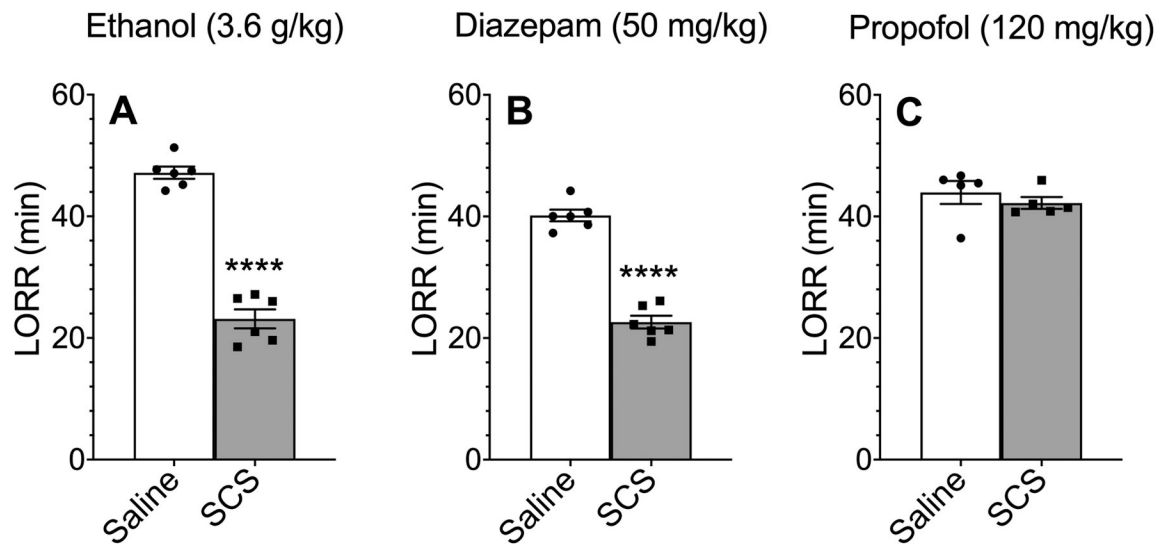


Figure 6.

Effect of SCS on the loss of righting reflex (LORR) induced by ethanol, diazepam, or propofol. Duration of LORR after i.p. injection of (A) ethanol (n = 6), (B) diazepam (n= 6), or (C) propofol (n = 5) in male C57BL/6J mice pretreated with saline (i.p.) or salicylidene salicylhydrazide (SCS, 40 mg/kg, i.p.). ****p < 0.0001 compared with the saline-treated group, two-tailed t-test.

