

# Control of Ethanol Sensitivity of the Glycine Receptor $\alpha 3$ Subunit by Transmembrane 2, the Intracellular Splice Cassette and C-Terminal Domain<sup>S</sup>

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## ABSTRACT

Previous studies have shown that the effect of ethanol on glycine receptors (GlyRs) containing the  $\alpha 1$  subunit is affected by interaction with heterotrimeric G proteins ( $G\beta\gamma$ ). GlyRs containing the  $\alpha 3$  subunit are involved in inflammatory pain sensitization and rhythmic breathing and have received much recent attention. For example, it is unknown whether ethanol affects the function of this important GlyR subtype. Electrophysiologic experiments showed that GlyR  $\alpha 3$  subunits were not potentiated by pharmacologic concentrations of ethanol or by  $G\beta\gamma$ . Thus, we studied GlyR  $\alpha 1$ – $\alpha 3$  chimeras and mutants to determine the molecular properties that confer ethanol insensitivity. Mutation of corresponding glycine 254 in transmembrane domain 2 (TM2) found in  $\alpha 1$  in the  $\alpha 3^{A254G}$ – $\alpha 1$  chimera induced a glycine-evoked current that displayed potentiation during application of ethanol

( $46 \pm 5\%$ , 100 mM) and  $G\beta\gamma$  activation ( $80 \pm 17\%$ ). Interestingly, insertion of the intracellular  $\alpha 3L$  splice cassette into GlyR  $\alpha 1$  abolished the enhancement of the glycine-activated current by ethanol ( $5 \pm 6\%$ ) and activation by  $G\beta\gamma$  ( $-1 \pm 7\%$ ). Incorporation of the GlyR  $\alpha 1$  C terminus into the ethanol-resistant  $\alpha 3S^{A254G}$  mutant produced a construct that displayed potentiation of the glycine-activated current with 100 mM ethanol ( $40 \pm 6\%$ ) together with a current enhancement after G protein activation ( $68 \pm 25\%$ ). Taken together, these data demonstrate that GlyR  $\alpha 3$  subunits are not modulated by ethanol. Residue A254 in TM2, the  $\alpha 3L$  splice cassette, and the C-terminal domain of  $\alpha 3$  GlyRs are determinants for low ethanol sensitivity and form the molecular basis of subtype-selective modulation of GlyRs by alcohol.

## Introduction

Glycine receptor (GlyR)-mediated neurotransmission is involved in key physiologic functions, such as motor rhythm generation, coordination of reflex responses, and sensory processing (Legendre, 2001; Dresbach et al., 2008; Lynch, 2009; Zeilhofer et al., 2012). These receptors are pentameric protein complexes arranged symmetrically as a ring around a central ion-conducting pore. Unlike nicotinic acetylcholine receptors (nAChRs) or GABA<sub>A</sub> receptors, only five GlyR isoforms have been identified to date ( $\alpha 1$ – $\alpha 4$  and  $\beta$ ). These subunits can form homomeric or heteromeric GlyRs (Betz et al., 1999; Legendre, 2001; Grudzinska et al., 2005). Each of the GlyR subunits comprises a large extracellular amino-terminal domain, four  $\alpha$ -helical transmembrane domains (TM1–TM4), and a large intracellular domain (ICD) between TM3 and TM4 (Laube et al., 2002; Lynch, 2009).

The  $\alpha$ -subunits of GlyRs share most of their structural, biochemical, and biophysical properties (Lynch, 2009) mainly due to extensive sequence identity. More interestingly, it has been reported that GlyR isoforms have different pharmacological properties (Yevenes and Zeilhofer, 2011). In this context, the alcohol pharmacology of GlyRs has been studied extensively, mostly focusing on the  $\alpha 1$  subunit (Malosio et al., 1991; Mascia et al., 1996b; Aguayo et al., 2004; Yevenes et al., 2008).

