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Title: An isoflurane- and alcohol-insensitive mutant $GABA_A$ receptor α_1 subunit with near normal apparent affinity for GABA: characterization in heterologous systems and production of knock-in mice.

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Running title: Isoflurane- and alcohol-insensitive $GABA_A$ receptor

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Abbreviations: CRC, concentration-response curve; EC, effective concentration; $GABA_AR$, γ-aminobutyric acid type A receptor; HA/HA, homozygous knock-in mice; HEK293, human embryonic kidney 293; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; mIPSC, miniature inhibitory postsynaptic current; SL/HA, heterozygous knock-in mice; SL/SL, control wild-type mice.

Abstract

Volatile anesthetics and alcohols enhance transmission mediated by γ-aminobutyric acid type A receptors ($GABA_ARs$) in the central nervous system, an effect that may underlie some of the behavioral actions of these agents. Substituting a critical serine residue within the $GABA_AR$ α_1 subunit at position 270 with the larger residue histidine, eliminated receptor modulation by isoflurane, but it also affected receptor gating (increased GABA sensitivity). To correct the shift in GABA sensitivity of this mutant, we mutated a second residue, leucine at position 277 to alanine. The double mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_AR$ was expressed in *Xenopus* oocytes and human embryonic kidney 293 (HEK293) cells, and it had near normal GABA sensitivity. However, rapid application of a brief GABA pulse to receptors expressed in HEK293 cells revealed that the deactivation was faster in double mutant than in wild-type receptors. In all heterologous systems, the enhancing effect of isoflurane and ethanol was greatly decreased in the double mutant receptor. Homozygous knock-in mice harboring the double mutation were viable and presented no overt abnormality, except hyperactivity. This knock-in mouse line should be useful in determining which behavioral actions of volatile anesthetics and ethanol are mediated by the $GABA_ARs$ containing the α_1 subunit.

Introduction

The γ-aminobutyric acid type A receptors ($GABA_A$Rs) are ligand-gated ion channels important for fast inhibitory synaptic transmission in the central nervous system. Clinically relevant concentrations of volatile anesthetics and alcohols potentiate $GABA_A$R function.

Using chimeric constructs, an alcohol and volatile anesthetic binding site was identified in $\alpha_2\beta_1$ $GABA_A$Rs (Mihic et al., 1997). Additional experiments showed that mutations in α subunits at serine (S) 270 decreased alcohol potentiation (Ueno et al., 1999; Findlay et al., 2000). Mutation of S270 to cysteine allowed irreversible labeling of mutant receptors with alcohol analogs (sulfhydryl-specific reagents) that blocked subsequent application of alcohols and volatile anesthetics (Mascia et al., 2000). Volatile anesthetics act in a common domain within the transmembrane region of $GABA_A$R α subunits, and their action depends on their molecular size (Jenkins et al., 2001; Kash et al., 2003).

One α subunit mutation, α_1 S270 to histidine (H) has been extensively tested using volatile anesthetics. Mutant $GABA_A$Rs [α_1(S270H)$\beta_2\gamma_{2S}$] expressed in human embryonic kidney 293 (HEK293) cells showed no potentiation by isoflurane, along with a significant increase of GABA sensitivity (Nishikawa et al., 2002). When expressed in *Xenopus laevis* oocytes, α_1(S270H)$\beta_2\gamma_{2S}$ $GABA_A$Rs provided a reduced isoflurane potentiation and a significant increase of GABA sensitivity (Hall et al., 2004). Knock-in mice were produced bearing the α_1(S270H) mutation; however, the mice presented several synaptic and behavioral abnormalities that limited their usefulness in studies of volatile anesthetic effects, probably as a consequence of the increased sensitivity to

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GABA (Homanics et al., 2005; Elsen et al., 2006). The α_1(S270H) knock-in mice presented an interesting feature, however: their miniature inhibitory postsynaptic currents in hippocampal slices showed a decreased sensitivity to isoflurane, but halothane produced the same effect as in wild-type mice (Elsen et al., 2006).

The present study aimed to create a knock-in mouse line expressing mutant α1 $GABA_AR$s that lack volatile anesthetic and alcohol sensitivity but present near normal response to GABA. Such a genetically engineered mouse would enable a definitive study of the relevance of α1-containing $GABA_AR$s to the cellular and behavioral effects of volatile anesthetics and alcohols. As mentioned above, replacement of S270 with H, eliminated potentiation by isoflurane, but the GABA concentration-response curve (CRC) shifted to the left. To correct for the enhanced sensitivity to GABA, a second mutation was needed. A variety of mutations [e.g., leucine (L) at position 277 to alanine (A)] in the trasmembrane 2-3 linker of the α subunit inhibit gating of the $GABA_AR$, increasing GABA EC_{50} and reducing the maximal GABA response (O'Shea and Harrison, 2000; Topf et al., 2003). Therefore, we constructed a double mutant that incorporated both mutations S270H and L277A in the same $\alpha_1$subunit [α_1(S270H;L277A)], and compared them to wild-type receptors. We co-expressed these α_1 subunits along with β and γ subunits in heterologous systems (HEK293 cells and *Xenopus* oocytes). We subsequently studied the pharmacology of the volatile anesthetics isoflurane and halothane at these receptors, along with other modulators of the $GABA_AR$ including alcohols. Drugs like etomidate and pentobarbital were included in the present experiments because they were also to be included in subsequent behavioral and electrophysiological studies of the knock-in mice, in order to determine if

the double mutation introduced in the α_1 subunit also produced any changes in the sensitivity to these drugs (Werner et al., in press). Based on these findings, we created a knock-in mouse line bearing the double mutation, and performed biochemical and behavioral analyses to assess the phenotype of these mice.

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Materials and Methods

Site-Directed Mutagenesis

To create the mutant α_1 subunit, we introduced two single point mutations into the cDNA encoding the human $GABA_AR$ α_1 subunit (Koltchine et al., 1996). Mutations were performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with commercially produced primers of 24-30 bases in length (Operon Technologies, Alameda, CA). Mutant cDNAs were confirmed by automated fluorescent DNA sequencing (Cornell DNA Sequencing Service, Ithaca, NY). All restriction enzymes were obtained from New England Biolabs (Beverley, MA).

Electrophysiology in HEK293 cells

Cell Culture and Transfection

Wild-type or mutant human α_1 subunit cDNAs were co-expressed with the rat $GABA_A$ β_2 and human γ_{2S} subunits via the plasmid vector pCIS2 in HEK293 cells, as previously described (Koltchine et al., 1999). HEK293 cells (American Type Tissue Culture Collection, Manassas, VA) were cultured on poly-D-lysine-treated coverslips (Sigma, St. Louis, MO) in Eagle's minimum essential medium (Sigma) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), L-glutamine (0.292 µg/ml, Life Technologies Inc.), penicillin G sodium (100 units/ml, Life Technologies Inc.) and streptomycin sulfate (100 µg/ml, Life Technologies Inc., Grand Island, NY). Cells were transfected using the calcium phosphate precipitation technique (Chen and Okayama, 1987) to achieve transient expression. Each coverslip of cells was transfected with

approximately 6 µg of total DNA. The transfected cells were cultured for 24 h in an atmosphere containing 3% CO_2 before being removed and replaced with fresh culture medium in an atmosphere of 5% CO_2.

Electrophysiological Recording In HEK293 Cells using Standard Application Methods

Coverslips with the transfected cells were transferred after 48-72 hrs to a bath that was continuously perfused with extracellular saline. The extracellular saline contained 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose and 10 mM HEPES, pH 7.4, at an osmolarity of 320-330 mOsm. Recordings were performed at room temperature using the whole cell patch clamp technique as described previously (Krasowski et al., 1997). The patch pipette solution contained 147 mM N-methyl-D-glucamine hydrochloride, 5 mM CsCl, 5 mM K_2ATP, 5 mM HEPES, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 1.1 mM EGTA, pH 7.2 at an osmolarity of 315 mOsm. The chloride equilibrium potential was therefore approximately 0 mV. Pipette-to-bath resistance was typically 5-10 MΩ. Cells were voltage-clamped at –60 mV. All drugs were dissolved in extracellular medium and rapidly applied to the cell by local perfusion with laminar flow using a multi-channel infusion pump (Stoelting; Wood Dale, IL) (Koltchine et al., 1996). The loss of isoflurane/halothane using this perfusion device has been measured using gas chromatography and represents only 5-10% of the total applied drug concentration (1998, Matthew D. Krasowski, M.D., University of Chicago, Chicago, IL, unpublished observations). The solution changer was driven by protocols in the acquisition program pCLAMP5 (Axon Instruments, Foster City, CA). Throughout the experiment, each cell was periodically challenged with a submaximal concentration of agonist to ensure that no cumulative desensitization or rundown of the $GABA_AR$

currents had occurred. Responses were digitized (TL1-125 interface; Axon Instruments) using pCLAMP5 (Axon Instruments).

GABA was dissolved directly into extracellular solution or stored overnight at 4°C in sealed Nalgene tubes (Nalge/Nunc, Rochester, NY). All reagents were purchased from Sigma, except for isoflurane (Abbott Laboratories, North Chicago, IL), and halothane (Halocarbon Laboratories, River Edge, NJ).

