Reversal of Ethanol-induced Intoxication by a Novel Modulator of Gβγ Protein Potentiation of the Glycine Receptor

Loreto San Martin, Fabian Cerda, Chunyang Jin, Veronica Jimenez, Gonzalo E. Yevenes, Daniela Nova, Jorge Fuentealba, Luis G. Aguayo, and Leonardo Guzman

The acute intoxicating effects of ethanol in the central nervous system result from the modulation of several molecular targets. It is widely accepted that ethanol enhances the activity of the glycine receptor (GlyR), thus enhancing inhibitory neurotransmission, leading to motor effects, sedation, and respiratory depression. We previously reported that small peptides interfered with the binding of Gβγ to the GlyR and consequently inhibited the ethanol-induced potentiation of the receptor. Now, using virtual screening, we identified a subset of small molecules capable of interacting with the binding site of Gβγ. One of these compounds, M554, inhibited the ethanol potentiation of the GlyR in both evoked currents and synaptic transmission in vitro. When this compound was tested in vivo in mice treated with ethanol (1–3.5 g/kg), it was found to induce a faster recovery of motor incoordination in rotarod experiments and a shorter sedative effect in loss of righting reflex assays. This study describes a novel molecule that might be relevant for the design of useful therapeutic compounds in the treatment of acute alcohol intoxication.

Ethanol is a brain-depressing drug possessing well-recognized acute physiological effects. Within the different molecular targets for ethanol, those that best explain the acute effects of ethanol are those affecting fast neurotransmission. It is believed that ethanol effects on sensorial transmission, motor control, respiratory rhythms, and cognitive processing are caused by changes in the activity of several ligand-gated ion channels (1) and particularly through the potentiation of the glycine receptor (GlyR) (2). The GlyR has been extensively studied as a molecular target for ethanol, and electrophysiologically experiments have demonstrated that ethanol potentiates GlyR activity (3), as determined by increased glycine-evoked currents (4, 5), an increased decay time constant in spontaneous synaptic events (6), increased probability of channel opening in single-channel analysis (7), and increased agonist affinity (8). It was shown previously that G proteins participate in the ethanol effect on the GlyR (4). In addition, more recent studies have determined that the G protein βγ dimer acts as an intermediary for the ethanol action on the GlyR (7, 10), where amino acids in the GlyR intracellular domain are essential for the interaction with Gβγ (11, 12). Interestingly, a small peptide (RQHe7) that binds Gβγ inhibited the potentiation effect of ethanol on both evoked and synaptic currents (6, 11). Using in silico analysis, an aspartic pocket (Asp-186, Asp-228, and Asp-246) in Gβ was identified as the binding region for RQHe7. Thus, the inhibition of the interaction between Gβγ and the GlyR intracellular domain prevented the ethanol effect in native and recombinant systems (6). Therefore, in this study, we aimed to identify small molecules capable of binding Gβγ and able to inhibit the potentiation of the glycine current induced by ethanol. One of these compounds was assayed in in vivo pharmacology in mice using increasing concentrations of ethanol, demonstrating that it was able to partly reverse some intoxicating effects. Thus, the rationale for designing pharmacological modulators that interfere with the Gβγ-GlyR protein-protein interaction is amenable for blocking acute ethanol intoxication.

Results

We performed in silico screening aimed at identifying small molecules able to block the effects of ethanol on the GlyR using the region of Gβγ that was proposed to interact with the GlyR and the RQHe7 peptide (6). The analysis resulted in a group of compounds possessing amide and polar groups that could take part in potential electrostatic and hydrogen bonding interactions with the protein receptor. Several selected compounds contained aromatic and heteroaromatic rings that could be involved in van der Waals and π-π stacking interactions with suitable amino acid side chains. Finally, a subset of 13 compounds was selected to examine their effect on the ethanol
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The glycine-evoked current at a concentration corresponding to EC_{10} (15 μM) in HEK293 cells expressing the GlyR_α1 subunit is potentiated by 48% ± 3% in the presence of ethanol (100 mM), as shown for the control condition in Fig. 1B (Gly + EtOH) or in the graph of Fig. 1C and as reported previously (7, 10, 11). The small molecules (200 μM) were included in the patch clamp recording pipette to allow them to freely diffuse into the cytoplasm. Recordings of the current with or without ethanol were done immediately after the whole cell configuration was established (minute 1) and after 15 min to allow complete diffusion of the compound. Glycine currents did not have a significant variation between minutes 1 and 15. The ethanol potentiation percentage was obtained from glycine-evoked currents at minute 15 in relation to glycine alone. C, the percentage of ethanol potentiation of chloride current evoked as described in the presence of different molecules (200 μM) selected from the virtual screening. Molecules were allowed to diffuse intracellularly. D, the percentage of ethanol potentiation of chloride current evoked as described in the presence of the extracellularly applied molecules M554 and M890 (200 μM, incubated for 45 min). The values represent the mean ± S.E. of the percentage of potentiation from at least six cells. *, p < 0.01; *, p < 0.05.