Although it is well accepted that the glycine-activated current through homomeric GlyRs containing the  $\alpha 1$  subunit is potentiated by ethanol (Aguayo and Pancetti, 1994; Mascia et al., 1996a; Mihic et al., 1997), the mechanisms underlying this phenomenon are still not completely understood. Indeed, several hypotheses have been proposed to explain the alcohol effects on GlyRs (Mihic et al., 1997; Perkins et al., 2008; Borghese et al., 2012). The existence of ethanol binding pockets and regulatory sites for ethanol actions within the TM of  $\alpha 1$  subunit GlyRs has been postulated. For instance, combining mutated GlyRs, molecular modeling, and covalent binding of alcohol analogs has determined that TM2 and TM3 residues jointly shape a water-filled cavity serving as an ethanol binding pocket (Ye et al., 1998; Mascia et al., 2000; Harris et al., 2008). Additional studies

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**ABBREVIATIONS:** GlyR, glycine receptor; GST, glutathione-S-transferase; GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); HEK, human embryonic kidney; ICD, large intracellular loop domain; INS, insertion of 15 amino acids; nAChR, nicotinic acetylcholine receptor; PKA, protein kinase A; TM, transmembrane domain.

showed that residues in extracellular loop 2 and TM1 might contribute to the alcohol binding pockets, or exert modulatory roles on ethanol potentiation (Crawford et al., 2008; Lobo et al., 2008). However, it is also well documented that determinants of the ethanol sensitivity of  $\alpha 1$  subunit GlyRs can be located intracellularly, such as protein kinase C phosphorylation (Mascia et al., 1998; Jiang and Ye, 2003) or direct modulation of the ion channel by G protein  $\beta\gamma$  subunits (Yevenes et al., 2003, 2006, 2008). In addition, the relevance of  $G\beta\gamma$  signaling has been recently shown using intracellular blocking peptides designed to alter the interaction between the TM3-TM4 intracellular domain of GlyR and  $G\beta\gamma$  (Guzman et al., 2009; San Martin et al., 2012).

Studies in recombinant and native GlyRs have consistently shown that receptors containing  $\alpha 1$  are more sensitive to ethanol than those containing  $\alpha 2$  (Mascia et al., 1996b; Tapia and Aguayo, 1998; Eggers et al., 2000; Yevenes et al., 2010). The low ethanol sensitivity of homomeric  $\alpha 2$  GlyRs has been associated with divergent alcohol binding sites (Ye et al., 1998; Mascia et al., 2000) and the absence of functional modulation by  $G\beta\gamma$  (Yevenes et al., 2010). Furthermore, we showed that all GlyR mutants that were sensitive to modulation by  $G\beta\gamma$  were also modulated by ethanol, establishing a high degree of positive correlation (Yevenes et al., 2010). On the other hand, little is known about the effects of ethanol on  $\alpha 3$  GlyRs (Lobo et al., 2008; Jonsson et al., 2009). Furthermore, it is also unknown whether this GlyR subtype is modulated by  $G\beta\gamma$ . This subunit has clinical significance because it is involved in the control of rhythmic breathing (Manzke et al., 2010), and ethanol modulation of this GlyR subtype could be responsible for the respiratory depression induced by excessive alcohol consumption. In addition, modulation of  $\alpha 3$  GlyRs by  $G\beta\gamma$  through the activation of G protein-coupled receptors could be relevant for the regulation of glycinergic inhibition in other physiologic processes, such as central pain sensitization (Harvey et al., 2004; Manzke et al., 2010).

Here, we examined the molecular mechanisms underlying the sensitivity of  $\alpha 3$  GlyRs to ethanol. We found that these GlyRs displayed a low sensitivity to ethanol and were not functionally modulated by  $G\beta\gamma$ . Therefore, we examined the molecular characteristics behind this insensitivity and found that they were quite different than those described for  $\alpha 2$  GlyRs. Thus, this study defines new sites that control the ethanol effects on GlyRs.

## Materials and Methods

**Cell Culture and Transfection.** Human embryonic kidney (HEK) 293 cells were cultured using standard methodologies. Cells were plated onto 18-mm glass coverslips in 20-mm culture plate wells and transfected using Xfect transfection reagent (Clontech, Mountain View, CA) with 0.5  $\mu\text{g}$  of DNA for each plasmid studied per well. Expression of enhanced green fluorescent protein was used as a marker of positively transfected cells, and recordings were made after 14–18 hours.