Electrophysiological Recording In HEK293 Cells Using Rapid Application Methods

The procedure was essentially as described previously (Li and Pearce, 2000). Briefly, human α_1 wild-type (α1) or double mutant [α1(S270H;L277A)], rat β_2 and human γ_{2S} $GABA_A$ subunits were transiently expressed in HEK293 cells (ratio 1:1:10) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Electrophysiological recordings were performed with rapid applications, using a two-barrel “theta” glass application pipette connected to a piezoelectric stacked translator (~2.5 ms solution exchange). GABA CRCs were determined using the Cellectricon™ Dynaflow df-16 chip (~25 ms whole cell 10-90% solution exchange, Dai and Pearce, unpublished observations).

Data Analysis

Concentration-response data were fitted (Kaleidograph, Reading, PA, or OriginPro 7.5, Microcal Software, Northampton, MA) using the following equation 1 (Hill equation):

$$I / I_{MAX} = \frac{[drug]^{n_H}}{[drug]^{n_H} + (EC_{50})^{n_H}} \qquad \text{(eq. 1)}$$

where I/I_{MAX} is the fraction of the maximally obtained GABA response, EC_{50} (effective concentration 50) is the concentration of agonist producing a half-maximal response, *[drug]* is drug concentration and n_H is the Hill coefficient. Agonist responses in each cell were normalized to the maximal current that could be elicited by GABA. Percent potentiation was then calculated as the percentage change from the control (EC_{20}) response to GABA in the presence of anesthetic. Pooled data were represented as mean ± standard error of the mean (S.E.M.). Statistical significance was determined at the $p< 0.05$ level by a two-tailed unpaired Student's *t*-test assuming unequal variance.

Electrophysiology in *Xenopus* Oocytes

Materials

Adult female *Xenopus laevis* frogs were obtained from Xenopus Express (Plant City, FL). GABA, ethanol, zinc chloride, flunitrazepam, and sodium pentobarbital were purchased from Sigma, etomidate was purchased from Tocris (Ellisville, MO), and isoflurane was purchased from Marsam Pharmaceuticals (Cherry Hill, NJ). All other reagents were of reagent grade. GABA, zinc chloride and pentobarbital sodium stocks were prepared in water; flunitrazepam and etomidate were dissolved in DMSO. The drug stocks were then dissolved in buffer; the final DMSO concentration was 0.1% V/V, which does not affect $GABA_A$-mediated current. Isoflurane solutions were prepared in buffer immediately before application.

Isolation and injection of oocytes

Xenopus laevis oocytes were manually isolated from a surgically removed portion of ovary. Oocytes were treated with collagenase (Type IA, 0.5 mg/ml) for 10 min,

and then placed in sterile Modified Barth's Solution (composition: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, adjusted to pH 7.5), supplemented with 10,000 units penicillin, 50 mg gentamicin, 90 mg theophylline and 220 mg sodium pyruvate per liter (Incubation medium). Oocytes were then injected into the nucleus with 30 nl of a solution containing cDNA encoding $GABA_A$ subunits (α:β:γ 0.5:0.5:1 in ng/oocyte). The cDNAs were human α_1 (wild-type and mutated), rat β_2 and human γ_{2S}, in vector pCIS2, and human β_3 in vector pcDNA1AMP (provided by Dr. P.J. Whiting). The injected oocytes were kept at 13°C in Incubation medium.

Electrophysiological recordings

Recordings were carried out 1-5 days after injection. The oocytes were placed in a rectangular chamber (approximately 100 µl) and continuously perfused with ND96 buffer (2 ml/min) at room temperature (24°C). The perfusion buffer composition was: 96 mM NaCl, 1 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). The whole-cell voltage clamp at –70 mV was achieved through two glass electrodes (1.5-10 MΩ) filled with 3 M KCl, using a Warner Instruments (Hamden, CT) oocyte clamp, model OC-725C.

All drugs were applied by bath-perfusion. All solutions were prepared the day of the experiment. The preapplication of volatile anesthetics and alcohol provided more reliable responses in previous studies, and was also used here.

The CRCs were obtained with increasing concentrations of GABA, applied for 20-30 s at intervals ranging from 5 to 15 min. From these CRCs, the concentration evoking a half-maximal response (EC_{50}) was calculated, along with the Hill coefficient

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(see the *Statistical analysis* section). To study the isoflurane (0.3 mM), ethanol (10-200 mM), butanol (10.8 mM), Zn^{++} (10 μM), flunitrazepam (1 μM), etomidate (1 μM), and pentobarbital (50 μM) modulation of GABA currents, the GABA concentration equivalent to EC_5 was determined after 1-3 mM GABA produced a maximal current. A washout of 5 min was observed in between all GABA applications, except after 1-3 mM GABA (15 min). After two applications of EC_5 GABA, each of the modulators was preapplied for 1 min and then coapplied with GABA for 30 s. EC_5 GABA was re-applied after coapplication of GABA and modulator. To observe the direct effect of etomidate on $GABA_A$Rs, 10 μM etomidate was applied for 1 min. All experiments shown include data obtained from oocytes taken from at least two different frogs. All oocytes that presented a maximal current > 20 μA were discarded.

Data analysis

Nonlinear regression analysis was performed with Prism (GraphPad Software Inc., San Diego, CA). CRCs were fitted to the equation 2:

$$I/I_{MAX} = \frac{1}{1+10^{(\log EC_{50} - \log[GABA])\times n_H}} \qquad \text{(eq. 2)}$$

where I/I_{MAX} is the fraction of the maximally obtained GABA response, EC_{50} is the concentration of agonist producing a half-maximal response, *[GABA]* is GABA concentration and n_H is the Hill coefficient. Agonist responses in each cell were normalized to the maximal current that could be elicited by GABA. Percent potentiation was then calculated as the percentage change from the control response to EC_5 GABA in the presence of anesthetic. Pooled data were represented as mean ± S.E.M. Statistical significance was determined using Student's *t*-test or ANOVA, as indicated.

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Knock-in Mouse Production

A targeting construct to modify the α_1 locus was created from a vector that was previously used to create gene targeted mice that harbored an α1 $GABA_A$-R S270 to H knock-in mutation (Homanics et al., 2005). Briefly, QuickChange XL Site Directed Mutagenesis (Stratagene) was used to replace the L277 codon (CTC) with an A codon (GCT) in a plasmid that already harbored the S270 to H substitution, and a silent 1 bp substitution that introduced a novel EcoR I site (see Fig. 6.A). Note that exon 9 in the present publication is identical to exon 8 in our previous publication . The recent identification of an untranslated exon at the 5' end of the α_1 gene resulted in the renumbering of exons. The gene targeting construct was completely linearized with Pvu I before introduction into mouse embryonic stem cells. The linearized construct was electroporated into Strain 129SvJ Go Germline embryonic stem cells (Genome Systems, Inc., St. Louis, MO) under previously described conditions (Homanics et al., 1997; Homanics, 2002). G418 (265 µg/ml; Invitrogen) resistant embryonic stem cell clones were screened for gene targeting by Southern blot analysis of EcoR I digested DNA and hybridization with a 3' external probe (Fig. 6.B) as previously described (Homanics et al., 2005). Targeted clones were also analyzed with additional enzymes and probes, and all results were consistent with correct targeting at the α_1 locus. The results presented here are from clone 107A5.

Correctly targeted embryonic stem cell clones were microinjected into C57BL/6J blastocysts to produce chimeric mice. Male chimeras were mated to C57BL/6J females to produce the F1 generation. Mice heterozygous for the neo-containing targeted locus were mated to FLPe deleter mice (Rodriguez et al., 2000) obtained from the Jackson

Laboratory (stock# 3800) to remove the neo cassette by FLPe mediated site specific recombination. The FLPe transgene was subsequently bred out. Mice heterozygous for the knock-in were interbred to produce controls (SL/SL), heterozygous (SL/HA), and homozygous (HA/HA) knock-ins. All mice were genotyped by Southern blot analysis of tail DNA as described (Homanics et al., 2005).

All mice were of a mixed C57BL/6J x Strain 129SvJ background of the F3-F6 generations. All animals were maintained under specific pathogen free conditions in a photoperiod-controlled environment (lights on at 7 am and off at 7 pm) with *ad lib* access to standard rodent chow and water. All experiments were approved by the institutional animal care and use committees and were conducted in accordance with NIH guidelines on the use of animals in research.

Whole brain RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and α_1 mRNA was amplified by RT-PCR as described (Homanics et al., 2005). Purified RT-PCR products from control and knock-in brain were sequenced and compared.

Quantitative Immunoblot Analysis

Cerebral cortices and cerebella of adult mice were rapidly dissected over ice, flash frozen on dry ice, and stored at -80^{o}C. P2 membrane fractions from cortex were processed and analyzed as pooled samples (3 pools per genotype, 8 mice per pool), whereas cerebellar samples were analyzed individually (n=8 per genotype). Aliquots of 25 µg of protein from each pooled sample were separated by electrophoresis on precast SDS-10% polyacrylamide gels (BioRad, Hercules, CA) and subsequently transferred to polyvinylidine difluoride membranes (BioRad) for detection by subunit specific

antibodies. $GABA_AR$ anti-α_1, -α_2, and -α_3 antibodies (Fritschy and Mohler, 1995) were generously donated by Dr. Jean-Marc Fritschy (University of Zurich, Zurich Switzerland). Anti-β_2 (NB 300-198), -β_3 (NB 300-119) and -γ_2 (NB 300-151) antibodies were obtained commercially (Novus Biologicals, Littleton, CO). Primary antibodies were detected with either HRP-conjugated goat anti-rabbit (α_1, β_2, β_3, γ_2, actin) or rabbit anti-guinea pig (α_2, α_3) (both from Abcam Inc., Cambridge, MA). IgG polyclonal antibodies and visualized by enhanced chemiluminescence (Western Lightning; Perkin Elmer, Boston, MA). To ensure equal loading, blots were stripped using Re-blot (Chemicon; Temecula, CA) and reprobed with an anti-β-actin polyclonal antibody (ab8227-50; AbCam) for normalization. Multiple exposures of each membrane were used to ensure that the measured signal was within the linear range of the film. Band intensity was measured densitometrically (Kodak 1-D software, v.3.6, Rochester, NY). Each sample was analyzed on 3-4 different blots. Data were analyzed by Student's *t*-test.

