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Supplemental Information

Human Hyperekplexic Mutations in Glycine Receptors Disinhibit the Brainstem by Hijacking $GABA_A$ Receptors

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Supplemental Figures

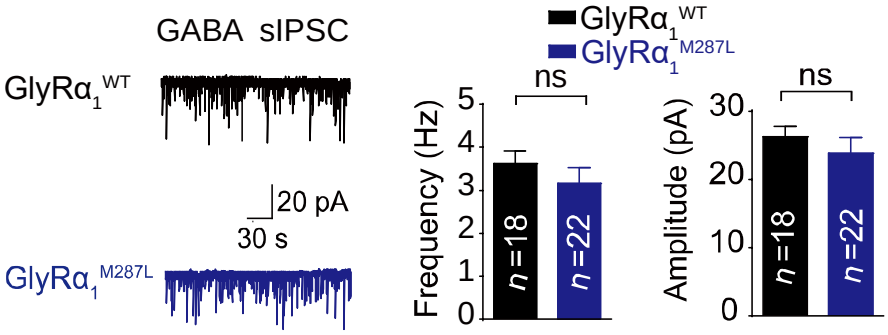

Figure S1. Trace records, average frequency and amplitude of GABAergic sIPSCs in brain-stem hypoglossal nucleus slices from WT and GlyRα$_1^{M287L}$ mutant mice (related to Figure 1). All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. ns, not significant ($P > 0.05$) based on unpaired t-tests.

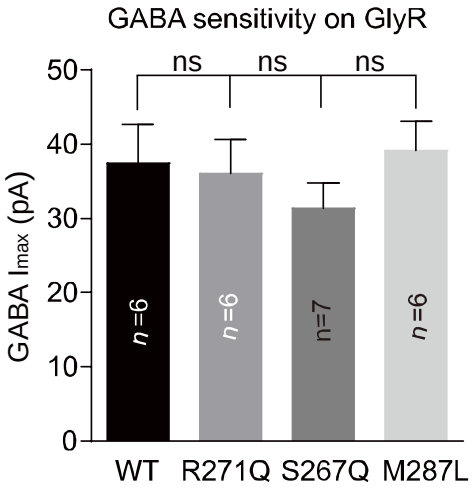

Figure S2. GABA-activated currents on GlyRs (related to Figure 2). Average values of currents activated by 1 mM GABA in HEK-293 cells expressing WT and mutant α_1 GlyRs. All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. ns, not significant ($P > 0.05$) based on unpaired t tests.

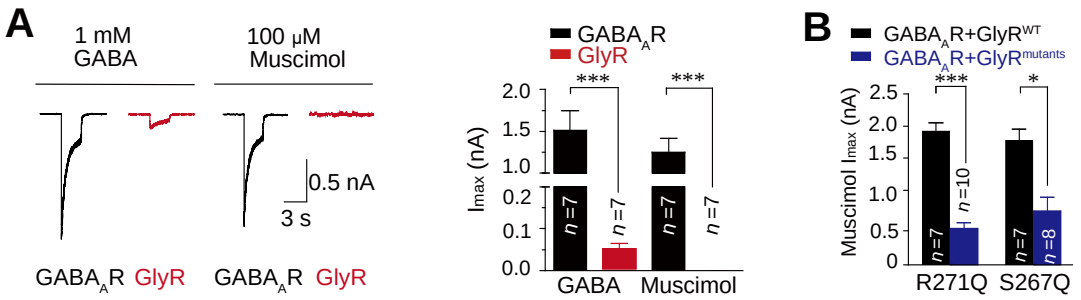

Figure S3. Effects of hyperekplexic mutations of GlyRα$_1$ on the currents activated by muscimol (related to Figure 2). (A) Trace records and average values of GABA and muscimol I_{max} in HEK-293 cells expressing GABA$_A$Rs ($\alpha_1\beta_2\gamma_2$) and GlyRs separately. **(B)** Average values of muscimol I_{max} activated by 100 μM muscimol in HEK-293 cells co-expressing GABA$_A$Rs ($\alpha_1\beta_2\gamma_2$) and mutant α_1 GlyRs. All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. * $P < 0.05$, *** $P < 0.001$ based on unpaired t tests.

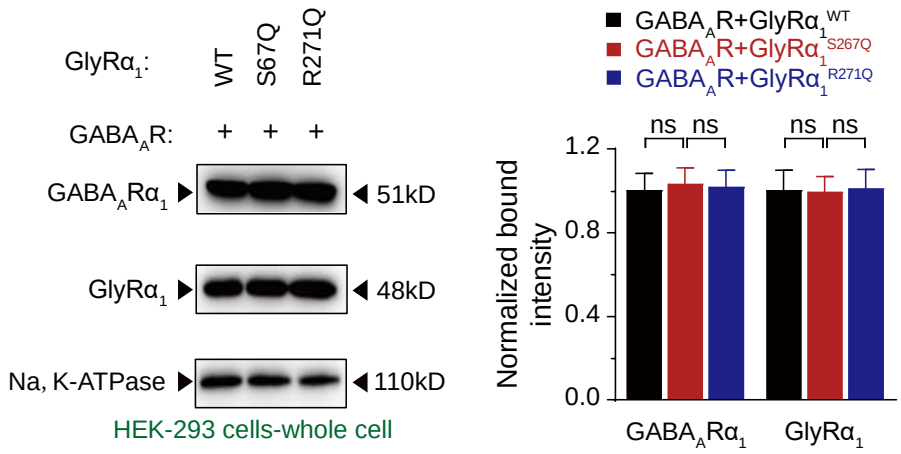

Figure S4. (related to Figure 3). Western blotting results showing protein expression levels of GlyR and $GABA_AR$ in plasma membranes extracted from HEK-293 cells co-transfected with the cDNA of $GlyR\alpha_1^{WT}$, $GlyR\alpha_1^{R271Q}$ and $GlyR\alpha_1^{S267Q}$ with $GABA_ARs$ ($\alpha_1\beta_2\gamma_2$). ns, not significant ($P > 0.05$) based on unpaired t tests.