To obtain data on the predictive intermolecular interaction between GlyR and the compounds M554 and M890, studies using molecular docking were done. This analysis provided information on the relative orientation and distances between the chemical groups involved in protein-ligand interactions (Fig. 3A). For example, the indole group of M554 was able to establish an electrostatic ion-dipole interaction with the carboxylate group of Asp-186. Potentially, this indole group could facilitate a van der Waals interaction with Cys-204. On the other hand, the amide group established either a hydrogen bond or an ion-dipole interaction with the carboxylate group of Asp-228 and Asp-246. The urea moiety of M890, similarly, appears to establish a hydrogen bond with the carboxylate...
group of Asp-228 and also forms an ion-dipole interaction with both Asp-228 and Asp-246. Finally, the hydroxyl group of M890 has the position and distance required to establish a hydrogen bond with Asp-246 (Fig. 3A).

Previous studies have shown that the sites where the diverse effectors in Gβγ bind are partially overlapped (14). Thus we thought that it was important to determine the existence of functional specificity for our best compounds, i.e. M554 and M890. For this, we first used HL60 cells that express the fMLP receptor coupled to Gi to perform a calcium assay linked to the activation of phospholipase Cβ (PLCβ) by Gβγ (fMLP column). Compounds M554, M119, and M890 were evaluated to determine any modulatory activity on this transduction system. C, modulation of GIRK channels activated by Gβγ. HEK cells overexpressing GIRK subunits and the GABA_B receptor were stimulated with baclofen in the presence or absence of molecules M554, M890, and M119. The values represent the mean ± S.E. from at least six cells. *, p < 0.05.

Together with this, HEK cells expressing the GABA_B receptor were used to study the effects of the compounds in the Gβγ regulation of the G protein-coupled inwardly rectifying potassium channel (GIRK). In this case, M554 and M890 were able to partly inhibit the GIRK activity induced by baclofen by 56% ± 8% and 63% ± 7%, respectively. The compound M119 also had an inhibitory effect in this system of about the same level as M554 (Fig. 3C).

Next we wanted to examine whether these compounds, besides blocking the potentiation of the glycine current by ethanol in GlyR-overexpressing cells, also affected the enhancement of currents caused by activation of the native synaptic GlyR in cultured spinal cord neurons. This was examined by recording miniature inhibitory postsynaptic currents after 15
min of intracellular dialysis with M554 and M890 (200 μM, Fig. 4A). The data show that both the frequency and the amplitude of the synaptic events were not affected by either ethanol or M554 and M890 (Fig. 4, B and C). On the other hand, ethanol (100 mM) caused an increase of 38% ± 8% above the control in the decay time constant, a parameter that indicates the relative time the channel spends in the open state (Fig. 4, D and E).

Interestingly, after application of M554 and M890 (Fig. 4E), ethanol elicited only a very low increment in the decay time constant: 8% ± 5% and 3% ± 7%, respectively. These results show that these molecules also act as inhibitors of the ethanol potentiation in postsynaptic glycinergic events.