^{3}H-Flunitrazepam Binding

Cortical tissue was harvested from male and female knock-in and wild-type mice, and $GABA_A$-R binding was performed using ^{3}H-flunitrazepam (Perkin Elmer Life Sciences, Boston, MA). Data from male and female mice were combined for statistical analyses. Tissue was homogenized in 25 ml ice-cold assay buffer (50 mM Tris, 25 mM HEPES; pH 7.4) and centrifuged twice at 47,500 x G for 10 min (4°C). Final pellets were re-suspended in ice-cold assay buffer. Binding was initiated by adding 200 µl aliquots of cortical tissue (100-200 µg protein) to a reaction mixture containing 200 µl ice-cold assay buffer, 50 µl ^{3}H-flunitrazepam (84.5 Ci/mmol; 1, 3, 10, 30, or 100 nM), and 50 µl

additional ice-cold assay buffer (non-specific binding) or 100 µM diazepam (total binding). The reaction mixture incubated for 60 min at 4°C and was quenched with 2 ml ice-cold assay buffer, and was then rapidly filtrated through a GB100R filter (Advantec MFS, Dublin, CA) and washed with ice-cold assay buffer. Filters were incubated overnight in 4 ml Biosafe II scintillation liquid (Research Products International, Mount Prospect, IL) before analysis in a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Specific binding was calculated by subtracting non-specific binding from total binding. K_D and B_{max} values were calculated using Prism 3.0 (GraphPad Software, San Diego, CA).

Motor activity

Spontaneous locomotor activity was measured in standard mouse cages by an Opto-microvarimex (Columbus Instruments, Columbus, OH, USA). Activity was monitored by 6 infrared light beams placed along the width of the cage at 2.5 cm intervals, 1.5 cm above the floor. Each cage contained bedding and food, and was covered by a heavy flat plastic lid equipped with ventilation holes and a bottle of water. The apparatus allowed the activity of 16 individual mice (8 of each genotype) to be monitored simultaneously. Each experimental cage contained one mouse. Motor response to novelty was monitored during a 3-hour session that began immediately after placing the mouse into the cage. Data was analyzed in 10-min time bins. For long term monitoring of locomotion, each mouse was habituated for 3 hours. After adaptation, activity levels were monitored for 24 hours, with data analyzed in 15-min bins.

Results

Pharmacology of Recombinant Receptors

Studies in HEK293 cells using standard application methods

$GABA_A$Rs harboring α_1 subunit mutations of S270 to H and L277 to A [α_1(S270H;L277A)$\beta_2\gamma_{2S}$], or only the S270 to H [α_1(S270H)$\beta_2\gamma_{2S}$] or only the L277 to A [α_1(L277A)$\beta_2\gamma_{2S}$] were studied by whole-cell patch clamp, after transient expression in HEK293 cells. Brief applications of agonist produced concentration-dependent inward currents (Fig. 1.A, B and C). The maximal amplitudes and Hill coefficients were similar in wild-type and mutant receptors (Table 1).

The currents activated by at least seven concentrations of GABA were expressed as a fraction of the maximal GABA response, and these normalized data were pooled and fitted by a Hill equation (see Methods). Inspection of the data (Fig. 1.E) revealed that the EC_{50} for the α_1(S270H)$\beta_2\gamma_{2S}$ mutant was decreased 5-fold compared to wild-type, expressed as a significant left shift in the CRC. In contrast, the α_1(L277A)$\beta_2\gamma_{2S}$ mutant increased the EC_{50} 17-fold, indicating that the receptor was significantly less sensitive to GABA than the wild-type receptor (Table 1).

As we hypothesized, introduction of both mutations into the same α_1 subunit neutralized the opposing effects of both single mutants on the CRC. Figures 1.A and D show typical current traces of the wild-type and the α_1(S270H;L277A)$\beta_2\gamma_{2S}$ mutant. Maximally elicited currents and Hill coefficients were similar (Table 1). Although the single mutations change the EC_{50} significantly, the receptor including both mutations

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yielded an EC_{50} that did not differ significantly from the wild-type $GABA_AR$ (Fig. 1.F and Table 1).

To examine the effect of volatile anesthetics on the wild-type and the mutant receptor, HEK293 cells expressing $\alpha_1\beta_2\gamma_{2S}$ or α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_ARs$ were exposed to the volatile anesthetics isoflurane and halothane. Clinically relevant concentrations of isoflurane (0.31 mM) increased the currents elicited by submaximal (EC_{20} concentration) GABA at the wild-type receptor by 100%. Another volatile anesthetic, halothane (0.21 mM), potentiated the GABA response to a lesser degree (36%) (Fig. 2.A and C). For the double mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ receptor, isoflurane had no enhancing effect on the GABA response compared to the wild-type receptor. In contrast, the enhancing effect of halothane was not significantly decreased in the double mutant receptor compared to the wild-type receptor (23% and 36%, respectively; Fig. 2.B and C).

Studies in HEK293 cells using rapid application methods

Application of 500 ms-pulses of varying concentrations of GABA produced CRCs with EC_{50} values that substantially exceeded those obtained using standard drug application techniques (Table 1). Presumably this was due to differences in exchange rates. The double mutation induced a significant 2-fold increase in the GABA EC_{50} of α_1(S270H;L277A)$\beta_2\gamma_{2S}$ compared to wild-type $\alpha_1\beta_2\gamma_{2S}$ $GABA_ARs$ (Table 1).

To analyze the deactivation kinetics, brief pulses of a high concentration of GABA (1 mM, 20 ms) were applied to wild-type and mutant $GABA_ARs$, in the absence or presence of 0.25 mM isoflurane (Fig. 3.A and B). The values for the decay time

constants are shown in Fig. 3.C. The double mutation caused receptors to deactivate approximately twice as fast as wild-type under control conditions ($p < 0.005$, unpaired t-test). While isoflurane significantly increased the decay time constants in wild-type $GABA_A$Rs ($p < 0.005$, paired t-test), mutant receptors were no longer sensitive to modulation by isoflurane ($p > 0.05$, paired t-test).

Studies in *Xenopus* oocytes

The immobilizing and sedative effects of the intravenous anesthetic etomidate are mediated by β_3 and β_2-containing $GABA_A$Rs, respectively (Jurd et al., 2003; Reynolds et al., 2003). Alcohols and volatile anesthetics appear to possess a larger range of targets than intravenous anesthetics, but the studies with etomidate suggest the possibility that $GABA_A$Rs with different subunit composition may also underlie different effects by alcohols and volatile anesthetics. Therefore, in the present study, the mutated α_1(S270H;L277A) was co-expressed in *Xenopus* oocytes with γ_{2S}, and with either β_2 or β_3 subunits.

The simultaneous replacement of α_1 S270 with H and of L277 with A produced no changes in the apparent GABA affinity compared to wild-type α1 when co-expressed with either $\beta_2\gamma_{2S}$ (Fig 4.A and Table 1) or $\beta_3\gamma_{2S}$ (Fig 4.C and Table 1). However, the maximal currents of both α_1(S270H;L277A)$\beta_2\gamma_{2S}$ (Fig 4.B) and α_1(S270H;L277A)$\beta_3\gamma_{2S}$ (Fig 4.D) were significantly decreased compared with the corresponding wild-type.

Responses to the volatile anesthetic isoflurane were significantly decreased in α_1(S270H;L277A)-containing receptors compared to wild-type controls. Isoflurane (0.3

mM) potentiation in α_1(S270H;L277A)-containing receptors was only half of the enhancement observed in wild-type receptors (Table 2).

The responses to alcohols were eliminated or significantly decreased in double mutant α_1-containing receptors. Analysis of the effect of ethanol on GABA responses in $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$ receptors (Fig 5.A) showed a significant effect of receptor ($F_{1,52}$=11.54, $p<0.005$), ethanol concentration ($F_{4,52}$=15.44, $p<0.0001$) and interaction ($F_{4,52}$ = 16.36, $p<0.0001$). A similar result was observed after the analysis of $\alpha_1\beta_3\gamma_{2S}$ and α_1(S270H;L277A)$\beta_3\gamma_{2S}$, (Fig 5.B) resulting in a significant effect of receptor ($F_{1,40}$=251, $p<0.0001$), ethanol concentration ($F_{4,40}$=56.7, $p<0.0001$), and interaction ($F_{4,40}$=120, $p<0.0001$). The effect of butanol on GABA responses in $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$ receptors was also tested. Butanol (11 mM) showed a robust potentiation in wild-type receptors, and only a very small increase in α_1(S270H;L277A)$\beta_2\gamma_{2S}$ receptors (Table 2).