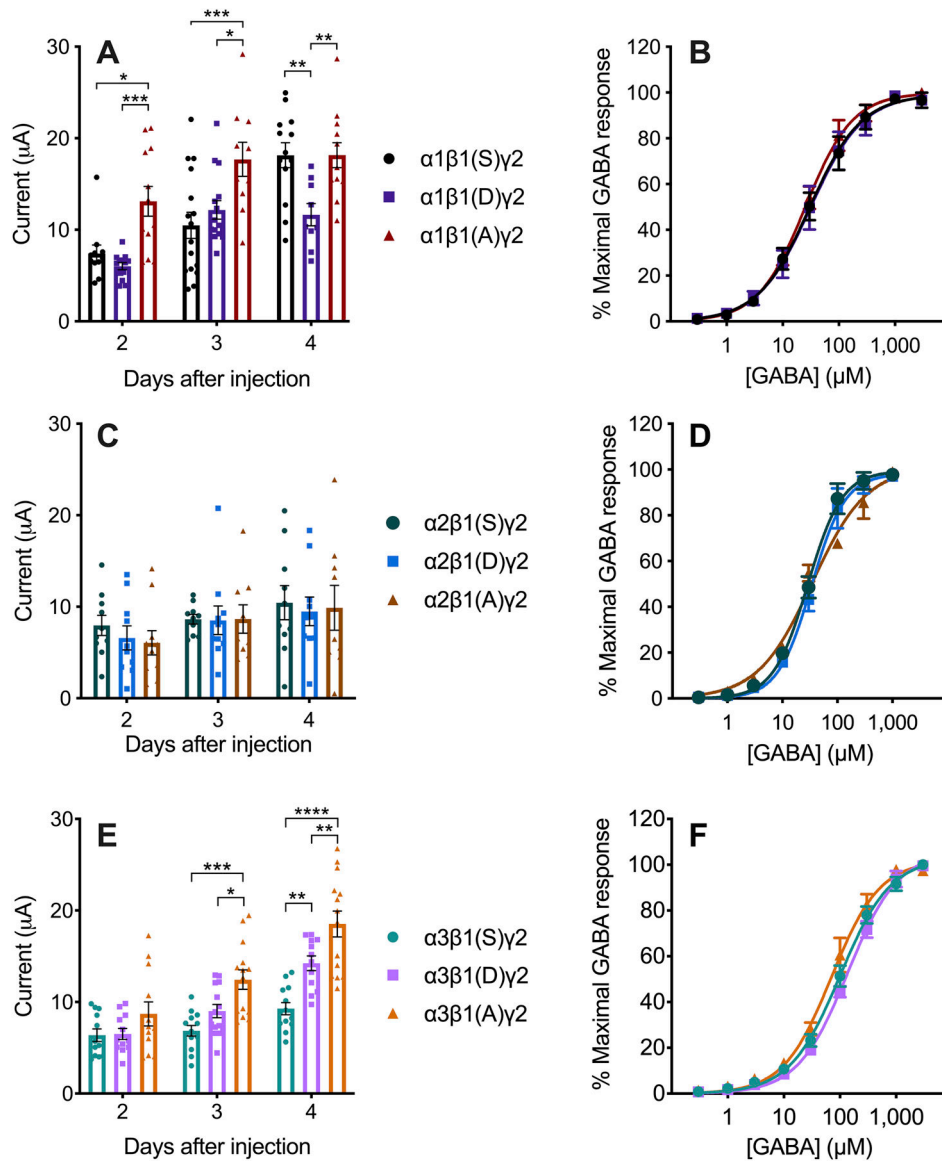


Figure 7. $\beta 1$ -containing GABA_A receptors expressed in *Xenopus laevis* oocytes. The letters in parentheses in the legend indicate the residues in position 409 of $\beta 1$. A) Currents induced by maximal GABA concentration (3 mM GABA, n = 9–16) and B) GABA concentration-response curves (n = 4–5) in $\alpha 1\beta 1\gamma 2$ GABA_A receptors. C) Currents induced by maximal GABA concentration (300 μ M GABA, n = 9–10) and D) GABA concentration-response curves (n = 4–5) in $\alpha 2\beta 1\gamma 2$ GABA_A receptors. E) Currents induced by maximal GABA concentration (3 mM GABA, n = 12–14) and F) GABA concentration-response curves (n = 4–6) in $\alpha 3\beta 1\gamma 2$ GABA_A receptors. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test, *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001.