**cDNA Constructs.** Mutations were inserted using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) in cDNA constructs encoding rat GlyRs in a pCI vector (Promega, Madison, WI). The GlyR  $\alpha 3$ - $\alpha 1$  chimera was constructed by the introduction of restriction sites into homologous regions of the cDNAs encoding the rat GlyRs  $\alpha 1$  and  $\alpha 3$  subunit cDNAs. XbaI sites were engineered into the nucleotide sequences of GlyR  $\alpha 1$  and  $\alpha 3\text{S}$  or  $\alpha 3\text{L}$ , corresponding to the region closest to the end of TM3, and Sall sites were incorporated into the cDNA sequences encoding the end of the C terminus, allowing us to combine DNA regions by standard subcloning. The  $\alpha 1$ - $\alpha 3$  ICD chimera

was constructed using a Sall site added previously and a new site for XbaI added for exchange of the C-terminal region of the loop between TM3 and TM4 in  $\alpha 1$  GlyRs. The replacement of the intracellular loop of  $\alpha 3\text{L}$  and  $\alpha 3\text{S}$  was accomplished by incorporating directed polymerase chain reaction products with the enzymes SacI and XbaI. Finally, the GlyR  $\alpha 1$ - $\alpha 3$ - $\alpha 1$  chimera was constructed by site-directed mutagenesis. All constructs were confirmed by Sanger DNA sequencing.

**Electrophysiology.** Whole-cell recordings were performed as previously described (Yevenes et al., 2003, 2008). A holding potential of  $-60$  mV was used. Patch electrodes were filled with 140 mM CsCl, 10 mM *N,N'*-[1,2-ethanediy]bis(oxy-2,1-phenylene)]bis[*N*-(carboxymethyl)glycine] tetraesium salt, 10 mM HEPES (pH 7.4), 4 mM  $\text{MgCl}_2$ , 2 mM ATP, and 0.5 mM GTP. The external solution contained 150 mM NaCl, 5.4 mM KCl, 2.0 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 10 mM HEPES (pH 7.4), and 10 mM glucose. For G protein activation experiments, guanosine 5'-*O*-(3-thiotriphosphate) (GTP $\gamma\text{S}$ ) (0.5 mM; Sigma-Aldrich, St. Louis, MO) was added directly to the internal solution, replacing GTP. The amplitude of the glycine current was assayed using a brief (1–6 seconds) pulse of glycine every 120 seconds. The modulation of the glycine current by ethanol (Sigma-Aldrich) was assayed using a pulse of glycine ( $\text{EC}_{10}$ ) co-applied with ethanol to each receptor studied, without any preapplication. In all experiments, a brief pulse of 1 mM glycine was performed at the end of the recording period to test that the glycine concentration corresponded to the actual  $\text{EC}_{10}$  in each single experiment. Cells that displayed responses  $\leq \text{EC}_5$  or  $\geq \text{EC}_{15}$  were discarded.

**Construction of Glutathione S-Transferase Fusion Proteins and Glutathione S-Transferase Pull-Down Assays.** DNA fragments encoding wild-type GlyR  $\alpha 1$ ,  $\alpha 3\text{S}$ , and  $\alpha 3\text{L}$  intracellular loops were first subcloned in the glutathione-S-transferase (GST) fusion vector pGEX-5X3 (GE Healthcare, Piscataway, NJ). GST fusion proteins were generated in *Escherichia coli* BL21 using 10 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside. After 3 hours, the cells were collected and sonicated in lysis buffer (1 $\times$  phosphate buffer, 1% Triton X-100, and protease inhibitor mixture set II; Calbiochem, San Diego, CA). Subsequently, proteins were purified using a glutathione resin (Novagen, Madison, WI), and normalized amounts of GST fusion proteins were incubated with purified bovine  $G\beta\gamma$  protein (Calbiochem). Incubations were made in 800  $\mu\text{l}$  of binding buffer [200 mM NaCl, 10 mM EDTA, 10 mM Tris (pH 7.4), 0.1% Triton X-100, and protease inhibitor mixture set II] at 4°C for 1 hour. The beads were then washed five times, and bound proteins were separated on 10% SDS-polyacrylamide gels. Bound  $G\beta\gamma$  was detected using a  $G\beta$  antibody (Santa Cruz Biotechnology, Dallas, TX) and a chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA). Finally, the relative amounts of  $G\beta\gamma$  were quantified by densitometry.