Considering the previous results demonstrating the inhibition of ethanol potentiation of glycine currents in recombinant and native GlyRs, we proceeded to examine whether these compounds were also able to attenuate some acute ethanol effects in vivo. Thus, C57BL6/J mice were intraperitoneally administered with ethanol in the absence and presence of the compounds. First, to study locomotor or exploratory activities, we examined the distance traveled in 5 min using an open field assay. After receiving an injection of ethanol (1.0 g/kg), the mice exhibited increased motor activity (p = 0.02541) that was not reversed in the presence of M554 or M890 at a dose of 100 mg/kg (p = 0.34584 and p = 0.81545, respectively; Fig. 5). Mice injected with the compounds alone did not show any change in locomotor activity. To assess the effects of a higher alcohol concentration, motor coordination was examined using an accelerating rotarod assay in the presence and absence of M554 or M890. First, the molecules M554 and M890 injected by themselves did not induce any significant change in motor coordination. The acute administration of ethanol (2.0 g/kg), on the other hand, produced a marked motor incoordination, as described previously (15), with a shorter latency to fall (28% ± 4% of control measured at 15 min post-injection, Fig. 6A). The ethanol-injected mice recovered to 60% ± 6% of pre-injection time, at 105 min. Interestingly, the administration of M554 (100 mg/kg) caused a faster recovery from ethanol-induced motor deficits, reaching 85% ± 3% of recovery at minute 75 and 100% ± 8% of recovery at minute 105 after ethanol injection (Fig. 6A). On the other hand, we found no differences in the M890-injected group. M554 did not only antagonize the motor incoordination induced by ethanol, but it also reduced its sedative effects, measured as the time of LORR (Fig. 6B). The data show the effect of ethanol alone and co-administered with different doses of M554 (0, 50, 100, and 200 mg/kg). The injection of 3.5 g/kg ethanol alone produced a sedative effect represented by a LORR of 45 ± 3 min (n = 8). The injection of a 50 mg/kg dose of M554 reduced the LORR to 40 ± 2 min (n = 6, p = 0.2446). Interestingly, increasing the dose to 100 mg/kg reduced LORR to 34 ± 3 min (n = 8, p = 0.04742). No larger effect was found with 200 mg/kg, possibly because of solubility issues. Blood alcohol concentration was determined after 30 min of receiving a dose of 3.5 g/kg of ethanol with or without M554 (100 mg/kg) to discard changes in
metabolic clearance that might explain the reduced intoxicating effects of ethanol. The measured blood alcohol concentration values were 3.64 ± 0.29 g/liter for the control (ethanol, n = 3) and 3.84 ± 0.17 g/liter (ethanol plus M554, n = 3), which did not represent any significant difference. Differences in the latency to LORR were not detected in any of the molecules studied (data not shown). Finally, we tested the duration of LORR in KI mice that had a reduced LORR in the presence of ethanol because of point mutations in the intracellular domain of the GlyR subunit (K385A/K386A GlyR1) important for the intracellular modulation of Gβγ (16). No differences were found in the duration of LORR with ethanol alone (29 ± 1 min) and ethanol plus M554 (31 ± 1 min, p = 0.27119) in these mice, supporting the notion that the effect of M554 is due to the interference in the intracellular signaling mechanism for the potentiation by ethanol in glycine currents (Fig. 6C).

Discussion

Ethanol is the most commonly abused drug in the world. It is socially accepted at moderate doses because of its anxiolytic and euphoric actions, but it produces serious intoxication at higher doses, leading to loss of motor coordination, sedation, and deep respiratory depression and death. Previous reports have shown the importance of the Gβγ-associated intracellular mechanism for modulation of the GlyR by ethanol and the importance of the intracellular domain in the Gβγ-GlyR interaction (7, 12, 16). With the aim of developing a pharmacologically active compound, we performed in silico studies followed by neurophysiological experiments and behavioral studies to identify small molecules capable of binding to Gβγ in the modulatory site for this channel and antagonizing the behavioral actions of ethanol. Supporting this rationale, we were able to identify one compound, named here M554.