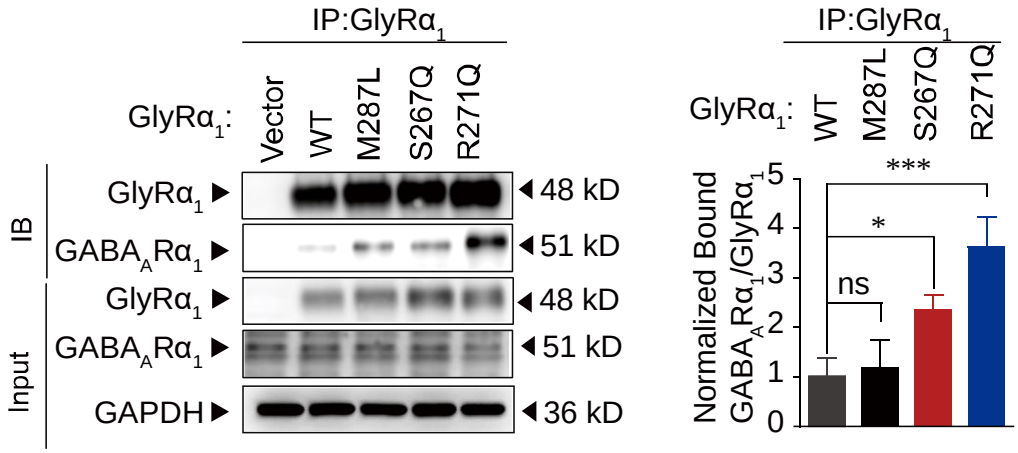

Figure S5. (related to Figure 3). The $GABA_AR\alpha_1$ was purified using $GlyR\alpha_1$ antibodies in HEK-293 cells co-expressing $GABA_ARs$ ($\alpha_1\beta_2\gamma_2$) and WT/mutant $GlyR\alpha_1$, and co-precipitating proteins were detected by immunoblotting. Inputs are immunoblots of the same protein in cell lysates prior to co-IP. Quantification of WT and mutant $GlyR\alpha_1$ binding to $GABA_AR\alpha_1$. All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. * $P < 0.05$, *** $P < 0.001$; ns, not significant ($P > 0.05$) based on unpaired t tests.

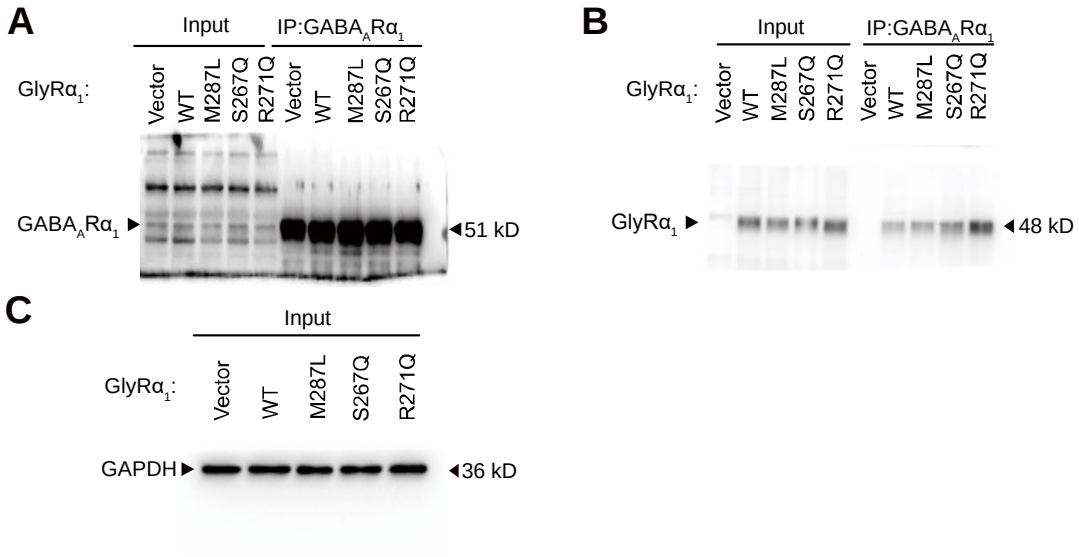

Figure S6. Whole gel images for Figure 3A (A-C) (related to Figure 3).

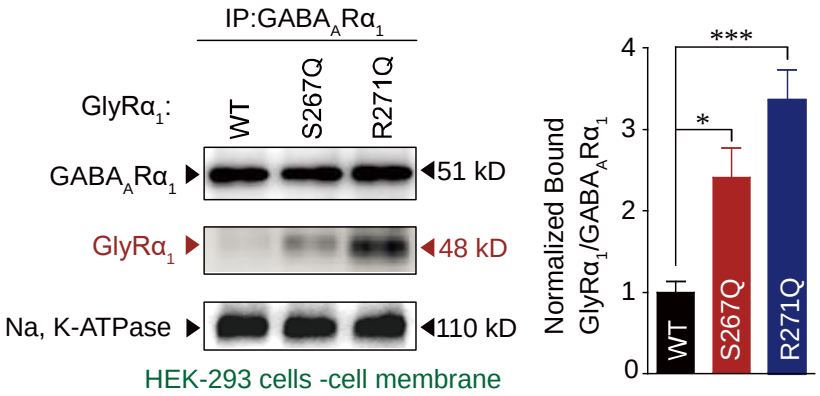

Figure S7. Identification of association between mutant GlyRα$_1$ and GABA$_A$Rα$_1$ in the plasma membrane (related to Figure 3). Plasma membrane GlyR α$_1$ was purified using GABA$_A$R α$_1$ antibodies in HEK-293 cells co-expressing GABA$_A$Rs ($\alpha_1\beta_2\gamma_2$) and WT/mutant α$_1$ GlyRs, and the co-precipitating proteins were detected by immunoblotting. Quantification of WT and mutant GlyR α$_1$ binding to GABA$_A$R α$_1$ (n = 3). The data were normalized to the WT group. Data are represented as mean ± SEM. * $P < 0.05$, *** $P < 0.001$ based on unpaired t tests.