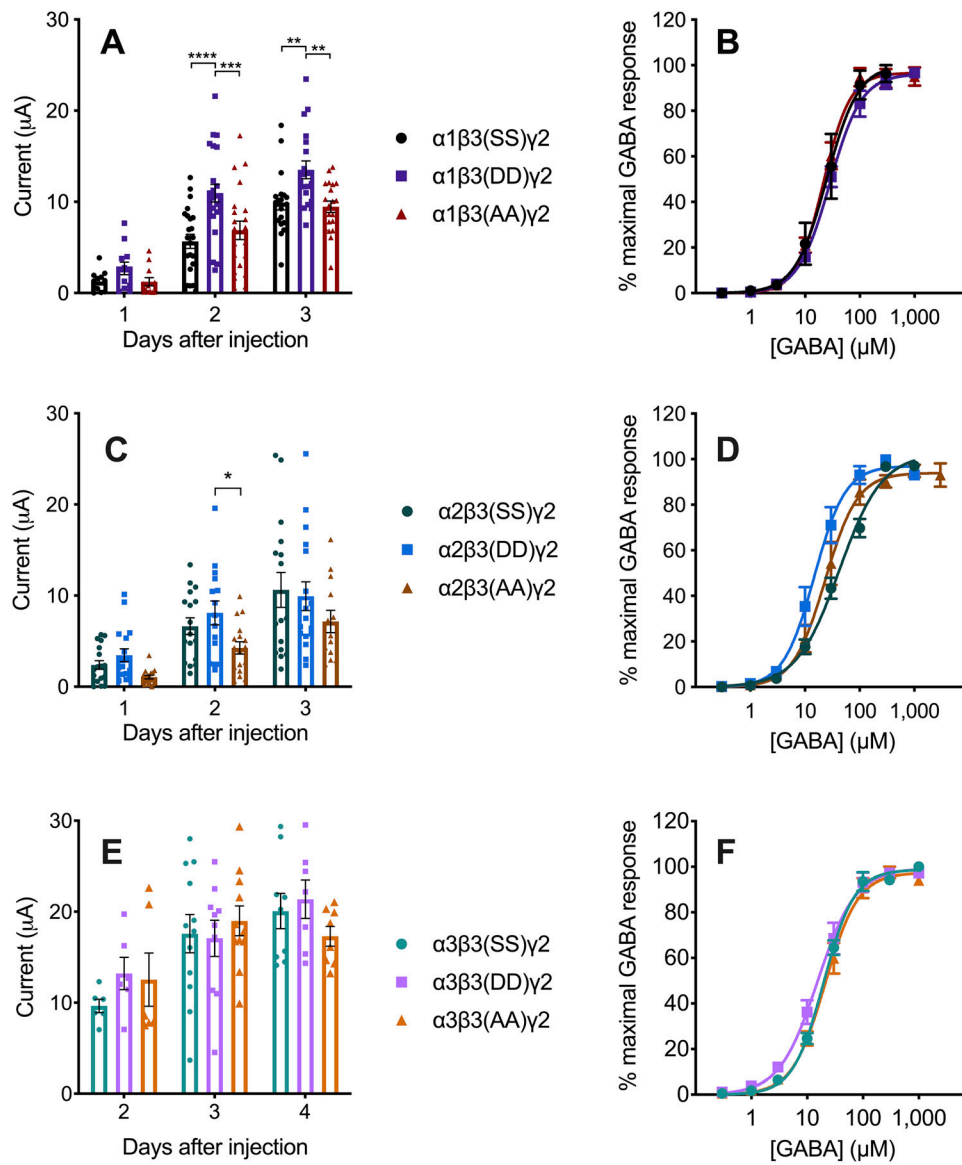


Figure 8. $\beta 3$ -containing GABA_A receptors expressed in *Xenopus laevis* oocytes. The letters in parentheses in the legend indicate the residues in positions 408 and 409 of $\beta 3$. A) Currents induced by maximal GABA concentration (1 mM GABA, n = 12–24) and B) GABA concentration-response curves (n = 3–5) in $\alpha 1\beta 3\gamma 2$ GABA_A receptors. C) Currents induced by maximal GABA concentration (1 mM GABA, n = 13–18) and D) GABA concentration-response curves (n = 5–7) in $\alpha 2\beta 3\gamma 2$ GABA_A receptors. E) Currents induced by maximal GABA concentration (300 μM GABA, n = 6–12) and F) GABA concentration-response curves in $\alpha 3\beta 3\gamma 2$ GABA_A receptors (n = 5–6). Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