The modulation of GABA responses by Zn^{++} (10 μM) was slightly decreased in α_1(S270H;L277A)$\beta_2\gamma_{2S}$ compared with $\alpha_1\beta_2\gamma_{2S}$ receptors, while the modulation by flunitrazepam (1 μM) showed no differences. However, there were no changes in the Zn^{++} or flunitrazepam effects in α_1(S270H;L277A)$\beta_3\gamma_{2S}$ compared with $\alpha_1\beta_3\gamma_{2S}$ $GABA_A$Rs (Table 2). Pentobarbital (50 μM) potentiation was decreased in α_1(S270H;L277A)-containing receptors (by -32% and –34% in β_2- and β_3-containing receptors, respectively; table 2). Etomidate can act either as a modulator or an agonist of $GABA_A$Rs, depending on the concentration. At a low concentration (1 μM), etomidate effects were decreased in α_1(S270H;L277A)$\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_3\gamma_{2S}$, compared with their respective wild-type receptors. At higher concentrations (10 μM), etomidate

can activate the $GABA_AR$ in the absence of GABA. When 10 μM etomidate was applied to $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$ receptors, the responses observed were not different, but the current induced by 10 μM etomidate was significantly decreased in α_1(S270H;L277A)$\beta_3\gamma_{2S}$ compared with $\alpha_1\beta_3\gamma_{2S}$ receptors (Table 2).

Characterization of knock-in mice

We used the targeting strategy depicted in Figure 6.A and B to modify the α1 locus in embryonic stem cells. This mutant locus was transferred through the germline, the neomycin selection marker was deleted by site-specific recombination, and homozygous wild-type (SL/SL) and homozygous knock-in (HA/HA) mice were produced and analyzed. To verify that the knock-in mutation was expressed, and no other unintended mutations were present in the α1 gene, brain α1 mRNA was amplified by RT-PCR and subsequently analyzed by DNA sequence analysis. As expected, only the intended mutations were present in the α1 gene product of HA/HA mice (Fig. 6.C). GABAAR number and binding affinity were unchanged by the mutation as assessed with 3H-flunitrazepam binding (Bmax= 1744 ± 66 vs. 1749 ± 74 fmol/mg protein; KD= 4.62 ± 0.71 nM vs. 5.90 ± 0.96 nM; SL/SL vs. HA/HA, respectively). Immunohistochemistry demonstrated normal distribution of α1 protein (not shown). Western blot of α1 protein revealed normal amounts of α1 in cerebellum of HA/HA mice (94 ± 9%) compared to SL/SL controls (100 ± 11%; Fig. 6.D). However, western blot anaylsis of cortex revealed an unexpected decrease in the amount of □1 present in HA/HA mice compared to SL/SL (Fig. 6.E). This analysis also revealed significant changes in the abundance of other GABAAR subunits in cortex, including an increase in

α3, β2, and γ2, and a decrease in β3 (Fig. 6.E). Analysis of genotype distributions at weaning indicated the expected (Mendelian) production of HA/HA mice (n=101) at approximately the same frequency as SL/SL controls (n=106).

We noticed a few (<10%) premature deaths of HA/HA mice between 7-12 weeks of age, but otherwise HA/HA mutants has normal life spans of well over a year of lifetime, indistinguishable from SL/SL littermates. Otherwise, the knock-in mice were normal in size, appearance, and overt behavior. HA/HA mutant mice did not show any obvious impairements compared to SL/SL littermates with respect to cage behavior, growth, weight, and fertility. HA/HA mice showed the typical pattern of light cycle-dependent activity, with wheel running and food consumption during the dark cycle (see data presented in next section). When observed in their home cages, HA/HA mice did not display any obvious behavioral abnormalities, such as spontaneous seizures, running in circles or other repetitive motions, aggression towards cage mates, or any increases or decreases in general locomotion compared to SL/SL littermates. There were no differences with respect to growth rate, body length, or body weight between HA/HA and SL/SL animals. For example, average body weight of male mice at 8 weeks of age was 24.8 ± 1.0 g for SL/SL, and 23.3 ± 1.0 g for HA/HA mice, and at 12 weeks of age was 27.8 ± 0.8 for SL/SL and 29.0 ± 1.0 for HA/HA mice. HA/HA mice show the normal (hunched) posture and gait as well as normal neurological reflexes such as righting reflex, postural reflex and whisker-orienting reflex. Breeder pairs of mutant males and females generated litters of normal sizes (58 pups) and at the expected frequency (every 34 weeks). Pups were cared for in a manner indistinguishable from SL/SL breeders with respect to nesting, grooming, and feeding. Additionally, we found

no differences in basal motor coordination, anxiety-like behavior, or thermal nociception (unpublished observations).

The normalcy of the double knock-in mice starkly contrasts with the abnormalities observed in mice that bear only the serine 270 to histidine mutation. Mice heterozygous for that single mutation are hyper-responsive, exhibit muscle tremors, have reduced body size, reduced motor coordination, are hypoactive in the home cage, hyperactive in the open field (Homanics et al., 2005) and have EEG abnormalities and anesthetic induced seizure-like activity (Elsen et al., 2006). The double mutant mice reported here have none of these abnormalities. Thus, inclusion of the leucine 277 to alanine mutation appears to have corrected the underlying cause of the behavioral abnormalities induced by the single mutation. The most likely explanation is the restoration of GABA sensitivity to near normal.

Motor activity

Wild-type and mutant mice demonstrated typical transient motor responses to novel situations ($F_{17,396}$= 17.7; p< 0.0001, dependence on time). However, the motor activity levels of knock-in HA/HA mutant mice were consistently higher than those of wild-type animals ($F_{1,396}$= 7.3; p <0.01, dependence on genotype) (Fig. 7.A).

After 24 hours of monitoring activity, main effects of genotype ($F_{1,2288}$= 81.9, p< 0.0001) and time ($F_{103,2288}$= 8.9, p< 0.0001) with strong interactions ($F_{103,2288}$= 1.5, p< 0.001) were observed (Fig. 7.B). HA/HA knock-in mutant mice were more active, both in the light and dark phases.

Discussion

This study showed that a $GABA_AR$ harboring two specific mutations within the α_1 subunit and expressed in heterologous systems exhibited a near normal GABA sensitivity but had a differential response to two commonly used volatile anesthetics. Isoflurane enhanced submaximal GABA currents in wild-type $\alpha_1\beta_2\gamma_{2S}$ $GABA_ARs$ but failed to potentiate them in receptors containing the double mutant α_1(S270H;L277A). On the other hand, halothane enhanced the GABA responses in both the wild-type and mutant receptors. In addition, when $GABA_ARs$ containing α_1(S270H;L277A) were expressed in *Xenopus* oocytes, they failed to show any ethanol-induced potentiation of the GABA responses.

S270 in the second transmembrane region of the α subunit of the $GABA_AR$ appears to be important for the binding of volatile anesthetics (Koltchine et al., 1999; Mascia et al., 2000). Mutation of S270 to amino acids of various side chain volumes has been shown to affect the GABA EC_{50} and anesthetic action. The differences in size between isoflurane (144 $Å^3$) and halothane (110 $Å^3$) could explain the differences in enhancing effects on the mutant $GABA_AR$: replacing S270 with H allowed potentiation by the smaller volatile anesthetic halothane but not by the larger anesthetic isoflurane. When S was replaced with even larger amino acids (e.g. tryptophan), the enhancing effects of halothane were abolished (Nishikawa et al., 2002). Substitution of S270 with a larger amino acid also decreased alcohol potentiation (Mihic et al., 1997; Ueno et al., 1999; Findlay et al., 2000). In α_1(S270H;L277A)-containing $GABA_ARs$, ethanol potentiation was abolished, and butanol potentiation in α_1(S270H;L277A)$\beta_2\gamma_{2S}$ was

greatly decreased. The absence or marked decrease of alcohol potentiation was most likely due to the S270 to H mutation in the alcohol and volatile anesthetic binding site.

Additional $GABA_AR$ modulators tested on the wild-type and mutant receptors expressed in *Xenopus* oocytes were Zn^{++} (endogenous modulator), flunitrazepam (benzodiazepine), pentobarbital and etomidate (intravenous anesthetics). The Zn^{++} inhibition of currents through the $GABA_AR$ is greatly reduced if the γ subunit is present in the receptor (Draguhn et al., 1990; Smart et al., 1991). Zn^{++} inhibition was minimal in all the expressed receptors, indicating expression of γ_{2S}. A small decrease in Zn^{++} inhibition was observed in α_1(S270H;L277A)$\beta_2\gamma_{2S}$, but not in α_1(S270H;L277A)$\beta_3\gamma_{2S}$. The flunitrazepam-induced potentiation was not altered in α_1(S270H;L277A)-containing receptors, suggesting that the level of expression of γ_{2S} was not modified in either combination. Discrete Zn^{++} binding sites have been described in the $GABA_ARs$ (Hosie et al., 2003). The small difference in the Zn^{++} sensitivity between wild-type and mutant receptors could be due to an allosteric effect of the α_1(L277A) mutation, which is near to a Zn^{++} binding site, or the α_1(S270H) mutation, that could be introducing a new Zn^{++} binding site by placing a H in a water-filled pocket. The pentobarbital-induced potentiation of GABA responses was reduced by about 30% in receptors containing the double mutation; however, there is no indication of any of these mutations being located in a pentobarbital binding site, and the observed change is likely due to an allosteric effect. The beta subunit of the $GABA_AR$ is crucial for etomidate action: etomidate is approximately 10 times more potent at $\beta_{2/3}$-containing receptors than at β_1-containing receptors due to the variable residue at 286, which is either Asn or Ser (Belelli et al., 1997; Hill-Venning et al., 1997), and substitution of Asn in $\beta_{2/3}$ can completely eliminate

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etomidate potentiation (Jurd et al., 2003; Reynolds et al., 2003). The etomidate-induced potentiation of GABA responses was decreased in α_1(S270H;L277A)-containing receptors, while the etomidate-induced currents through the $GABA_AR$ were decreased only in α_1(S270H;L277A)$\beta_3\gamma_{2S}$ receptors. This suggests that the conformational changes necessary for etomidate action are impeded by the mutations in α_1, even though they do not modify the action of other agonists (GABA) or modulators (flunitrazepam). In summary, the effect of most of the anesthetics tested was decreased (pentobarbital and etomidate, isoflurane) or abolished (ethanol) in the α_1(S270H;L277A)-containing receptors expressed in *Xenopus* oocytes.