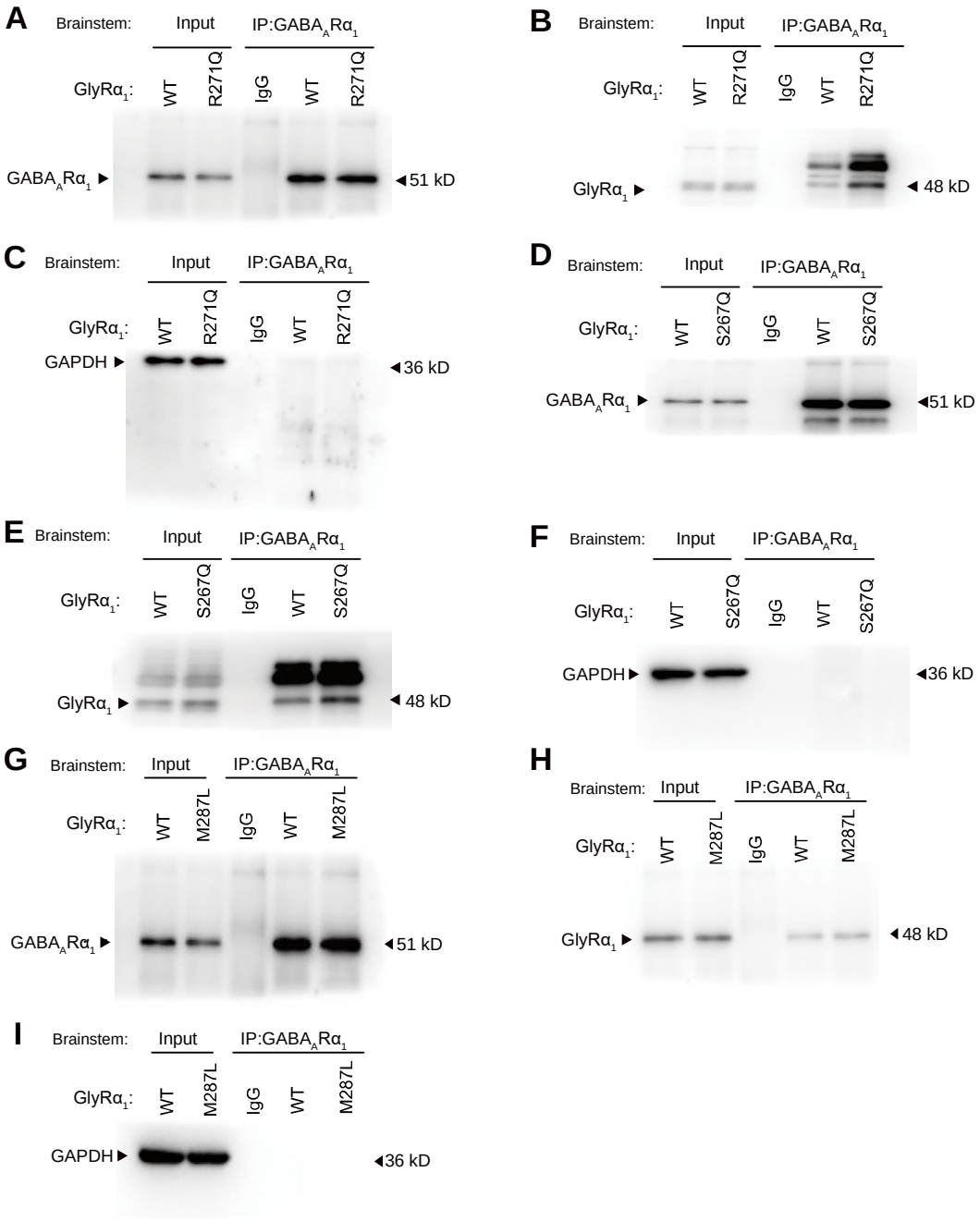

Figure S8. Whole gel images for Figure 3B (A-C); whole gel images for Figure 3C (D-F) and whole gel images for Figure 3D (G-I) (related to Figure 3).

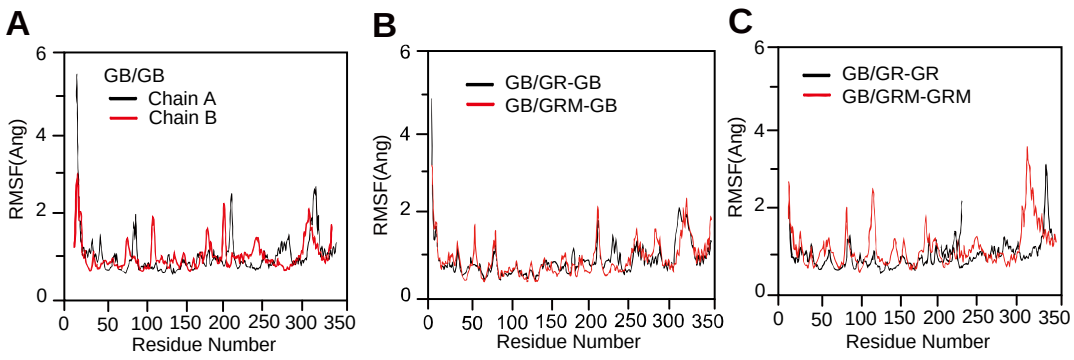

Figure S9. Root mean square fluctuation (RMSF) of three protein systems with respect to the starting structure (related to Figure 4). (A-C) Preparation and Molecular dynamic simulation of three protein bound systems. Protonation status of residues in three systems: Four histidine residues (His107, His 119, His 191 and His267 in chain A) and four histidine residues (His107, His 119, His 191 and His267 in chain B) were protonated at Nε in GB/GB system. Four histidine residues (His107, His 119, His 191 and His267 in chain A) and three histidine residues (His109 , His 201 and His215 in chain B) were protonated at Nε in GB/GR system. Four histidine residues (His107, His 119, His 191 and His267 in chain A) and three histidine residues (His109 , His 201 and His215 in chain B) were protonated at Nε in GB/GRM system. All other residues were configured under the standard protonation states at pH 7.