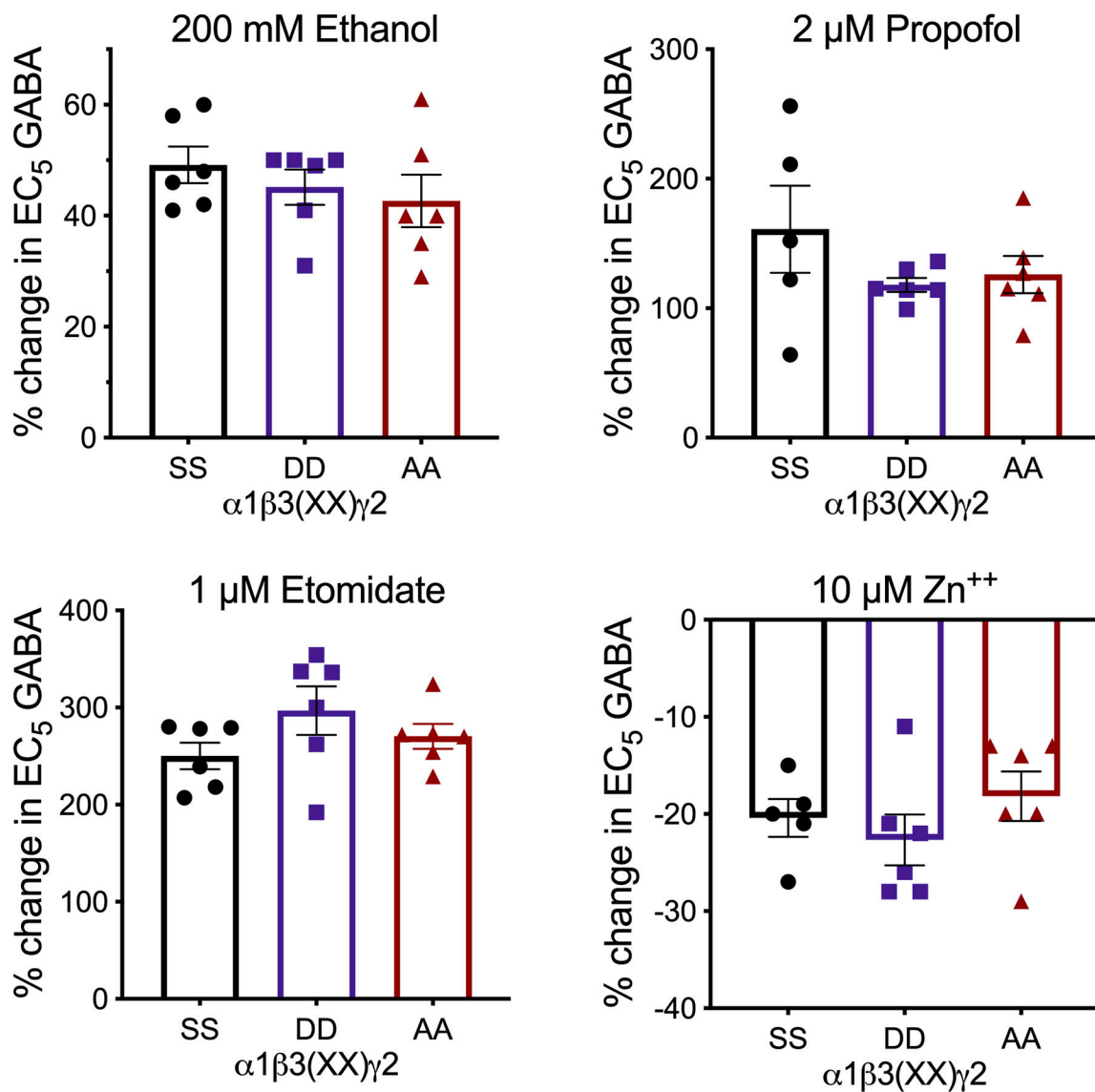


Figure 9. Allosteric modulators of submaximal GABA currents in $\alpha 1\beta 3\gamma 2$ GABA_A receptors with different phosphorylation states of $\beta 3$ subunits. Data were analyzed by one-way ANOVA ($n = 5-6$).