Our virtual screening produced a subset of small molecule leads that were examined as inhibitors for the ethanol effects on the GlyR using electrophysiological studies as a first approach to identify active compounds. The strategy was to use the Gβγ site that binds to the GlyR for the analysis of a small molecule library (6). In this way, the first discrimination parameter was the interaction energy of the molecules to the site in Gβγ. The presence of amine and amide groups in the compounds was expected because of the number of aspartic acid residues in the Gβγ site used as a receptor in the virtual screening. This type of approach is not unique because other reasonably specific and
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potent γ1 inhibitors have also been identified following similar approaches (13, 17). One of these, M119, was found to bind with good affinity to the γ1 subunit and inhibited PLCβ3 signaling in vitro. Additionally, co-administration of M119 with morphine resulted in a leftward shift in the morphine antinociceptive dose-response curve (13), similar to the shift observed with PLCβ3 knockout mice (18). These and other studies have determined that M119 and its related compound Gallenin™ are effective in animal models of inflammation (19), analgesia (20), and heart failure (21). Interestingly, the M119 binding site is similar to the binding site for the SIGK peptide, which is near the binding site of M554. Because M119 was unable to affect the ethanol potentiation of the glycine current, we concluded that the binding site of M119 in Gβγ does not overlap with the binding site for the GlyR, where M554 and M890 bind. These data are important because the Gβγ binding sites include those for PLCβ, adenylyl cyclase, and GIRK, among others. M554 and M890 had no effect on PLCβ activity under the same conditions where M119 was inhibitory for the intracellular Ca2+ increase mediated by this enzyme (13). On the other hand, all assayed compounds, including M119, had inhibitory activity on GIRK channels, possibly because of the critical presence of Asp-228 on the Gβγ surface (22). The fact that M554 can inhibit a potassium channel is relevant because it might enhance excitability, which is already low in an intoxicated brain. Notably, we did not observe any increase in mouse excitability or behavior, suggesting that the compound is not toxic on its own (Fig. 6A). This is also interesting because future chemical modifications should be expected to improve the selectivity of M554.

The compounds M554 and M890 were also effective at inhibiting the effects of ethanol on synaptic transmission. Glycinergic miniature inhibitory postsynaptic currents were analyzed to evaluate kinetic aspects of the current in the presence and absence of ethanol and to determine their inhibiting effects. In this study, we found that ethanol increased the decay time constant in agreement with previous studies (16, 23). Thus, the effect of M554 and M890 of reducing the ethanol enhancement in decay time constant is in agreement with the working model that predicts that the inhibition of the Gβγ-GlyR interaction inhibits the ethanol potentiation of the glycine current.

Behavioral studies have demonstrated that the GlyR is involved in some of the acute effects of ethanol (24, 25). Accordingly, intracerebroventricular administration of glycine, or its precursor serine, augmented ethanol-induced LORR in mice (26), and these effects were blocked by strychnine (27). Additionally, KI mice with mutations (Q266I, M287L, or D80A) in the a1 subunit demonstrated changes in ethanol-induced incoordination and LORR (15, 28). Interestingly, it has been shown in our laboratory that a KI mouse with a mutation that reduces ethanol sensitivity of the GlyRα1 subunit reduced ethanol-induced LORR duration (16). Based on this evidence, we assayed different doses of ethanol in animal models, finding that M554 had an inhibitory effect at high ethanol concentrations. M554 did not reverse the enhancing effect of a low dose of ethanol in exploratory activity (Fig. 5), probably because the euphoric effects associated with low concentrations of ethanol are related to modulation of ion channels or proteins that are not regulated by Gβγ (29). However, when we evaluated motor incoordination using a high dose of ethanol, we found a shorter LORR in the presence of M554, which is in agreement with results indicating the importance of the GlyR in recovery from rotarod ataxia (15). Possible effects of these molecules that could be affecting PLCβ and GIRK channel activity did not have any influence in behavioral studies because the compounds alone did not show significant changes in both locomotor activity and motor coordination. LORR assays were performed in mice to induce acute intoxication and the impairment of the righting reflex in the presence of M554. The lack of effect of M890 in behavior assays could be determined by an increased renal clearance, liver modification, or for a peripheral distribution, which would be interesting to study in more detail. According to the rationale followed in this work, the ethanol effect induced by the interaction of Gβγ with the intracellular domain of GlyR is inhibited by the presence of a compound that binds to the same site in Gβγ, acting as a competitive inhibitor of this regulation. Thus, the faster recovery of motor deficits and sedation supports this idea. Indeed, the sedation time induced by ethanol was ~25% shorter in the presence of M554. Interestingly, a KI mouse with a mutation in the Gβγ binding site of the GlyR that reduces ethanol-mediated intracellular modulation of the GlyRα1 subunit (16) showed no difference in duration in ethanol-induced LORR with M554, indicating that the inhibitory effect of M554 is mediated by the GlyR and the signal transduction protein Gβγ.