The optimization of the solvent, equilibration of the whole systems and the molecular dynamic simulation of the equilibrated systems were conducted following the steps listed below: After applying a position restraint of 100 mol−1 Å−2 on all solute atoms, solvent and ions were optimized by three steps: a. energy minimization for 1000 cycles; b. dynamic simulation of 10ps with the temperature increased from 10K to 298K; c. dynamic simulation of 10ps under pressure of 1 bar to equilibrate the density. After applying a restraint weight of 2.0 mol−1 Å−2 on proteins, the whole systems were equilibrated. First, 1000 cycles of energy minimization were applied. Second, the temperature was increased from 10K to 298 K over a period of 5ps dynamic simulation. Third, a dynamic simulation of 200ps under the constant pressure of 1 bar was applied. Finally, the whole system was equilibrated by 100ps dynamic simulation under constant temperature of 298 K and pressure of 1 bar. 1 ns MD production simulations were carried out under the constant temperature and pressure of 298K and 1 bar. Periodic boundary conditions were applied in the NPT ensemble using langevin dynamics. The SHAKE algorithm was applied to fix all bond lengths involving hydrogen atoms. A time step of 2 fs and a direct non-bond interaction cut off radius of 8.0 Å were used with particle-mesh Ewald for long-range electrostatic interactions. Three parallel runs were carried out for each system.

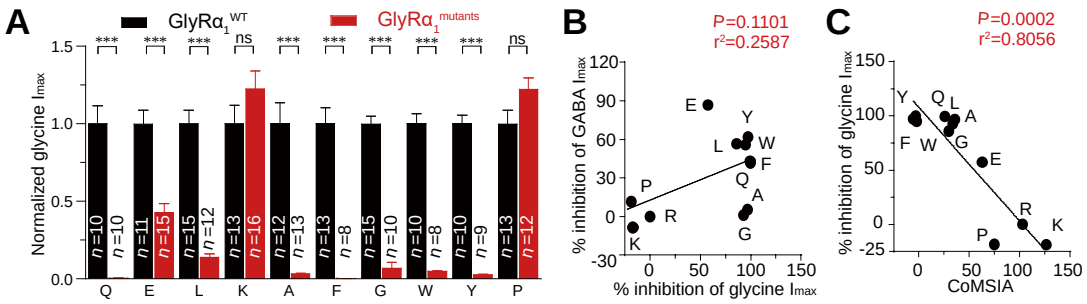

Figure S10. Mutagenesis and correlation analysis of GlyRα$_1^{R271}$ site (related to Figure 4).

(A) The average values of glycine I_{max} activated by 1 mM glycine in HEK-293 cells expressing various R271 site mutant GlyRα$_1$ subunits. All data were normalized to their respective controls (WT group). All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. *** $P < 0.001$; ns, not significant ($P > 0.05$) based on unpaired t tests.

(B) Correlation analysis of R271 mutations-induced percentage inhibition of glycine and GABA I_{max} (linear regression).

(C) Correlation analysis of CoMSIA values of various amino acids at 271 and percentage inhibition of glycine I_{max} (linear regression).

Residues used for 3D-QSAR analysis were generated in SYBYL8.1 software. The structures were minimized and charged with MMFF94 force field. Comparative molecular similarity index analysis (CoMSIA) was conducted to model the correlation between residues structures and inhibition activity. Both electrostatic field and steric field were generated. Final computed CoMSIA value of residues were plotted with inhibition activity.

Figure S11. Whole gel images for Figure 4G (related to Figure 4).

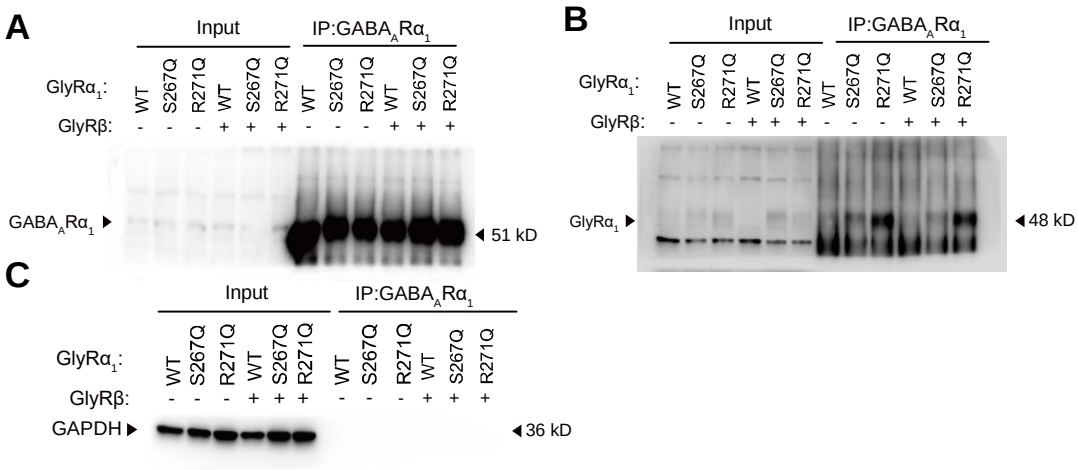

Figure S12. Whole gel images for Figure 5C (related to Figure 5).