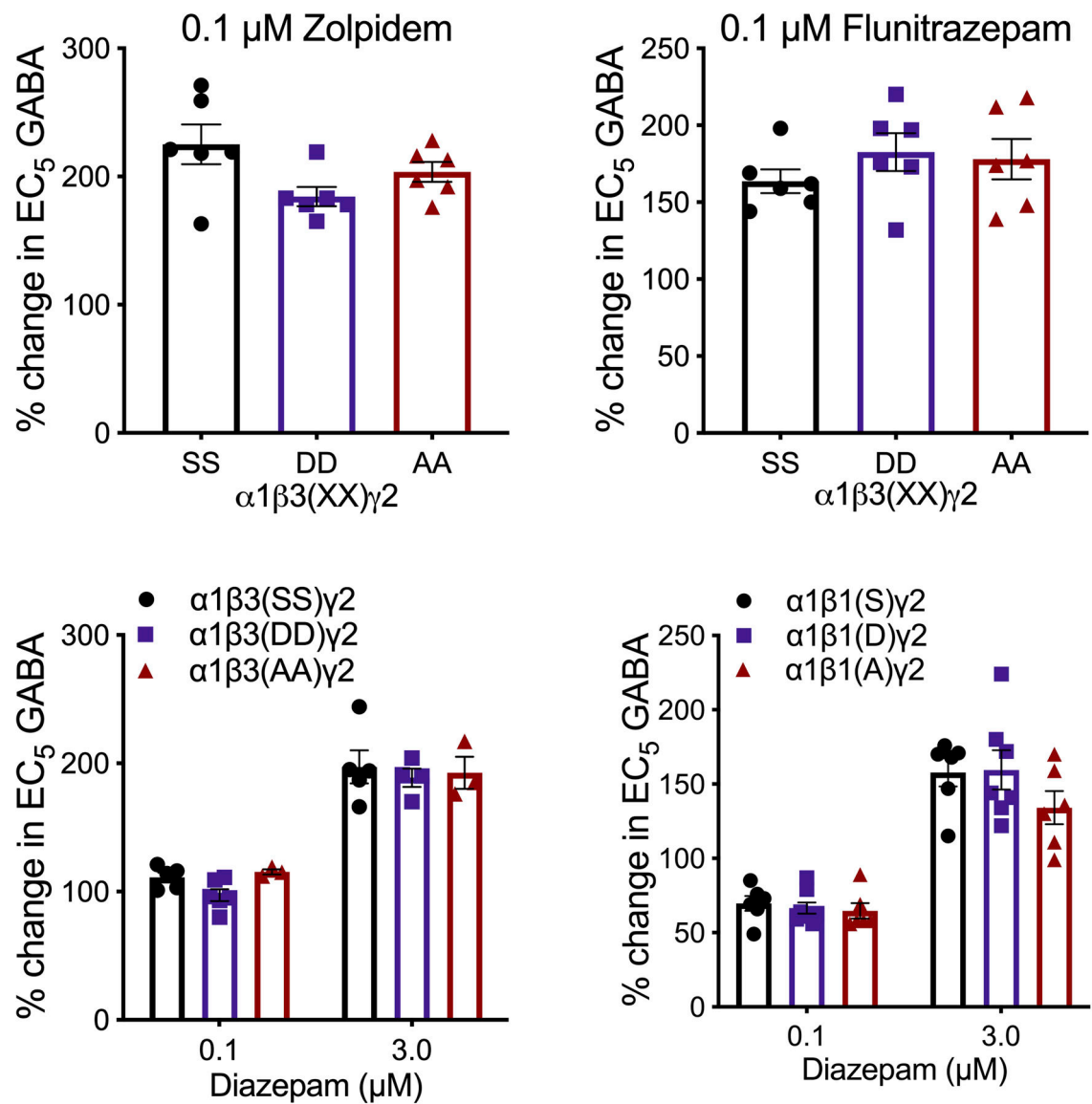


Figure 10.