When $GABA_ARs$ expressed in *Xenopus* oocytes and HEK293 cells were tested using standard solution exchange techniques, the double mutation α_1(S270H;L277A) did not affect the GABA sensitivity. However, when tested in HEK293 cells using rapid application methods, α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_ARs$ showed a lower sensitivity to GABA and a faster rate of deactivation at the termination of the GABA pulse than wild-type. Nevertheless, the effect of isoflurane was substantially reduced in the double mutant when tested by rapid application. We do not have a ready explanation for the influence of solution exchange rates on EC_{50}, or why this differs in degree for the mutant *versus* wild-type receptors. Nevertheless, it is apparent that the characteristics of the GABA transient influence the impact of the mutation on receptor activation. One implication of this finding is that the effect of the double mutation on drug-free responses at receptors *in situ* might depend on the conditions of receptor activation, with little or no influence on tonic currents produced by low concentrations of GABA, but with changes in synaptic currents produced by transient receptor activation, and the

elimination of isoflurane sensitivity under both conditions. This prediction can now be tested since the mice that bear the double mutation are viable.

Mice bearing the double mutation S270 to H and L277 to A in the $GABA_AR$ α_1 subunit were successfully generated, and with no overt abnormal characteristics. In biochemical analysis, wild-type and knock-in mice presented no differences; for instance, flunitrazepam binding was similar between wild-type and knock-in mice, and the α_1 protein distribution was normal in brain of HA/HA mice. However, differences in receptor subunit levels in cortex, and locomotor activity were observed between SL/SL and HA/HA mice. The maximal currents of wild-type and double mutant receptors did not differ when the receptors were expressed in HEK293 cells, but there was a decrease in the maximal currents for the double mutant α_1(S270H;L277A)$\beta_{2/3}\gamma_{2S}$ expressed in *Xenopus* oocytes. Different expression systems may reflect more or less accurately the receptor characteristics *in vivo*. Even though the current amplitudes observed in oocytes better reflected the effects of the mutation on protein levels in cortex (the levels of mutant α_1 protein were decreased by 48% in HA/HA mice compared with wild-type SL/SL mice), and these results are consistent with decreased protein levels in the oocyte expression system, they do not definitively address this issue. The flunitrazepam binding was not modified in HA/HA mice, probably because of compensatory changes in other subunits (the levels of α_3 were increased by 44%, while α_2 showed an increase of 34%).

We found that HA/HA knock-in mutant mice demonstrate a hyperactive behavioral phenotype. These mice exhibited a slightly greater motor response to novelty; this response was transient, and after 150-180 min the activity of mutant and

wild-type mice was similar. Over 24 hrs of continuous monitoring of spontaneous locomotion, HA/HA knock-in mutant mice demonstrated normal circadian motor rhythm, but were more active than control mice. $GABA_A$Rs are known to be important for regulation of exploratory activity (File, 1985), and mice lacking $GABA_A$R α_1 or α_2 subunits demonstrated reduction of spontaneous locomotion (Blednov et al., 2003; Boehm et al., 2004). In contrast, mice lacking the $GABA_A$R β_2 subunit showed high levels of locomotor activity (Sur et al., 2001; Blednov et al., 2003), similar to the β3 knock-out mice (DeLorey et al., 1998). Thus, the reduction of α_1 protein found in the HA/HA mice is unlikely to account for the motor activity phenotype.

In conclusion, we were able to design a $GABA_A$R containing a mutant α_1 subunit that maintained a near normal GABA response *in vitro*, but showed a differential response to volatile anesthetics and alcohols. This receptor provides a promising candidate for an *in vivo* model of altered volatile anesthetic sensitivity, because it did not markedly affect the normal response to GABA but showed a decreased response to isoflurane in both HEK293 cells and *Xenopus* oocytes, and a normal potentiation by halothane in HEK293 cells. It will also be useful in the study of alcohol effects through α_1-containing $GABA_A$Rs, since the double mutant showed insensitivity to ethanol. The homozygous knock-in mice engineered with this mutation presented no striking differences from the wild-type mice except for behavioral hyperactivity. These knock-in mice are a unique resource that will allow for an unprecedented dissection of the role of the α_1 subunit of the $GABA_A$R in clinically relevant whole-animal behavioral responses to volatile anesthetics and ethanol. For example, if α_1-containing $GABA_A$Rs are

important mediators of anesthetic action, then knock-in mice bearing the double mutation should resist the anesthetizing effects of isoflurane but not halothane.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. GABA EC_{50} was higher for α_1(L277A)$\beta_2\gamma_{2S}$, decreased for α_1(S270H)$\beta_2\gamma_{2S}$ and restored by the double mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ compared to wild-type $\alpha_1\beta_2\gamma_{2S}$ $GABA_AR$ expressed in HEK293 cells. GABA-induced responses from individual HEK293 cells expressing either wild-type or mutant α subunits are shown (A, B, C, D). Bars over individual current traces indicate the duration of GABA application with the concentration of GABA in μM. Pooled data are plotted in CRCs (E, F) for wild-type and mutant $GABA_A$Rs. *Data* points shown are the means of multiple normalized experiments. *Bars* indicate S.E.M.

Fig. 2. The mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_AR$ expressed in HEK293 cells lacked potentiation by isoflurane but halothane potentiated the response to GABA. Bars over individual current traces indicate the duration of GABA/isoflurane/halothane application. (A) Traces show the response of the wild-type receptor to submaximal doses of GABA (EC_{20}) and subsequently in the presence of either isoflurane (0.31 mM) or halothane (0.21 mM). The potentiation of the GABA response by the volatile anesthetics was reversible. (B) The mutant receptor lacked the potentiating effects of isoflurane but retained the potentiating effects of halothane on submaximal GABA applications. (C) Summary bar graphs of GABA-induced responses at EC_{20} concentrations from multiple HEK293 cells expressing wild-type or mutant α subunits in the absence or presence of isoflurane or halothane are shown. The potentiation of the GABA response by isoflurane was significantly reduced in the

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α_1(S270H;L277A)$\beta_2\gamma_{2S}$ mutant compared to wild-type ($p<0.05$, Student's *t*-test). The potentiating effects of halothane were reduced in the mutant receptor but not significantly different from the wild-type receptor. *Bars* indicate means ± S.E.M. (N= 8-10).

Figure 3. Effect of isoflurane in deactivation of the wild-type $\alpha_1\beta_2\gamma_{2S}$ and the mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_A$Rs expressed in HEK293 cells and studied through rapid application. Currents were induced in response to a brief pulse of GABA (1 mM, 20 ms). Isoflurane (0.25 mM) slowed deactivation in (A) the wild-type $\alpha_1\beta_2\gamma_{2S}$ but not in (B) the double mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_A$Rs. Currents were normalized to peak amplitude for comparison of the time course of deactivation. Bars indicate 20 ms. (C) Graphic summary of the isoflurane effect on the weighted decay time constant (τ_{decay}) for wild-type $\alpha_1\beta_2\gamma_{2S}$ and the double mutant α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_A$Rs. #$p< 0.05$ statistically significant difference from wild-type, *$p< 0.005$ statistically significant difference from control (Student's *t*-test). *Bars* indicate means ± S.E.M. (N= 3-4).

Figure 4. No difference was observed in the GABA affinity in α_1- and double mutant α_1(S270H;L277A)-containing $GABA_A$Rs expressed in *Xenopus* oocytes, but the maximal current was decreased in the double mutant. (A) GABA CRC in $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_A$Rs. *Data* points shown are the means of multiple normalized experiments; *bars* indicate S.E.M. (B) GABA maximal responses in $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$ $GABA_A$Rs (N= 13-31 per bar). *$p< 0.05$, **$p< 0.001$,

statistically significant difference from wild-type (Two-way ANOVA). (C) GABA CRC in $\alpha_1\beta_3\gamma_{2S}$ and α_1(S270H;L277A)$\beta_3\gamma_{2S}$ $GABA_A$Rs. *Data* points shown are the means of multiple normalized experiments; *bars* indicate S.E.M. (D) GABA maximal responses in $\alpha_1\beta_3\gamma_{2S}$ and α_1(S270H;L277A)$\beta_3\gamma_{2S}$ $GABA_A$Rs (N= 2-10 per bar). *p< 0.01 statistically significant difference from wild-type (Two-way ANOVA).

Figure 5. Ethanol enhanced GABA responses in $\alpha_1\beta_{2/3}\gamma_{2S}$ $GABA_A$Rs but not in α_1(S270H;L277A)$\beta_{2/3}\gamma_{2S}$ $GABA_A$Rs expressed in *Xenopus* oocytes. Effect of increasing concentrations of ethanol on EC_5 GABA responses in: A. $\alpha_1\beta_2\gamma_{2S}$ and α_1(S270H;L277A)$\beta_2\gamma_{2S}$. * p< 0.05, ** p< 0.01, (n= 7-8), and B. $\alpha_1\beta_3\gamma_{2S}$ and α_1(S270H;L277A)$\beta_3\gamma_{2S}$. ** p< 0.01, *** p< 0.001, (n=6).