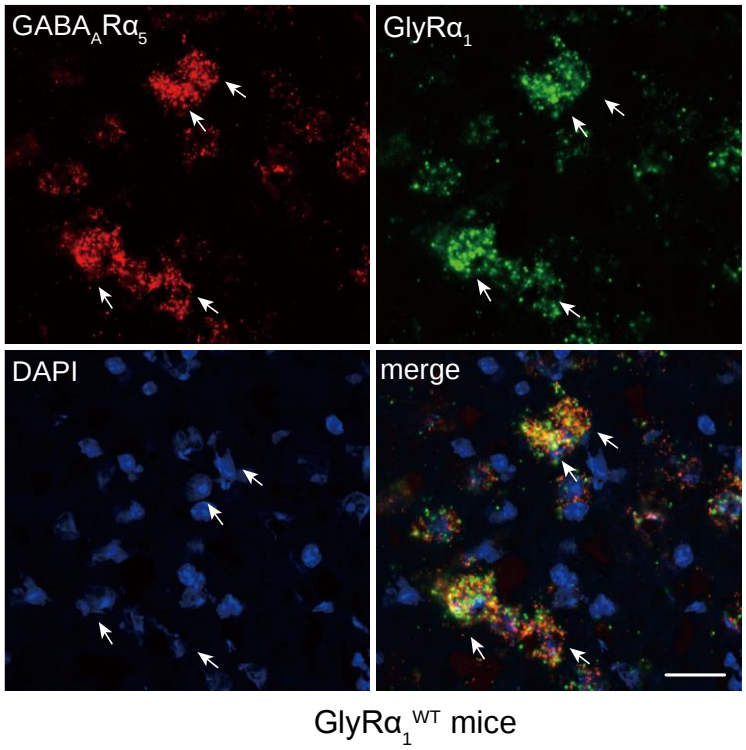

Figure S13. (related to Figure 6). Representative confocal imaging showing colocalization of $GABA_ARα_5$ and $GlyRα_1$ subunits mRNAs in the $GlyRα_1^{WT}$ mouse brainstem hypoglossal nucleus using RNAscope technology. Scale bar, 25µm.

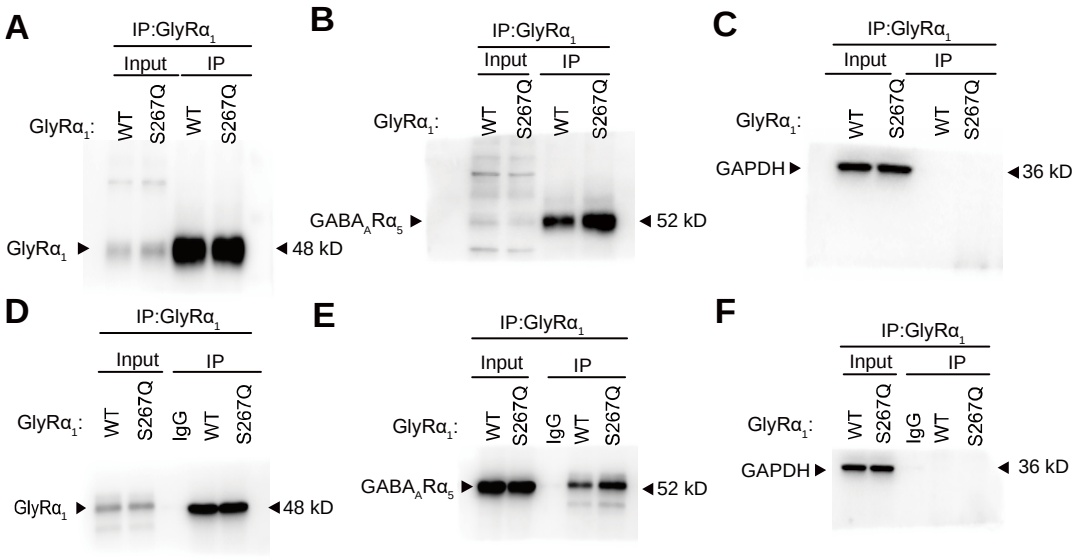

Figure S14. Whole gel images for Figure 6D (A-C) and whole gel images for Figure 6E (D-F) (related to Figure 6).

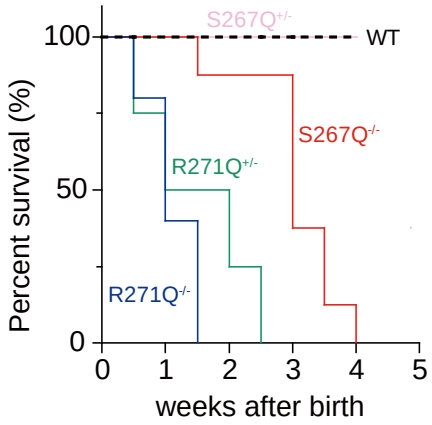

Figure S15. Survival curves of WT, S267Q and R271Q GlyRα$_1$ mutant transgenic mice (related to Figure 7) (WT, n= 6; R271Q$^{+/-}$, n=4; R271Q$^{-/-}$, n=6; S267Q$^{-/-}$, n=8; S267Q$^{+/-}$, n=6). All R271Q$^{+/-}$, R271Q$^{-/-}$ and S267Q$^{-/-}$ mice died within 4 weeks of life.

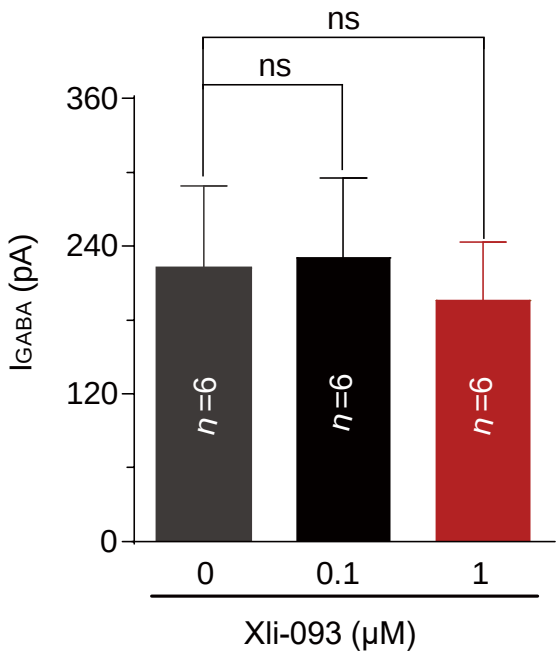

Figure S16. Average values of currents activated by 1 μM GABA in HEK-293 cells with or without pre-incubation of 0.1 or 1 μM Xli-093 in HEK-293 cells expressing $GABA_ARs$ ($\alpha_5\beta_2\gamma_2$) (related to Figure 7). All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. ns, not significant ($P > 0.05$) based on unpaired t tests.