**Immunofluorescence, Image Visualization, and Analysis.** HEK293 cells on 18-mm glass coverslips were first fixed with 4% paraformaldehyde (0.1 M phosphate buffer, pH 7.4) and were then permeabilized (0.1% Triton X-100) and blocked (10% normal horse serum). Subsequently, all-night incubation with a monoclonal anti-FLAG M2 (Stratagene) and polyclonal anti-GlyR  $\alpha 1$  (Synaptic Systems, Goettingen, Germany) or anti-GlyR  $\alpha 3$  antibodies (Millipore, Billerica, MA) was carried out. Epitope visualization was performed by incubating the sample with two secondary antibodies conjugated to fluorescein isothiocyanate and cyanine dye (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the coverslips were then mounted onto microscope slides using Fluorescence Mounting Medium (Dako Cytomation, Carpinteria, CA). For quantitative analysis, cells were chosen randomly for imaging using a Nikon confocal microscope (TE2000; Nikon, Tokyo, Japan). Single stacks of optical sections in the *z*-axis were acquired, and dual-color immunofluorescent images were captured in simultaneous two-channel mode. Colocalization was studied by superimposing both color channels. The cross-correlation coefficient (*r*) between both fluorescence channels was measured using computer software ImageJ (National Institutes of Health, Bethesda, MD) starting from separate immunoreactivity for GlyR and  $G\beta_1$ -FLAG in the same cell. The theoretical maximum was 1 for identical images. Subsequently, the obtained data were compiled, analyzed, and plotted.

**Molecular Modeling.** The GlyR  $\alpha 1$  and  $\alpha 3$  subunits were constructed by homology modeling using Modeller 9v10 software (<http://salilab.org/modeller/>) and the *Caenorhabditis elegans* glutamate-gated chloride channel structure (PDB ID 3RIF) as template (Hibbs and Gouaux, 2011). Due to the lack of sequence identity of ICDs with a known structure protein, these regions were predicted by ab initio technique using the QUARK server (Ann Arbor, MI; Xu and Zhang, 2012). All models were relaxed by energy minimization using a conjugate-gradient protocol with Maestro v9.3 (Schrödinger, LLC, New York, NY). The energy calculation and structural validation were performed by Prosa (Wiederstein and Sippl, 2007) and Procheck (Laskowski et al., 1993), respectively. All figures presented were created by PyMOL (Schrödinger, LLC).

**Data Analysis.** Statistical analyses were performed using analysis of variance with values of  $P < 0.05$  considered statistically significant. The values are expressed as arithmetic mean  $\pm$  S.E.M. For all statistical analyses and plots, Origin 9.0 software (MicroCal, Northampton, MA) was used. Normalized values were obtained by dividing the current amplitude obtained with time of GTP $\gamma$ S dialysis by the current at minute 1. Although the effects of ethanol start at 10 mM (Aguayo and Pancetti, 1994), we and other groups routinely used 100 mM to facilitate statistical comparisons.

## Results

**Ethanol Sensitivity of Homomeric  $\alpha 3S$  and  $\alpha 3L$  Glycine Receptors.** In the mammalian central nervous system, the GlyR  $\alpha 3$  subunit is expressed in two splice variants denoted here as GlyR  $\alpha 3S$  and  $\alpha 3L$  (Nikolic et al., 1998). These receptors differ in the presence of a 15-amino-acid insertion within the TM3-TM4 intracellular domain of the  $\alpha 3L$  GlyR variant (KTEAFALEKFYRFSD, denoted as  $\alpha 3L$  INS). Using an EC<sub>10</sub> glycine obtained from concentration-response curves (Table 1), we found that neither  $\alpha 3S$  nor  $\alpha 3L$  GlyRs were potentiated by 100 mM ethanol (Fig. 1) or by any ethanol concentration used (with incremental applications between 0.01 and 200 mM) (Supplemental Fig. 1). The glycine-evoked current through  $\alpha 3L$  GlyRs was slightly reduced by  $-19 \pm 5\%$ , whereas the current through  $\alpha 3S$  was not affected by a single application of 100 mM ethanol ( $7 \pm 2\%$ ) (Fig. 1A). As described previously (Aguayo and Pancetti, 1994),  $\alpha 1$  GlyRs

were significantly potentiated by 100 mM ethanol ( $55 \pm 7\%$ ). Intriguingly, the insensitivity of  $\alpha 3$  subunits resembled the resistance of  $\alpha 2$  GlyRs to ethanol, which has been previously reported by our group and others (Fig. 1B) (Mascia et al., 1996b; Perkins et al., 2008, 2012; Yevenes et al., 2010). These results suggest the existence of critical structural differences between  $\alpha 3$  and  $\alpha 1$  GlyRs associated with their differential alcohol sensitivity. In addition, due to the similar ethanol sensitivity and high sequence identity between  $\alpha 3S$  and  $\alpha 2$  GlyRs (85 and 82% for  $\alpha 3S$  and  $\alpha 3L$ , respectively), it is likely that both GlyR isoforms share common molecular elements that explain their similar ethanol pharmacology.