Figure 6. Production of α_1 knock-in mice by gene targeting. (A) Partial DNA sequence of wild-type and knock-in α_1 genes. Note that the codons for Ser at position 270 and Leu at position 277 were changed to codons for His and Ala, respectively. Also changed was one base pair that introduced an EcoR I restriction site in the mutated exon that did not change any codon. Mutations introduced are highlighted in yellow in the knock-in sequence. (B) Gene targeting strategy used to modify the α_1 locus in mouse embryonic stem cells. Exon 9 corresponds to nucleotides 1308-1510 of the published mouse α1 cDNA (Keir et al., 1991). (C) DNA sequence analysis of α_1 RT-PCR products from whole brain of SL/SL (upper panel) and HA/HA (lower panel) mice that confirm the intended mutations are expressed from the α_1 gene in the brain of knock-in animals. (D) Western blot analysis of $GABA_A$R α_1 subunit protein in

cerebellum of individual SL/SL and HA/HA mice. Shown are representative samples. Amount of α_1 in HA/HA samples did not differ from SL/SL. All samples were normalized for equal loading by probing with an actin antibody. (E) Western blot analysis of $GABA_AR$ subunit proteins in cortex of SL/SL and HA/HA mice. Shown are representative pooled samples. Also shown is the percent change in band intensity of HA/HA mice compared to SL/SL (data presented as mean ± S.E.M.). *p< 0.05; ** p< 0.01.

Figure 7. HA/HA knock-in mutant mice showed higher spontaneous activity in a novel environment and in the home cage. Open symbols are SL/SL wild-type mice, filled symbols are HA/HA knock-in mutant mice. (A) Motor activity when first placed into the unfamiliar cage (N = 12 for each genotype). (B) Motor activity of HA/HA knock-in mutant mice in the same cage over a period of 24 hours. Dark phase is indicated by the bar (N = 13 for each genotype). Values are means ± S.E.M.

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TABLE 1. Summary of the characteristics of GABA responses in the wild-type and mutant $\alpha_1\beta_{2/3}\gamma_{2S}$ $GABA_A$Rs expressed in HEK293 cells and *Xenopus* oocytes

$GABA_A$R	EC_{50} (μM)	n_H	I_{MAX} (pA)	N
HEK293 cells-Standard application				
$\alpha_1\beta_2\gamma_{2S}$	16.0 ± 1.8	1.27 ± 0.05	-198 ± 24	63
α_1(S270H)$\beta_2\gamma_{2S}$	3.2 ± 0.4**	1.82 ± 0.14*	-183 ± 45	28
α_1(L277A)$\beta_2\gamma_{2S}$	278 ± 23**	1.00 ± 0.04*	-162 ± 10	54
α_1(S270H;L277A)$\beta_2\gamma_{2S}$	14.0 ± 1.8	1.33 ± 0.07	-214 ± 16	38
HEK293 cells – Rapid application			I_{MAX} (nA)	
$\alpha_1\beta_2\gamma_{2S}$	112 ± 8	1.2 ± 0.04	-5.0 ± 1.2	5
α_1(S270H;L277A)$\beta_2\gamma_{2S}$	251 ± 60**	1.6 ± 0.16*	-6.3 ± 1.9	5
***Xenopus* oocytes**				
$\alpha_1\beta_2\gamma_{2S}$	64.4 (44.6 to 92.7)	1.07 ± 0.16	See Fig. 4.B	6
α_1(S270H;L277A)$\beta_2\gamma_{2S}$	56.3 (45.3 to 70.0)	0.96 ± 0.08	See Fig. 4.B	6
$\alpha_1\beta_3\gamma_{2S}$	43.9 (36.3 to 53.1)	1.29 ± 0.12	See Fig. 4.D	7
α_1(S270H;L277A)$\beta_3\gamma_{2S}$	31.9 (27.2 to 37.5)	1.16 ± 0.09	See Fig. 4.D	6

EC_{50} concentrations, Hill coefficient (n_H), and maximal current (I_{MAX}) are given for each receptor as mean ± S.E.M. for "N" HEK293 cells. EC_{50} concentrations are given for each receptor as mean (95% Confidence Interval), and Hill coefficients (n_H) are given for each receptor as mean ± S.E.M., for "N" oocytes. Statistical significance was assessed using ANOVA or Student's *t*-test. *p< 0.05, **p <0.001 compared to wild-type receptor.

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TABLE 2. Summary of the characteristics of modulators of GABA responses in the wild-type and mutant $\alpha_1\beta_{2/3}\gamma_{2S}$ $GABA_A$Rs expressed in *Xenopus* oocytes

	$\alpha_1\beta_2\gamma_{2S}$	α_1(S270H; L277A)$\beta_2\gamma_{2S}$	$\alpha_1\beta_3\gamma_{2S}$	α_1(S270H; L277A)$\beta_3\gamma_{2S}$
EC_5 GABA + Drug				
Isoflurane (0.30 mM)	283 ± 28 (7)	148 ± 9 *** (8)	352 ± 25 (5)	153 ± 18 *** (6)
Butanol (10.8 mM)	184 ± 16 (8)	29 ± 7 *** (8)	Not tested	Not tested
Zn^{++} (10 μM)	-27.5 ± 4.6 (21)	-11.9 ± 2.9 * (22)	-26.5 ± 1.6 (17)	-25.2 ± 1.3 (18)
Flunitrazepam (1 μM)	89 ± 19 (15)	108 ± 8 (16)	230 ± 22 (5)	226 ± 10 (6)
Pentobarbital (50 μM)	621 ± 75 (6)	425 ± 42 * (6)	803 ± 67 (5)	533 ± 23 ** (6)
Etomidate (1 μM)	368 ± 37 (8)	165 ± 17 *** (8)	338 ± 29 (5)	165 ± 14 *** (6)
Drug alone				
Etomidate (10 μM)	23.6 ± 9.4	19.8 ± 5.2	9.1 ± 2.2	2.9 ± 0.7 *

	(8)	(8)	(7)	(8)

Changes in EC_5 GABA in the presence of drug are given as percentage mean ± S.E.M. for each receptor. The direct responses to etomidate are given as percentage of the maximal GABA response. The number of oocytes is given between parentheses. Statistical significance was assessed using Student's *t*-test. *p <0.05, **p <0.005, ***p <0.0005 compared to the corresponding wild-type receptor.