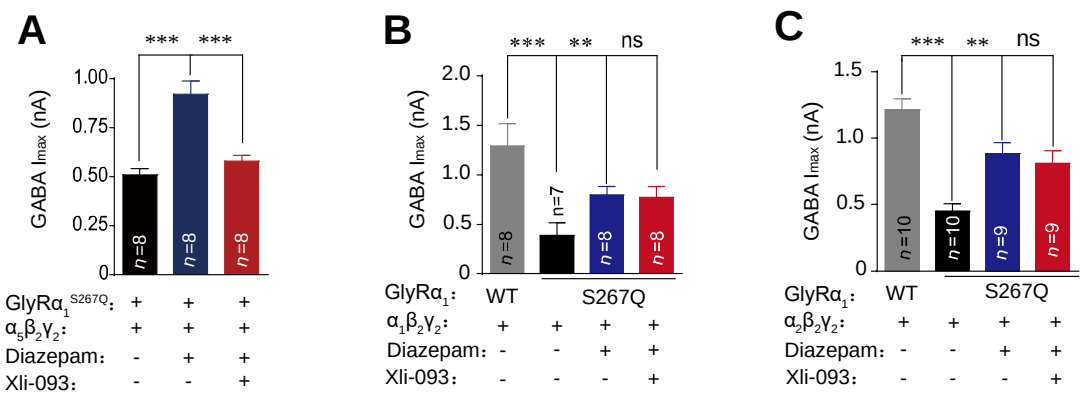

Figure S17. Effects of Xli-093 on diazepam-induced potentiation of $GABA_A$Rs (related to Figure 7). (A-C) Average values of currents activated by 1 mM GABA in HEK-293 cells co-expressing $GlyR\alpha_1^{S267Q}$ and α_5- **(A)**, α_1- **(B)** or α_2- **(C)** containing $GABA_A$Rs with or without pre-incubation of 10 μM diazepam and 1 μM Xli-093. All digits within the columns represent numbers of cells measured. Data are represented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$; ns, not significant ($P > 0.05$) based on unpaired t tests.

Transparent Methods

Animals.

GlyRα_1^{S267Q} and GlyRα_1^{M287L} transgenic mice were from Yuri Blednov and Adron Harris (University of Texas at Austin, Texas) (Findlay et al., 2003; Borghese et al., 2012). GlyRα_1^{R271Q} transgenic mice were from Hans Weiher (University of Applied Sciences Bonn-Rhein-Sieg, Germany) (O'Shea et al., 2004). Hyperekplexic GlyR mutant mice and their wild-type littermates (P12-P21) were used in all recording, western blot and co-immunoprecipitation experiments. Hyperekplexic GlyR mutant mice and their wild-type littermates (7-8 weeks old) were used in startle reflex and RNAscope tests. All mice were housed under a semi-natural dark/light cycle of 12:12 h. All genetically engineered mice studied were homozygous and heterozygous for the mutant α_1 subunit. Genotyping of the α_1 M287L mutant mice was done using the following primers: forward: 5′-GAATCTTCCAGGCAACATTTCAG-3′; reverse: 5′-AGTATCCCACCAAGCC AGTCTTT-3′. Genotyping of the α_1 S267Q mutant mice were done using the following primers: forward: 5′-GCTTTAACTTCTGCCCTATGG-3′; reverse: 5′-GTTGTTGTTAACTTGTTTATTG-3′. Genotyping of the α_1 R271Q mutant mice was done using the following primers: forward: 5′-CTCATCTTTGAGTGGCAGG A-3′; reverse: 5′-GCATCCATGTTGAT CCAGAA-3′. Wild-type littermates and mutant (α_1 M287L, α_1 S267Q and α_1 R271Q) homozygous mice used for the electrophysiological recording were produced from heterozygous breeding pairs. Mice used in this study are all male unless otherwise indicated. All procedures were approved by the Institutional Animal Use and Care Committee of School of Life Sciences, University of Science & Technology of China.

Site-directed mutagenesis.

All point mutations for $\alpha_{1\text{-}3}$ GlyR were introduced using a QuikChange Site-Directed Mutagenesis kit (Takara, Inc.). Sequence of DNA mutants were confirmed through double-stranded DNA sequencing with Genetic Analysis System (Sangon, Inc.).

Electrophysiological recording.

HEK-293 cells (ATCC) were cultured using Dulbecco`s Modified Eagle Media with 10 % fetal bovine serum in 37°C and 5 % CO2. Cells were plated at a density of 10^6 cells/ml in 35-mm dishes and allowed to grow to 70 % confluence before transfection (Hu et al., 2006). Plasmids coding $GABA_A$ R and GlyR were co-transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen) reagents. 2 days after transfection, electrophysiological recordings were carried out. HEK-293 cells were treated with 0.25 % (w/v) Trypsin 2 hours before recording. HEK-293 cells were then lifted and recorded with external solution containing 140 mM NaCl, 5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES (pH 7.4 with NaOH, ~320 mOsm with sucrose). Patch pipettes (3–5 MΩ) were filled with intracellular solution contained 140 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 0.5 mM Na-GTP and 2 mM Mg-ATP (pH 7.2 with CsOH, ~280 mOsm). Membrane currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier (Axon) at 20–25 °C. Cells were held at –60 mV unless otherwise indicated. Data were acquired using pClamp 10.4 software (Molecular Devices, Sunnyvale, CA). Drugs were applied using a Warner fast-step stepper motor–driven system.

Brainstem hypoglossal nucleus slice preparation and recording.