**G Protein Modulation of  $\alpha 3S$  and  $\alpha 3L$  GlyRs.** An important number of reports have shown that several intracellular signaling pathways can influence the ethanol sensitivity of several Cys-loop ion channels (Smart, 1997; Yevenes et al., 2003; Harvey et al., 2004; Fischer et al., 2005). For example, previous studies demonstrated that the ethanol potentiation of  $\alpha 1$  GlyRs depends on the direct protein interaction between G $\beta\gamma$  with two basic motifs (<sup>316-320</sup>RFRRK and <sup>385-386</sup>KK) of the TM3-TM4 ICD (Yevenes et al., 2003, 2006). Therefore, we first performed a sequence alignment between the large intracellular domains of  $\alpha 1$ ,  $\alpha 3S$ , and  $\alpha 3L$  to examine these basic motifs. This sequence analysis revealed 100% identity of the relevant basic residues, suggesting that  $\alpha 3S$  and  $\alpha 3L$  TM3-TM4 intracellular domains are able to interact with G $\beta\gamma$ . To evaluate this potential ICD-G $\beta\gamma$  interaction, we designed and constructed GST fusion proteins with the ICD of  $\alpha 3S$  and  $\alpha 3L$  subunits. Once expressed and purified, these proteins were used for in vitro interaction assays with purified G $\beta\gamma$  protein. Similar to the results obtained with the  $\alpha 1$  and  $\alpha 2$  subunits (Yevenes et al., 2006, 2008), the intracellular loops of  $\alpha 3S$  and  $\alpha 3L$  were able to bind to G $\beta\gamma$  (Fig. 2A). To further evaluate these interactions in a more intact cellular context, immunocytochemical and confocal microscopy analyses were performed in HEK293 cells transfected with GlyRs and G $\beta\gamma$ . The cellular distribution of GlyRs and G $\beta\gamma$  showed a membrane pattern of colocalization in their expression (Fig. 2B). Correlation analysis yielded high coefficient values (Fig. 2C), giving support for a physical

TABLE 1

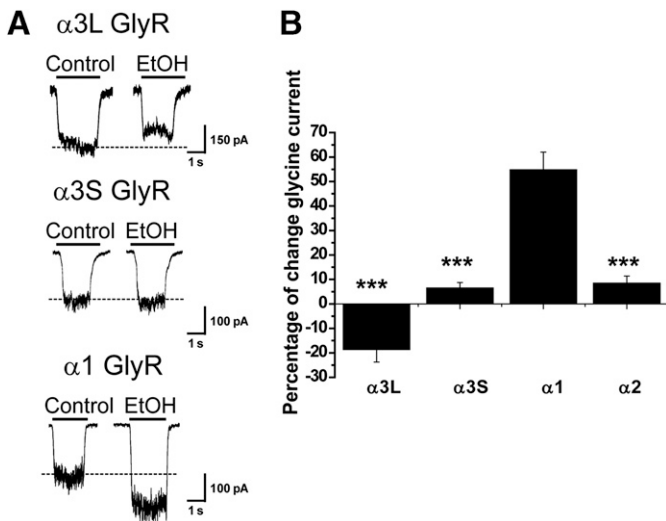
Electrophysiologic properties of wild-type, mutated, and chimeric GlyRs

Values are given as the mean  $\pm$  S.E.M. Values were obtained by fitting the data to the equation  $\text{glycine} = I_{\max}(\text{glycine})^n/(\text{glycine})^n + (EC_{50})^n$  using computer software.  $\Delta$  of current change (%) corresponds to change in the presence of 100 mM ethanol with respect to control.  $\Delta$  of current change (%) at 15 minutes corresponds to change in the presence of intracellular GTP $\gamma$ S (500  $\mu$ M) measured after 15 minutes of dialysis with respect to control current.