In summary, a novel, small molecule capable of inhibiting intoxicating ethanol effects in vitro and vivo assays by an intracellular mechanism involving Gβγ as a target was identified following a rational design strategy. To the best of our knowledge, this is the first reported molecule that partially reverses the acute effects of alcohol. Our findings provide information that could be helpful in the design of improved molecules with therapeutic use in the treatment of ethanol-related medical problems.

Experimental Procedures

Virtual Screening and Molecular Docking—A library of molecules was retrieved from the ZINC database (5,548,741 molecules) (9). Property prediction was performed with QikProp (Schrödinger) and filtered with Maestro (Schrödinger), aimed at retaining molecules that satisfy Lipinski and Jorgensen rules, with a >1LogBB value prediction. As a result of this procedure, 18,691 molecules were selected. After that, high-throughput virtual screening was performed based on Standard Precision docking (GlideSP) and Extra Precision docking (GlideXP) analysis using the three-dimensional structure of Gβγ taken from the Protein Data Bank (PDB code 1TBG). For the grid receptor generation, the receptor structure of molecular docking was used to generate a 23 × 19 × 23 Å grid containing the Asp-186, Asp-228, and Asp-246 protein residues that have a predicted participation in binding the RQHc7 peptide. A subset of 176 molecules was obtained from this process. Molecular mechanics generalized born solvent accessibility calculation was performed using Prime (Schrödinger) to obtain a preliminary estimation of the binding affinity of the small molecules with the Gβγ site. Estimated binding free energies were used to select an enriched subset of 13 molecules with highly specific binding
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and desired properties for further experimental evaluation (supplemental Fig. 1).

Small Molecules—Compounds identified in the virtual screening and used in our experiments were synthesized by the Research Triangle Institute. M119 was obtained from the NCI, National Institutes of Health. The two-dimensional structure of the molecules used in this study was created using Marvin Sketch (ChemAxon, 2013).

Animals—C57BL/6J mice from 9–14 weeks of age and weighing 20–30 g were used in behavioral studies. In LORR assays, knockin (KI) mice with a mutation that reduced the intracellular signal modulation of the GlyRα1 subunit were also used (16). All mice were housed 1 week prior to experimentation and had ad libitum access to food and water. Mice were maintained on a 12-h light/dark cycle. Care of the animals and the experimental protocols of this study were approved by the Institutional Animal Use Committee of the University of Concepción and conducted according to the ethical protocols established in the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health and the National Committee of Science and Technology (CONICYT).

Cell Culture and Transfection—For glycine-evoked current recording, HEK293 cells were transfected with the human GlyRα1 subunit using an Xfct™ transfection reagent kit (Clontech). Expression of GFP was used as a marker of positively transfected cells, and recordings were made after 18–36 h. For experiments with GIRK channels, HEK293 cells were cultured using standard methodologies and co-transfected with plasmids encoding the GABA_B receptor subunits GABA_B1 (fused to GFP), GABA_B2, GIRK1, and GIRK4 using an Xfect transfection reagent kit (Clontech).

Spinal Cord Neuron Cultures—Spinal cord neurons were obtained from five or six C57BL/6 mouse embryos (embryonic days 13–14) and plated at 250,000 cells/ml onto 18-mm glass coverslips coated with poly-L-lysine (70–150 kDa, Trevigen, Gaithersburg, MD) in plating medium. The neuronal feeding medium consisted of 90% minimal essential medium (Gibco), 5% horse serum (inactivated by heat, Hyclone), 5% fetal bovine serum (Gibco), and a mixture of nutrient supplements. Experiments were performed on 13–17 days of culture in vitro.