For brainstem slice neuron recording, hyperekplexic GlyR mutant mice and their wild-type littermates (P12-P21) were used. Brainstem slices were prepared as followings: parasagittal brainstem slices (260-µm thick) were prepared from P_{12} to P_{21} mice with Leica Vibratome in ice-cold cutting solution containing (in mM) 30 NaCl, 26 $NaHCO_3$, 10 glucose, 194 sucrose, 4.5 KCl, 1.2 NaH_2PO_4, 1 $MgCl_2$ and continuously bubbled with carbogen (95 % O_2-5 % CO_2). Slices were transferred to a perfusion chamber containing artificial cerebrospinal fluid (ACSF) (in mM): 124 NaCl, 4.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, and 26 $NaHCO_3$, continuously bubbled in carbogen. After 60 min recovery at room temperature, slices were transferred to a recording chamber continuously perfused with ACSF (2-3ml/min). All recordings were performed at 34 °C using glass pipettes filled with internal solution containing 120 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 0.5 mM Na-GTP and 2 mM Mg-ATP (pH 7.2 with CsOH,

~280 mOsm). For sIPSCs recording, 4 mM kynurenic acid and 1 μM strychnine were added in continuously perfused ACSF solution. For mIPSCs recording, 10 μM TTX was additionally added in continuously perfused ACSF. Maximum current of $GABA_ARs$ induced by 1mM GABA was recorded in brainstem slices of $GlyR\alpha_1$ mutant mice and littermate wild type mice. Extra-synaptic current of $GABA_ARs$ was recorded with bicuculline. 80 μM bicuculline,10 μM Diazepam and 1 μM Xli-093 was applied by puff application directly to the recorded neuron using a positive pressure system (4 PSI, 15 ms; Toohey Company, Fairfield, NJ). The input resistance was monitored continuously, and the recording was abandoned if the resistance changed more than 15 %. All brainstem slice recordings were performed under a double-blind condition.

Computational investigation of three protein bound systems.

Three protein bound systems were prepared to investigate the binding affinity between $GABA_AR$ and GlyR. Crystal structures of protein $GABA_AR\ \beta_3$ obtained at a 2.7 Å resolution (Miller et al., 2014) (PDB ID: 4COF) and protein GlyR α_3 obtained at a 2.5 Å resolution (Huang et al., 2017) (PDB ID: 5VDH) were obtained from the RCSB Protein Data Bank (RCSB PDB: www.rcsb.org). Each system contains the following two chains: GB/GB - two chains (A; B) extracted from the crystal structure of $GABA_AR\ \beta_3$ (PDB ID: 4COF); GB/GR - one chain (A) extracted from the $GABA_AR$ and one chain extracted (A) from the GlyR; GB/GRM – same complex as GB/GR, except for Arg271 of GlyR is mutated to Glutamine. The initial binding conformation of the GB/GR complex was obtained using Z-dock software (Pierce et al., 2014).

All crystallographic water molecules and ligands were removed. The protonation states were investigated using the H++ Server (Anandakrishnan et al., 2012) (protonation status is listed in Supporting Information). The protein was charged using an AMBER ff12SB force field. The proteins were solvated in a rectangular box of TIP3P water with a minimum distance between the protein and the box edge of 11 Å. The initial density of the systems was set as 0.9 $g \cdot mL^{-1}$.

The optimization of the solvent, equilibration of the whole systems and the molecular dynamic simulation of the equilibrated systems were conducted in all three systems.

The trajectory was sampled every 1 ps for the analysis using the ptraj and cpptraj programs. The protein structures and snapshots were visualized using VMD (Humphrey et al., 1996). The RMSF values of the protein systems were calculated after aligning to the first structure during the entire 1 ns. Using the MM/GBSA (Molecular Mechanics/Generalized Born Surface Area) method, the binding free energy of two chains was calculated during the entire simulation time. The distances between the residue and atom pairs were obtained using the WORDOM program (Seeber et al., 2007) and mapped using the Gnuplot program (http://www.gnuplot.info/).

Western blotting.

The GlyR and $GABA_AR$ plasmids were transfected into HEK-293 cells using lipo2000 (Invitrogen) reagents. After 48–72 h, whole cell proteins were prepared using buffer containing 1 M Tris-HCL (pH 7.5), 1 % protease inhibitor cocktail (Roche), 1 M NaCl and 5 % sodium deoxycholate. The membrane protein was collected using a Membrane Protein Extraction Kit (89842, Thermo Fisher) according to the manufacturer's instructions. Equal amounts of protein were loaded on 12 % SDS-PAGE gels and transferred to PVDF membranes (NEN, Boston, MA, USA) for 90 min. After the transfer, the membranes were blocked by incubation with TBS containing 0.1 % Tween-20 and 5 % (wt/vol) nonfat milk for 1 h and with primary antibodies against $GABA_AR$ α_1 (1:100, 06-868, Merck), $GABA_AR$ α_5 (1:500, ab10098, Abcam), GlyR α_1 (1:500, NB300-113, Novus), GAPDH (1:5000, 60004-1-AP, Proteintech), and Na, K-ATPase (1:1000, #3010, CST) overnight. After three 5-min washes with TBS plus Tween-20, the membranes were incubated with secondary antibodies against rabbit (1:5000, ab6721, Abcam) or mouse (1:5000, ab6789, Abcam) for 1 h at room temperature. The membranes were washed three times with TBS plus Tween-20 for 5 min, and the protein bands were imaged using ECL reagent (Thermo Fisher Scientific). For western blot analysis of tissue samples, hyperekplexic GlyR mutant mice and their wild-type littermates (P12-P21) were used. The other procedures were consistent with those used for the HEK-293 cells.

Co-Immunoprecipitation.

The cell lysates were collected using methods similar to those used for the Western blotting. To show whether there is any change in protein expression level, 60 μL cell or tissue lysates was extracted and mixed with same volume loading buffer as input before immunoprecipitation. The "input" always performed as a necessary control in all co-immunoprecipitation experiments. IgG-agarose beads were incubated with primary antibodies against $GABA_AR$ α_1 or GlyR α_1 protein overnight at 4°C. The mixture was washed and centrifuged 5 times for 1 min at 12,000 rpm with PBS. The samples were collected, and the centrifugal mixture with the cell lysates was blended and then incubated overnight. After washing and centrifuging the mixtures 5 times for 1 min with cell lysis buffer, 100 μL loading buffer were added, and then the mixture was boiled for 5 min. The samples and inputs were then used for the SDS-PAGE and Western blotting analysis. The primary antibodies were the same as those used in the Western blotting analysis. A mouse anti-rabbit IgG (light-chain specific) (L57A3) mAb reacting with the light chain of rabbit IgG was used to confirm the specific protein band. Normal rabbit IgG (sc-2027, Santa Cruz) was used as a negative control in the immunoprecipitation experiments. For co-immunoprecipitation of tissue samples, hyperekplexic GlyR mutant mice and their wild-type littermates (P12-P21) were used. To completely grind the tissue samples, an automatic lapping machine and ultrasonic homogenizers were used. The other procedures were consistent with those used for the HEK-293 cells.