GlyR	EC <sub>50</sub>	I <sub>max</sub>	n <sub>H</sub>	n	$\Delta$ of Current Change	$\Delta$ of Current Change at 15 Min
	$\mu$ M	pA			%	%
$\alpha 1$ WT	45 $\pm$ 1	2771 $\pm$ 563	2.8 $\pm$ 0.1	5	55 $\pm$ 7	80 $\pm$ 13
$\alpha 3L$ WT	109 $\pm$ 3**	2041 $\pm$ 390	2.6 $\pm$ 0.2	4	-19 $\pm$ 5**	-59 $\pm$ 6***
$\alpha 3S$ WT	92 $\pm$ 3**	3510 $\pm$ 420	2.2 $\pm$ 0.2	6	7 $\pm$ 2**	-46 $\pm$ 6***
$\alpha 3L$ A254G	251 $\pm$ 9**	2849 $\pm$ 692	2.9 $\pm$ 0.3	5	1 $\pm$ 3**	ND
$\alpha 3S$ A254G	92 $\pm$ 3**	3422 $\pm$ 889	2.7 $\pm$ 0.2	6	11 $\pm$ 3**	ND
$\alpha 3$ - $\alpha 1$	31 $\pm$ 1**	3840 $\pm$ 691	2.3 $\pm$ 0.1	11	8 $\pm$ 2**	10 $\pm$ 8***
$\alpha 3$ - $\alpha 1$ A254G	50 $\pm$ 2	2831 $\pm$ 592	2.7 $\pm$ 0.2	8	46 $\pm$ 5	80 $\pm$ 5
$\alpha 1$ - $\alpha 3L$ ICD	61 $\pm$ 3**	3545 $\pm$ 669	2.2 $\pm$ 0.1	5	10 $\pm$ 3**	18 $\pm$ 10***
$\alpha 1$ - $\alpha 3S$ ICD	50 $\pm$ 1**	3914 $\pm$ 760	2.5 $\pm$ 0.1	5	45 $\pm$ 5	72 $\pm$ 17
$\alpha 1$ + $\alpha 3L$ INS	65 $\pm$ 2**	3600 $\pm$ 650	2.0 $\pm$ 0.1	6	5 $\pm$ 6**	-1 $\pm$ 7***
$\alpha 1$ - $\alpha 3S$ - $\alpha 1$ <sub>CT</sub>	51 $\pm$ 1**	3278 $\pm$ 280	2.5 $\pm$ 0.1	7	49 $\pm$ 5	70 $\pm$ 16
$\alpha 3S$ A254G- $\alpha 1$ <sub>CT</sub>	49 $\pm$ 4	4232 $\pm$ 881	2.4 $\pm$ 0.4	5	40 $\pm$ 6	68 $\pm$ 15
$\alpha 1$ <sub>ACT</sub>	116 $\pm$ 3**	7650 $\pm$ 425	3.0 $\pm$ 0.2	7	51 $\pm$ 8	ND

INS, 15-amino-acid insert; ND, not determined; WT, wild type.

\*\* $P < 0.01$ ; \*\*\* $P < 0.001$  with respect to  $\alpha 1$  WT.



**Fig. 1.** Ethanol sensitivity of homomeric  $\alpha 3$  GlyRs expressed in HEK293 cells. (A) Representative current traces evoked by glycine ( $EC_{10}$ ) in control and in the presence of 100 mM ethanol for GlyR  $\alpha 1$  and  $\alpha 3$  subunits. The segmented line shows the control current amplitude value. (B) Summary of the percentage potentiation obtained after application of a single concentration of 100 mM ethanol. Data with the  $\alpha 2$  subunit were included for comparison. The results are the mean  $\pm$  S.E.M., and all data were significant (\*\*\*)  $P < 0.001$ , analysis of variance with respect to GlyR  $\alpha 1$ . EtOH, ethanol.