Electrophysiology—Whole cell recordings were performed using a holding potential of −60 mV. Patch electrodes were filled with 140 mM KCl, 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 10 mM HEPES (pH 7.4), 4 mM MgCl2, 2 mM ATP, and 0.5 mM GTP with or without small molecules at 200 μM following the concentration used for the RQHc7 peptide (6). The external solution contained 150 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 10 mM HEPES (pH 7.4), and 10 mM glucose. For recording ethanol-mediated potentiation of the glycine current, a methodology described previously was used (6). Briefly, glycine-evoked currents (glycine 15 μM) were registered in the presence and absence of ethanol (100 μM) at minutes 1 and 15 after obtaining the whole cell configuration. The maintenance of the glycine-evoked current was always checked. Ethanol potentiation was expressed as a percentage between the glycine plus ethanol currents (glycine−only current at minute 15. Isolated glycineric miniature inhibitory postsynaptic currents from synaptic activity were recorded in the absence and presence of ethanol and molecules (intracellularly applied) after 13–17 days in vitro in spinal neurons. 6-Cyano-7-nitroquinoxaline-2,3-dione (4 μM), bicuculline (4 μM), and tetrodotoxin (TTX) (100 nM) were added to the external solution to isolate spontaneous glycineric events. For registering GIRK activity, whole cell recordings were performed in HEK cells expressing the GABA_B receptor with a holding potential of −60 mV. Patch electrodes were filled with 120 mM KCl, 10 mM BAPTA (pH 7.4), 4 mM MgCl2, 2 mM ATP, and 0.5 mM GTP with or without small molecules. The external solution contained 150 mM NaCl, 30 mM KCl, 2.0 mM CaCl2, 1.0 mM MgCl2, 10 mM HEPES (pH 7.4), and 10 mM glucose. Baclofen was applied in short pulses (4–5 s) of 10 μM every 2 min for 17 min.

Cytosolic Ca2+ Release in Differentiated HL-60 Cells—HL-60 cells expressing the fMLP receptor coupled to G1 were seeded in 75-mm2 flasks at a density of 0.1 × 10^6 cells/ml in 35 ml of RPMI 1640 medium containing 10% FBS, penicillin, and streptomycin. DMSO was added to 1.2%, and cells were allowed to differentiate to neutrophils at 37 °C (5% CO2) for 72 h. After differentiation, cells were centrifuged and resuspended in HEPES-buffered saline solution to 250,000 cells/well/100 μl. Then cells were loaded with Fluo8 NW for 30 min at 37 °C. M554, M890, or M119 was added to the cells for 5 min at room temperature, and then cells were stimulated with formyl-methionyl-leucyl-phenylalanine (fMLP). Intracellular Ca2+ levels were measured using a NOVOstar microplate reader (BMG Labtech).

Open Field Assay—Male and female mice were tested for basal locomotor activity and ethanol-induced locomotor stimulation in a novel open field using a video tracking system (ANY-maze, Stoelting Co.) in C57BL/6J mice. Mice were injected with saline or ethanol (1 g/kg i.p.) in the presence or absence of small molecules 10 min prior to being placed into the 50 × 50 cm test area. Mice were allowed to freely explore the chamber for 5 min, during which time and distance traveled were recorded.

Accelerating Rotarod—Basal motor skill performance was tested in male C57BL/6J mice using an accelerating rotarod assay. Briefly, mice were placed on a non-rotating rod (IITT, Life Science). A timer was started when the rod began accelerating from 4 to 40 rpm in 120 s, and the latency to fall was recorded. Each mouse was tested five times on day 1, day 2, and before injection and four times after injection (every 30 min) on day 3. The latency to fall was normalized for each animal to the time registered immediately before injection. Injections consisted of saline or ethanol (2 g/kg i.p.) in the presence or absence of small molecules.

Loss of Righting Reflex—The sensitivity to ethanol (3.5 g/kg) was determined by using the standard duration of the LORR assay in male C57BL/6J mice (age 9–13 weeks). Ethanol was diluted in 0.9% saline (20% v/v) and administered i.p. in doses adjusted by injected volumes. Mice were injected with ethanol or ethanol wash M554 (100 mg/kg or 200 mg/kg), and when they 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. LORR was defined as the time from being placed in the supine position until the mice regained their righting reflex. HP-β-cyclodextrin was used as a carrier because of the low water solubility of M554. During all LORR assays, normothermia was maintained with the aid of a heat lamp.

Data Analysis—Results are expressed as the mean ± S.E., and statistical analyses were performed using one-way or two-way analysis of variance followed by Bonferroni or Tukey post hoc tests. \( p < 0.05 \) was considered statistically significant. Origin 6.0 (MicroCal) software was used for all statistical analyses.

Author Contributions—L. S. M. conducted most of the electrophysiological experiments, analyzed the results, and contributed to writing the paper. F. C. and V. J. conducted the in silico analysis, analyzed the results, and contributed to writing the paper. C. J. synthesized the small molecules tested and contributed to writing the paper. G. E. Y. contributed to standardizing the behavioral experiments. T. H., D. N., and J. F. conducted the fluorescence measurements. L. G. A. contributed the electrophysiological and behavioral analysis and advice for the project. L. G. conceived the idea for the project, designed the experiments, and wrote most of the paper.