Modulation by ligands of the benzodiazepine binding site of submaximal GABA currents in $\alpha 1\beta 2$ GABA_A receptors with differing phosphorylation states of β subunits. Data were analyzed by one-way (flunitrazepam, zolpidem; $n = 6$) and two-way (diazepam) ANOVA ($\beta 3$, $n = 3-6$; $\beta 1$, $n = 6-8$).

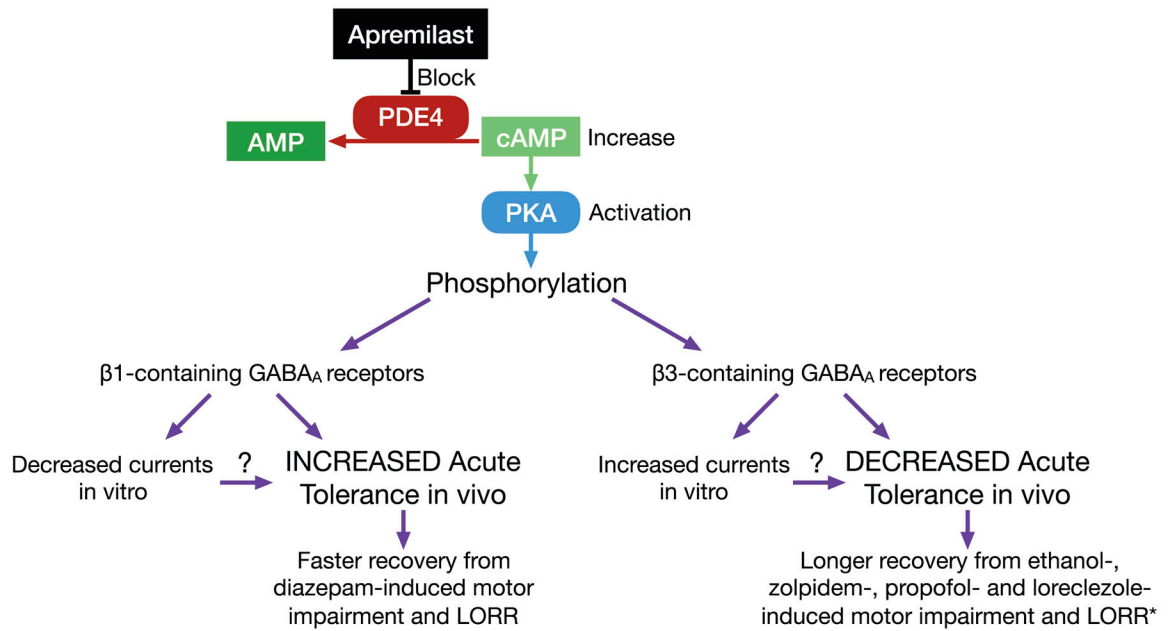


Figure 11.

Hypothetical mechanism of action for apremilast. The phosphodiesterase type 4 (PDE4) inhibitor, apremilast, blocks hydrolysis of cAMP (cyclic adenosine monophosphate) to AMP, thus increasing levels of cAMP and activation of protein kinase A (PKA). PKA-induced phosphorylation differentially regulates β 1- and β 3-containing GABA_A receptors (as shown in both HEK293 cells and *Xenopus* oocytes), producing specific effects on acute tolerance to the ataxic (rotarod) and sedative-hypnotic (LORR) effects of ethanol and other GABAergic drugs in mice. *Loreclezole-induced LORR could not be determined.

Table 1.

Summary of behavioral effects in male and female C57BL/6J mice.