RNAscope method.

For RNAscope tests, hyperekplexic GlyR mutant mice and their wild-type littermates (7-8 weeks old) were used. Whole brain tissues were removed and frozen on dry ice. The fresh frozen tissue sections (12 μm thick) were mounted on positively charged microscopic glass slides (Thermo Fisher Scientific, Waltham, MA). Both the GlyRα_1 (Glra1) RNA probe (NM_001290821) and $GABA_AR\alpha_5$ subunit (GABra5) probe (NM_176942) were designed and provided by Advanced Cell Diagnostics, Inc. (Hayward, CA). The experimental procedures followed the manufacturer's instructions of RNAscope Fluorescent Multiplex V2 Assay. Stained slides were cover-slipped with fluorescent mounting medium (ProLong Gold Antifade Reagent, P36930, Thermo Fisher

Scientific, Waltham, MA) and scanned using Zeiss LSM880 confocal microscope (Zeiss, USA, San Diego, CA). For each sample, three adjacent sections were stained using the Glra1 and GABra5 RNAscope probes. "GlyRα_1 mRNA–positive neurons and $GABA_AR\alpha_5$ mRNA–positive neurons were counted using ImageJ software (National Institutes of Health, NIH. https://imagej.nih.gov/ij/). The percentage of GlyRα_1 mRNA–positive neurons that co-expressing $GABA_AR\alpha_5$ mRNA were calculated using the following formula: Proportion of $GABA_AR\alpha_5^+$ neurons among GlyRα_1^+ neurons (%) = amount of neurons expressing both $GABA_AR\alpha_5$ and GlyRα_1 / amount of neurons expressing GlyRα_1 alone. The percentage of $GABA_AR\alpha_5$ mRNA–positive neurons that co-expressing GlyRα_1 mRNA were calculated using the following formula: Proportion of GlyRα_1^+ neurons among $GABA_AR\alpha_5^+$ neurons (%) = amount of neurons expressing both $GABA_AR\alpha_5$ and GlyRα_1 / amount of neurons expressing $GABA_AR\alpha_5$ alone."

The synthesis of Xli-093.

Xli-093 were synthesized according to a previous study (Li et al., 2003) as shown in the following steps. A solution of carbonyldiimidazole (90.7 mg, 0.56 mmol) and 8-ethynyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[l,5-a][l,4]-benzod iazepi-ne-3-car-boxylic acid (148.9 mg, 0.53 mmol) in anhydrous DMF (10 mL) was stirred for 3 h at room temperature. After the starting material was converted by TLC (silica gel), to the solution was then added 1,3-propanediol (21.3 mg, 0.28 mmol) and DBU (102.1 mg, 0.67 mmol) in dry DMF (1 mL). The mixture was stirred at room temperature over night until the reaction was complete by TLC (silica gel). The reaction mixture was then poured into water (60 mL) and extracted with DCM (3 × 50 mL). The combined organic layer was washed with water (50 mL), brine and dried with Na_2SO_4. The solu-tion was filtered and the filtrate was condensed. The residue was purified by flash chro-matography (silica gel, EtOAc/ petroleum ether (60-90°C), 1:2) to provide Xli-093 (81.3 mg) as a white solid in 51 % yield. 1H NMR (400 MHz, CDCl3): δ 8.19 (2H, d), 7.90 (2H, s), 7.73 (2H, dd), 7.41 (2H, d), 5.29-5.15 (2H, br), 4.56 (4H, t), 4.37 (2H, br), 3.26 (6H, s), 3.24 (2H, s), 2.42-2.27 (2H, m).

Drugs.

Most chemicals including GABA and glycine were achieved from Sigma-Aldrich. All solutions were prepared the day before experiment with ultrapure water. Agonist, modulator and antagonist were diluted before experiment with external solution or ACSF. Diazepam and Xli-093 was dissolved in ethanol before further dilution by external solution. Diazepam was sourced from Sigma-Aldrich. All the final concentration of ethanol in working solution was less than 8 mM, which had no potential effect on I_{Gly} and I_{GABA}. All the vehicles used in experiments had no latency responses when used alone.

Startle reflex test.

The mice were placed in Med Associates Startle Reflex System (Med Associates Inc.) chambers and allowed to habituate for 5 min. Then, the mice were tested to measure their level of startle using a series of pseudorandom white noise startle stimuli (10 presentations of each sound intensity, 85 dB, 90 dB, and 95 dB) with a 58-63 s inter-trial interval (ITI). Male heterozygous S267Q transgenic mice and their wild-type littermates (7–8 weeks old) were used in the startle test. The mice were injected with diazepam (10 mg/kg, i.p.) and Xli-093 (5 μg/2 μL, intra-brainstem hypoglossal nucleus.) before being placed in the startle device.

Statistical analysis.

In our study, no statistical methods were used to predetermine sample sizes, all experiments and data analysis were conducted in a blinded way. For behavioral experiments, animals from different genotypes were picked randomly for testing. For electrophysiological experiments, brainstem hypoglossal neurons or transfected HEK-293 cells were randomly picked for patch-clamp recordings. Statistical analysis of the concentration-response data is performed with the use of a nonlinear curve-fitting program. Data were fit using the Hill equation, I/I_{max} = bottom + (top − bottom)/(1 + 10^(logEC_{50} − log[agonist]) × Hill slope), where I is the current amplitude activated by a given concentration of agonist ([agonist]), *Imax* is the maximum response of the cell, and EC_{50} is the concentration eliciting a half-maximal response. Correlation analysis were performed with linear regression. Data were statistically compared by unpaired t test using GraphPad Prism 6.0 (GraphPad Software), as indicated in the specific figure legends. Average

values are expressed as the mean ± SEM and mean ± SD. $P < 0.05$ was considered significant. The data distribution was assumed to be normal, but this was not formally tested.

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