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Figure 1

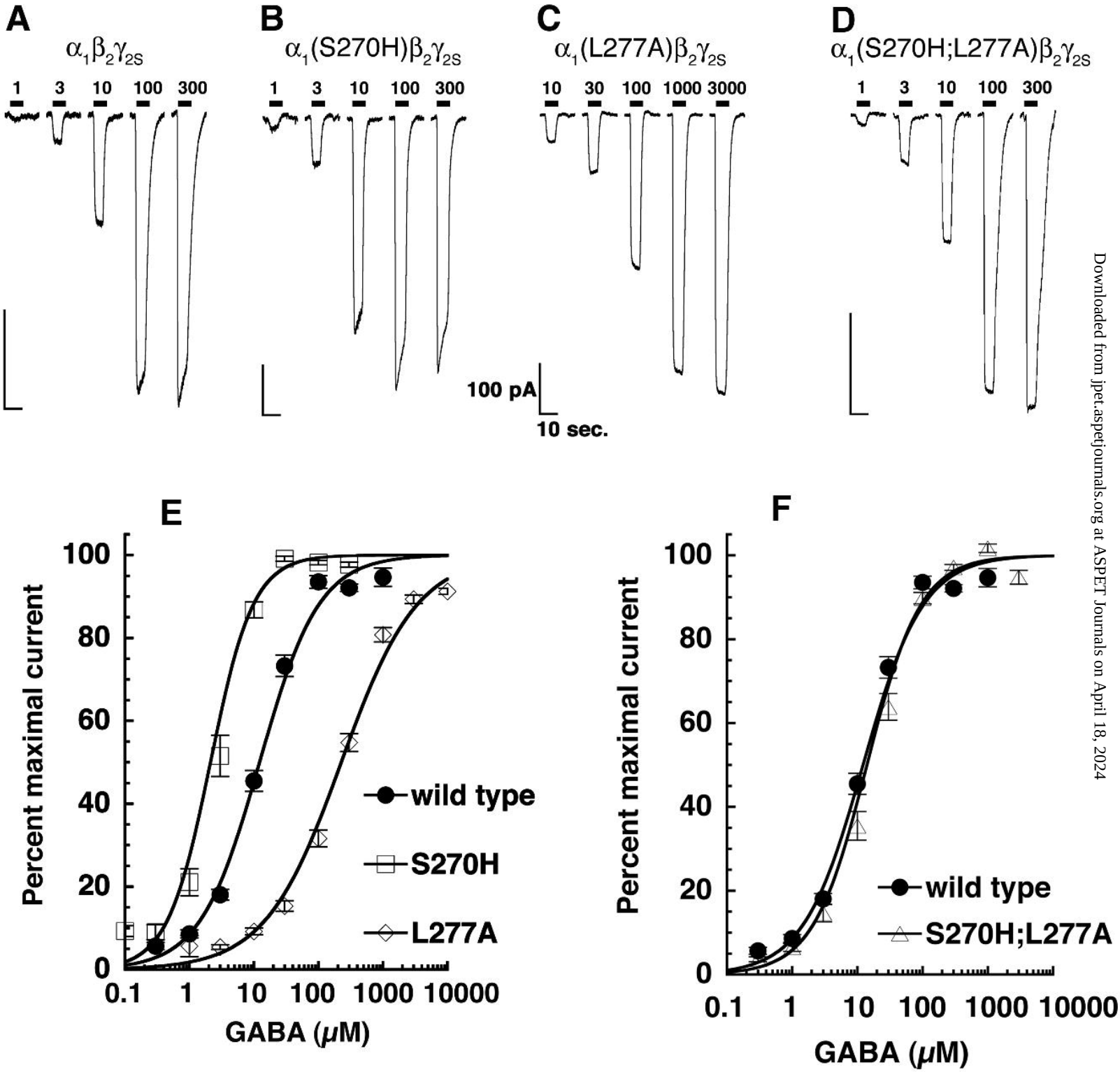

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Figure 2

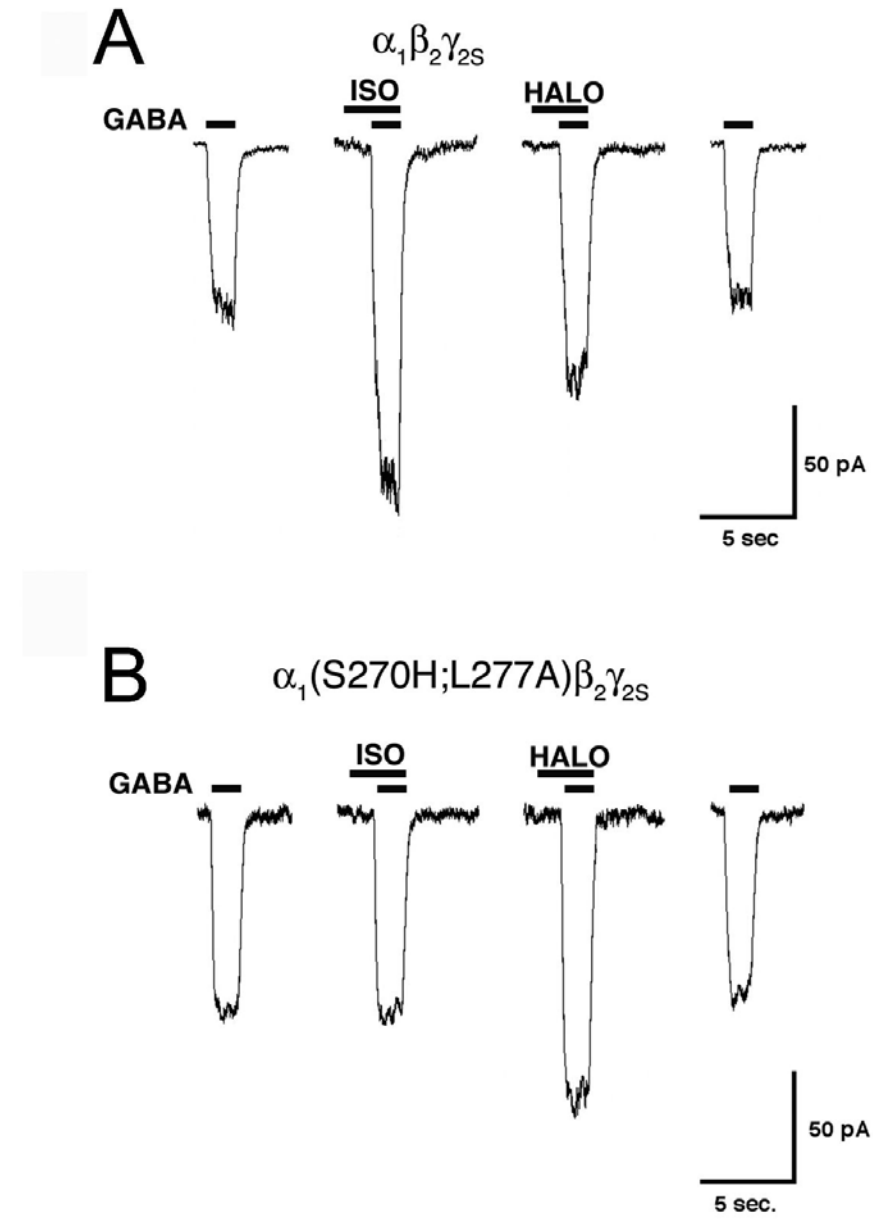

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Figure 3

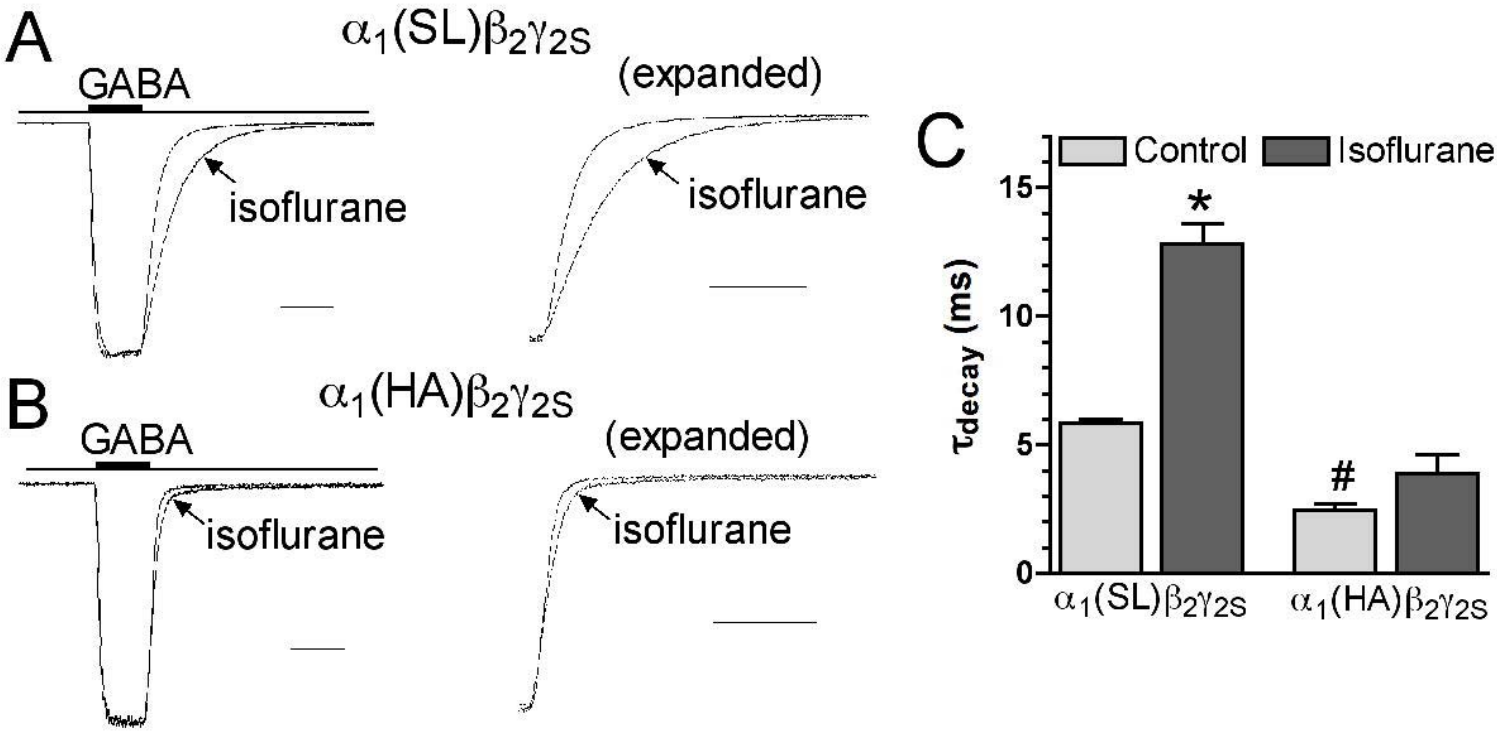