Behavior	Modulator	Dose	+ Apremilast	+ Apremilast + H-89	+ SCS ^a
Ethanol intake	Ethanol		↓ <i>b</i>		
LORR duration	Ethanol	3.6 g/kg	↑ <i>b</i>	↓	↓
	Gaboxadol Non-specific	55 mg/kg	↑	↓	
	Etomidate β2-β3 specific	25 and 50 mg/kg	=		
	Propofol Non β-specific	120 mg/kg	↑	↓	=
	Diazepam α3/2/1/5 γ2	50 mg/kg	↓	↑	↓
	Zolpidem Non-specific	60 mg/kg	↑		
Rotarod recovery	Ethanol	2 g/kg	→ <i>b</i>	←	←
	Gaboxadol α4 δ specific	10 mg/kg	=		
	Etomidate β2-β3 specific	10 mg/kg	=		
	Etomidate β2-β3 specific	15 mg/kg			=
	Propofol Non β-specific	30 mg/kg	→	←	=
	Diazepam α3/2/1/5 γ2	6 mg/kg	←	→	←
	Zolpidem α1 γ2-specific	5 mg/kg	→		
	Loreclezole β2-β3 specific	60 mg/kg	→		

Effects of apremilast (20 mg/kg), apremilast (20 mg/kg) + PKA inhibitor H-89 (10 mg/kg), or a β1-specific antagonist salicylidene salicylhydrazide (SCS, 40 mg/kg) on ethanol- and GABAergic-mediated behaviors are summarized as follows: = no change from saline control; ↑ (increased) or ↓ (decreased) response from saline control; → (longer) or ← (shorter) recovery from rotarod ataxia compared with saline control. GABAergic drugs have subunit-specific or non-specific actions depending on the dose. Etomidate mediates ataxia mainly through β2-containing receptors.

^aResults are from male mice only.

^bResults are from (Blednov et al., 2018b). LORR, loss of the righting reflex.

Table 2.

Parameters determined by nonlinear regression of GABA concentration-response curves (Figures 7 and 8). GABA EC₅₀, GABA effective concentration 50 (μM); n_H, Hill slope; n, number of oocytes.

Receptor	GABA EC ₅₀ (95% confidence intervals)	n _H ± SEM	n
α.1β1(S)γ2	31.1 (26.2 to 37.0)	0.92 ± 0.05	5
α.1β1(D)γ2	25.3 (23.4 to 27.4)	1.03 ± 0.03	5
α.1β1(A)γ2	29.8 (26.1 to 34.1)	0.94 ± 0.04	4
α.2β1(S)γ2	28.8 (24.8 to 33.7)	1.39 ± 0.10	4
α.2β1(D)γ2	33.7 (29.8 to 38.1)	1.42 ± 0.08	5
α.2β1(A)γ2	34.9 (18.4 to 65.9)	0.88 ± 0.14	4
α.3β1(S)γ2	100 (82 to 127)	0.96 ± 0.07	6
α.3β1(D)γ2	137 (117 to 165)	0.97 ± 0.06	4
α.3β1(A)γ2	70.4 (55.8 to 91.5)	1.01 ± 0.10	5
α.1β3(SS)γ2	24.7 (21.4 to 28.6)	1.50 ± 0.10	3
α.1β3(DD)γ2	28.2 (26.6 to 30.0)	1.50 ± 0.05	6
α.1β3(AA)γ2	21.5 (18.7 to 24.8)	1.77 ± 0.15	5
α.2β3(SS)γ2	42.3 (30.4 to 59.1)	1.11 ± 0.12	5
α.2β3(DD)γ2	14.8 (12.5 to 17.6)	1.53 ± 0.13	5
α.2β3(AA)γ2	23.6 (21.8 to 25.5)	1.47 ± 0.06	7
α.3β3(SS)γ2	19.8 (17.4 to 22.5)	1.57 ± 0.10	5
α.3β3(DD)γ2	15.3 (13.7 to 17.1)	1.26 ± 0.06	5
α.3β3(AA)γ2	21.0 (18.0 to 24.5)	1.47 ± 0.11	6