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References

1. Lovinger, D. M., and Roberto, M. (2013) Synaptic effects induced by alcohol. Curr. Top. Behav. Neurosci. 13, 31–86
2. Legnarde, P. (2001) The glycineergic inhibitory synapse. Cell Mol. Life Sci. 58, 760–793
3. Aguayo, L. G., and Pancetti, F. C. (1994) Ethanol modulation of the γ-aminobutyric acidA and glycine-activated Cl– current in cultured mouse neurons. J. Pharmacol. Exp. Ther. 270, 61–69
4. Aguayo, L. G., Tapia, J. C., and Pancetti, F. C. (1996) Potentiation of the glycine-activated Cl– current by ethanol in cultured mouse spinal neurons. J. Pharmacol. Exp. Ther. 279, 1116–1122
5. Zhu, L., and Ye, J. H. (2005) The role of G proteins in the activity and ethanol modulation of glycine-induced currents in rat neurons freshly isolated from the ventral tegmental area. Brain Res. 1033, 102–108
6. San Martin, L., Cerda, F., Jimenez, V., Fuentelba, J., Muñoz, B., Aguayo, L. G., and Guzman, L. (2012) Inhibition of the ethanol-induced potentiation of α1 glycine receptor by a small peptide that interferes with G\( \beta \gamma \) binding. J. Biol. Chem. 287, 40713–40721
7. Yevenes, G. E., Moraga-Cid, G., Peoples, R. W., Schmalzing, G., and Aguayo, L. G. (2008) A selective G \( \beta \)–\( \gamma \)-linked intracellular mechanism for modulation of a ligand-gated ion channel by ethanol. Proc. Natl. Acad. Sci. U.S.A. 105, 20523–20528
8. Welsh, B. T., Goldstein, B. E., and Mihic, S. J. (2009) Single-channel analysis of ethanol enhancement of glycine receptor function. J. Pharmacol. Exp. Ther. 330, 198–205
9. Irwin, J. J., Sterling, T., Mysinger, M. M., Bolstad, E. S., and Coleman, R. G. (2012) ZINC: a free tool to discover chemistry for biology. J. Chem. Inf. Model. 52, 1757–1768
10. Yevenes, G. E., Peoples, R. W., Tapia, J. C., Parodi, J., Soto, X., Olate, J., and Aguayo, L. G. (2003) Modulation of glycine-activated ion channel function by G-protein \( \beta \gamma \) subunits. Nat. Neurosci. 6, 819–824
11. Guzman, L., Moraga-Cid, G., Avila, A., Figueroa, M., Yevenes, G. E., Fuentelba, J., and Aguayo, L. G. (2009) Blockade of ethanol-induced potentiation of glycine receptors by a peptide that interferes with G\( \beta \gamma \) binding. J. Pharmacol. Exp. Ther. 331, 933–939
12. Yevenes, G. E., Moraga-Cid, G., Guzmán, L., Haeger, S., Oliveira, L., Olate, J., Schmalzing, G., and Aguayo, L. G. (2006) Molecular determinants for G protein \( \beta \gamma \) modulation of ionotropic glycine receptors. J. Biol. Chem. 281, 39390–39307
13. Bonacci, T. M., Mathews, J. L., Yuan, C., Lehmann, D. M., Malik, S., Wu, D., Font, J. L., Bidlack, J. M., and Smrcka, A. V. (2006) Differential targeting of G\( \beta \gamma \)-subunit signaling with small molecules. Science 312, 443–446
14. Davis, T. L., Bonacci, T. M., Sprang, S. R., and Smrcka, A. V. (2005) Structural and molecular characterization of a preferred protein interaction surface on G protein \( \beta \)\( \gamma \) subunits. Biochemistry 44, 10593–10604
15. Blednov, Y. A., Benavidez, J. M., Homanics, G. E., and Harris, R. A. (2012) Behavioral characterization of knockin mice with mutations M287L and Q266I in the glycine receptor α1 subunit. J. Pharmacol. Exp. Ther. 340, 317–329