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Figure 4

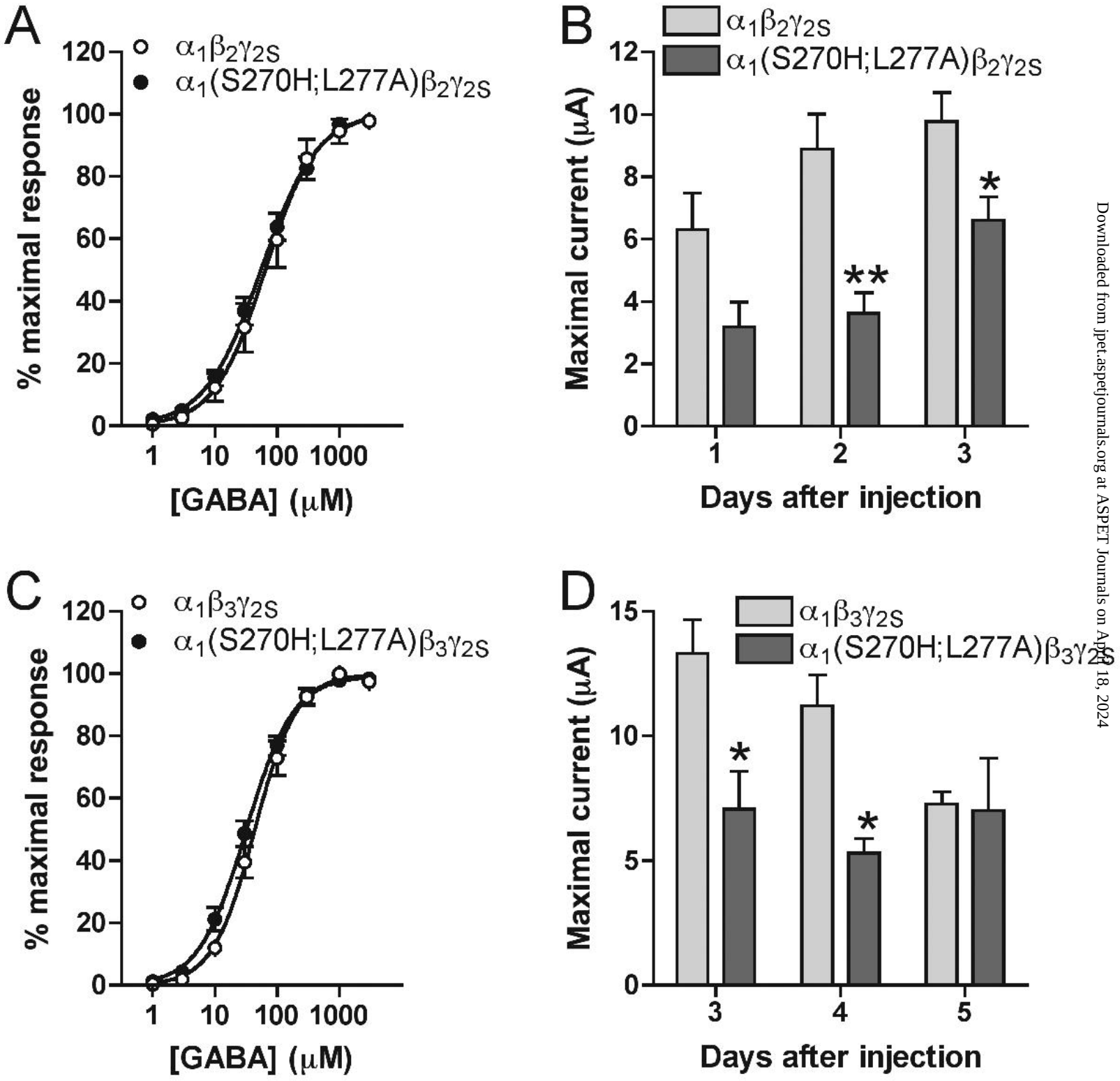

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Figure 5

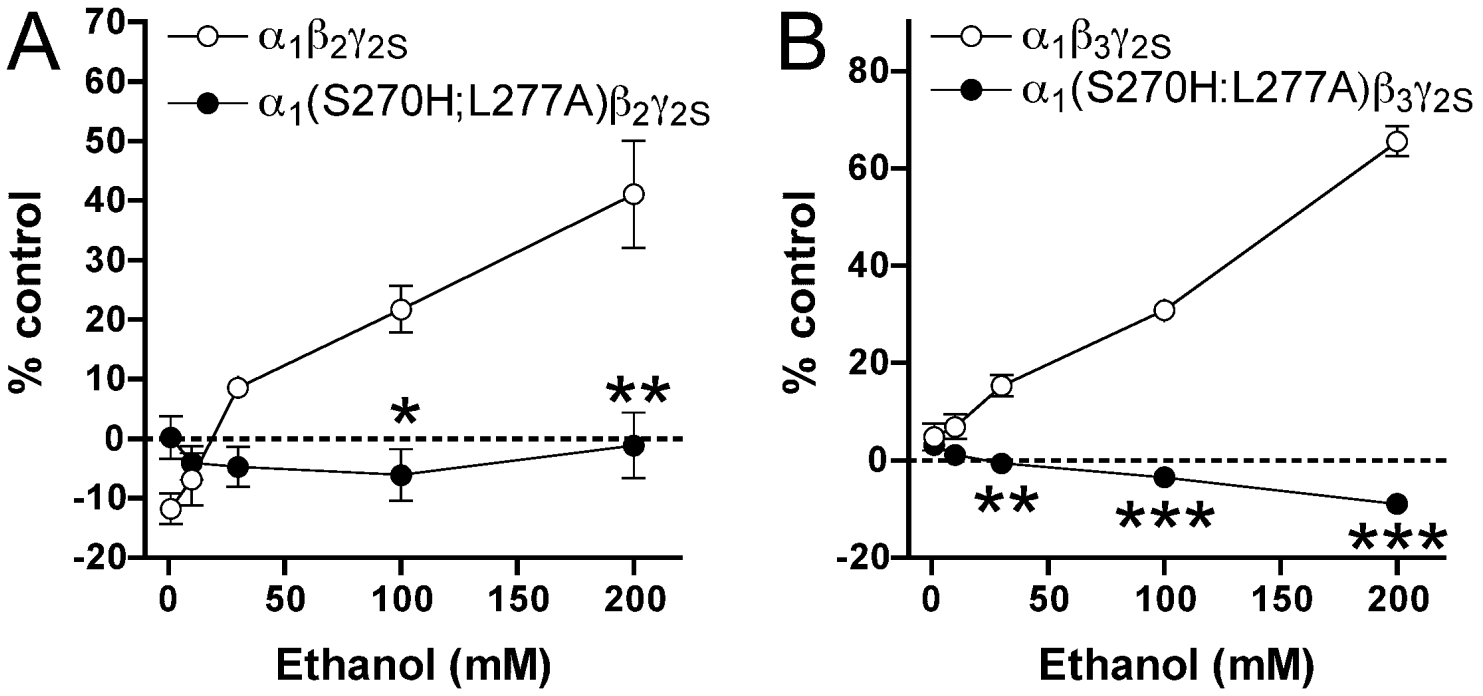

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Figure 6

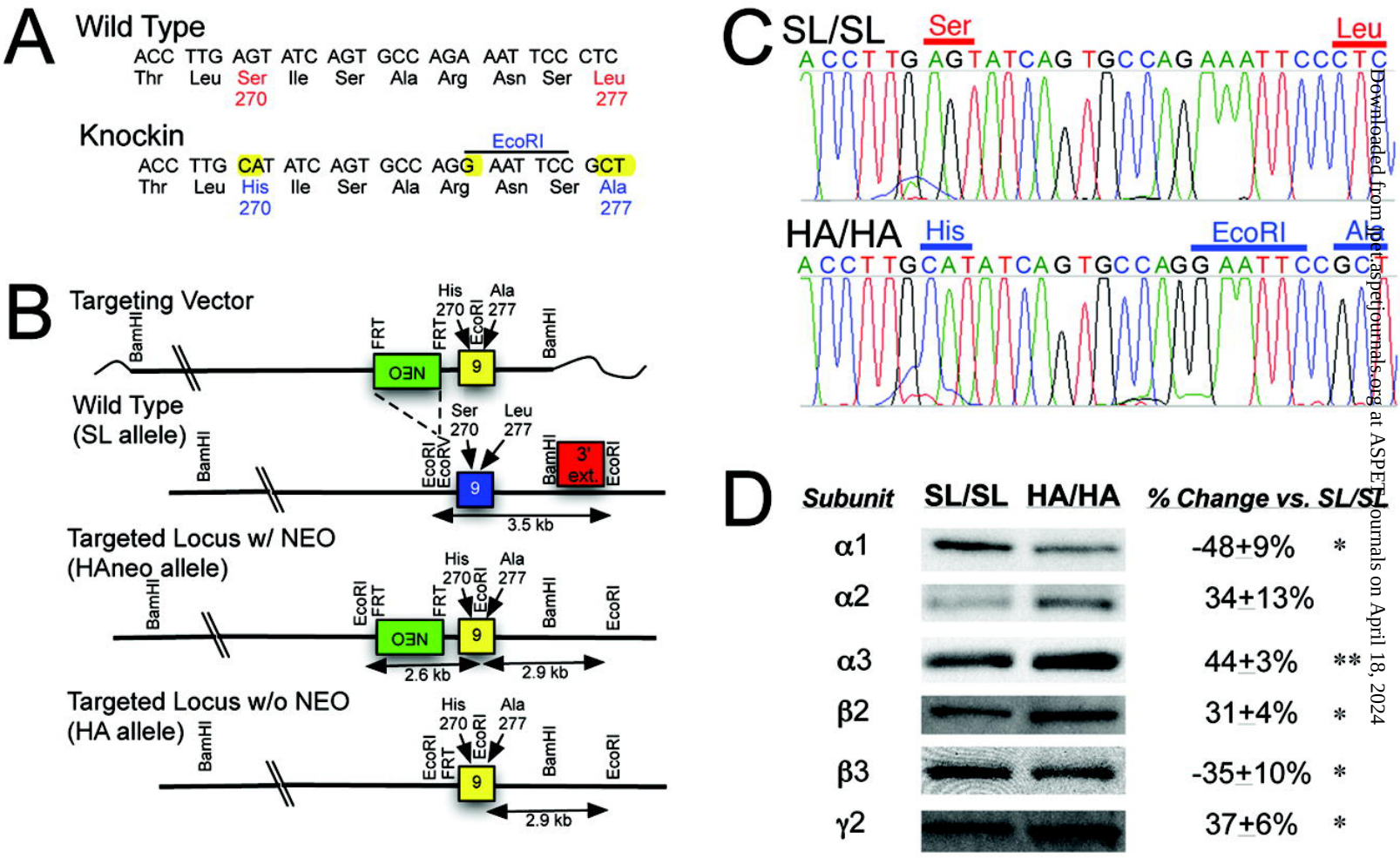

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Figure 7