16. Aguayo, L. G., Castro, P., Mariqueo, T., Muñoz, B., Xiong, W., Zhang, L., Lovinger, D. M., and Homanics, G. E. (2014) Altered sedative effects of ethanol in mice with α1 glycine receptor subunits that are insensitive to G\( \beta \gamma \) modulation. Neuropsychopharmacology 39, 2538–2545
17. Wells, C. A., Betke, K. M., Lindsley, C. W., and Hamm, H. E. (2012) Label-free detection of G protein-SNARE interactions and screening for small molecule modulators. ACS Chem. Neurosci. 3, 69–78
18. Xie, W., Samoriski, G. M., McLaughlin, J. P., Romoser, V. A., Smrcka, A., Hinkle, P. M., Bidlack, J. M., Gross, R. A., Jiang, H., and Wu, D. (1999) Genetic alteration of phospholipase C \( \beta \)3 expression modulates behavioral and cellular responses to \( \mu \) opioids. Proc. Natl. Acad. Sci. U.S.A. 96, 10385–10390
19. Lehmann, D. M., Seneviratne, A. M., and Smrcka, A. V. (2008) Small molecule disruption of G protein \( \beta \gamma \) subunit signaling inhibits neutrophil chemotaxis and inflammation. Mol. Pharmacol. 73, 410–418
20. Mathews, J. L., Smrcka, A. V., and Bidlack, J. M. (2008) A novel G\( \beta \gamma \)-subunit inhibitor selectively modulates \( \mu \)-opioid-dependent antinociception and attenuates acute morphine-induced antinociceptive tolerance and dependence. J. Neurosci. 28, 12183–12189
21. Casey, L. M., Pintner, A. R., Belmonte, S. L., Migdalovich, D., Stolpnik, O., Nwakanna, F. E., Vorobiof, G., Dunaevsky, O., Matafel, A., Lopes, C. M., Smrcka, A. V., and Blaxall, B. C. (2010) Small molecule disruption of G\( \beta \)\( \gamma \) signaling inhibits the progression of heart failure. Circ. Res. 107, 532–539
22. Ford, C. E., Skiba, N. P., Bae, H., Daaka, Y., Reveney, E., Shelker, L. R., Rosal, R., Weng, G., Yang, C. S., Iyengar, R., Miller, R. J., Jan, L. Y., Lekowitz, R. J., and Hamm, H. E. (1998) Molecular basis for interactions of G protein \( \beta \gamma \) subunits with effectors. Science 280, 1271–1274
23. Mariqueo, T. A., Aguerto, A., Muñoz, B., San Martin, L., Coronado, C., Fernández-Pérez, E. J., Murath, P., Sánchez, A., Homanics, G. E., and Aguayo, L. G. (2014) Effects of ethanol on glycineergic synaptic currents in mouse spinal cord neurons. J. Neurophysiol. 111, 1940–1948
24. Findlay, G. S., Wick, M. J., Mascia, M. P., Wallace, D., Miller, G. W., Harris, R. A., and Blednov, Y. A. (2002) Transgenic expression of a mutant glycine receptor decreases alcohol sensitivity of mice. J. Pharmacol. Exp. Ther. 306, 526–534
25. Blednov, Y. A., Benavidez, J. M., Black, M. C., Leiter, C. R., Osterndorff-Kahanek, E., and Harris, R. A. (2015) Glycine receptors containing \( \alpha2 \) or \( \alpha3 \) subunits regulate specific ethanol-mediated behaviors. J. Pharmacol. Exp. Ther. 353, 181–191
26. Williams, K. L., Fenko, A. P., Barbieri, E. J., and DiGregorio, G. J. (1995) Glycine enhances the central depressant properties of ethanol in mice. Pharmacol. Biochem. Behav. 50, 199–205
27. Ye, J. H., Sokol, K. A., and Bhavsar, U. (2009) Glycine receptors contribute to hypnosis induced by ethanol. Alcohol Clin. Exp. Res. 33, 1069–1074
28. McCracken, L. M., Blednov, Y. A., Trudell, J. R., Benavidez, J. M., Betz, H., and Harris, R. A. (2013) Mutation of a zinc-binding residue in the glycine receptor α1 subunit changes ethanol sensitivity in vitro and alcohol consumption in vivo. J. Pharmacol. Exp. Ther. 344, 489–500
29. Harris, R. A., Trudell, J. R., and Mihic, S. J. (2008) Ethanol’s molecular targets. Sci. Signal. 1, re7