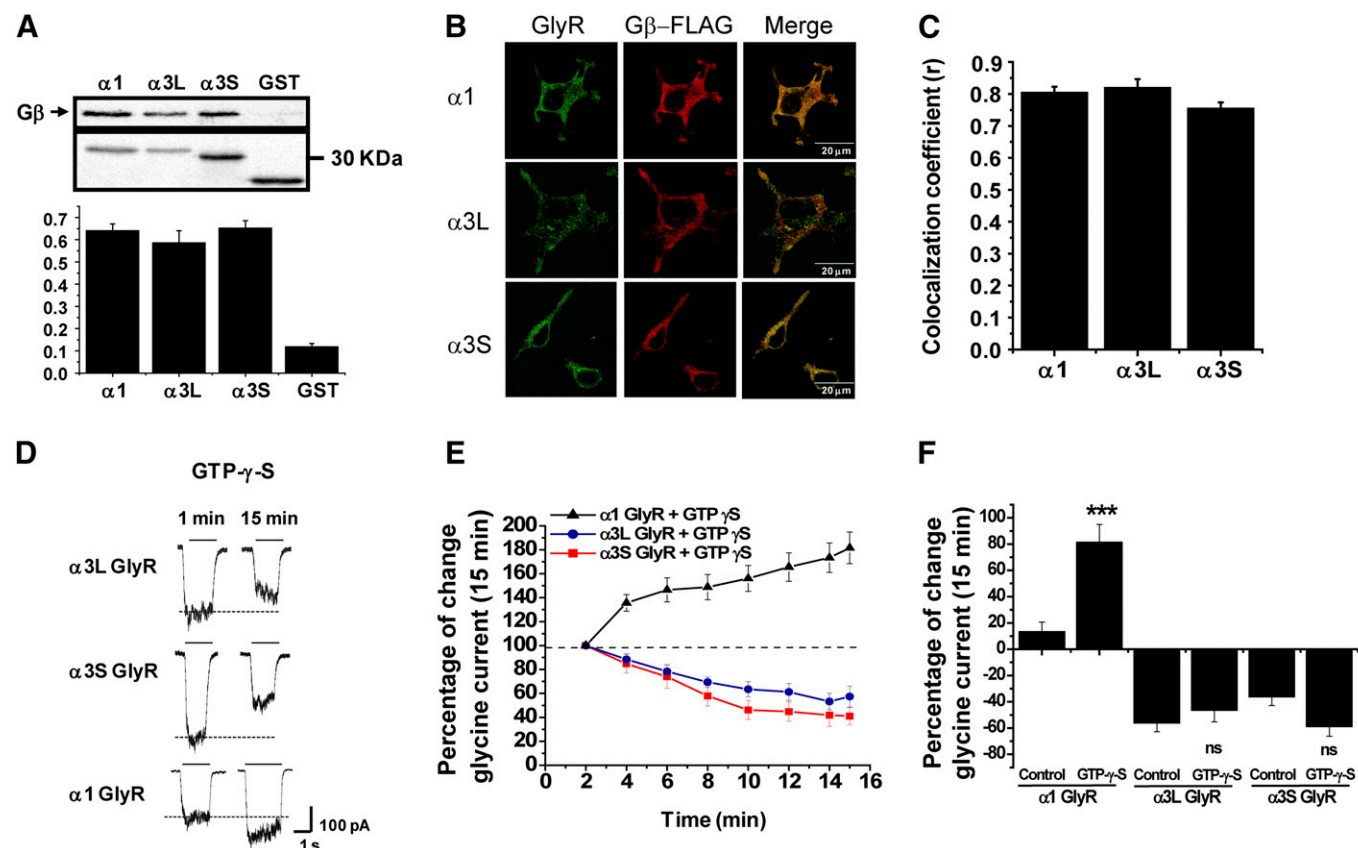
interaction between these GlyRs and  $G\beta\gamma$  in a cellular context.

We next examined the effect of the nonhydrolyzable GTP analog  $GTP\gamma S$  on glycine-evoked currents in  $\alpha 3$  GlyRs to evaluate the presence of functional modulation by G proteins. In  $\alpha 1$  GlyRs, intracellular dialysis of  $GTP\gamma S$  significantly enhanced the glycine-evoked currents by  $80 \pm 13\%$  after 15 minutes of dialysis (Fig. 2, D and E, solid triangles). This response has been shown to be linked to  $G\beta\gamma$  modulation and independent of signaling pathways involving protein kinases (Yevenes et al., 2003, 2006). The experiments showed that  $\alpha 3S$  and  $\alpha 3L$  GlyRs were not potentiated by G protein activation (Fig. 2, E and F). After 15 minutes of intracellular  $GTP\gamma S$ , the glycine-evoked currents of  $\alpha 3S$  and  $\alpha 3L$  GlyRs were diminished to  $-59 \pm 6\%$  and  $-46 \pm 6\%$ , respectively. These results could be suggesting a negative modulation of G protein activation on  $\alpha 3$  GlyRs (Harvey et al., 2004). However, experiments using control internal solution (i.e., without  $GTP\gamma S$ ) showed similar percentages of current inhibition (i.e., current run-down) of  $\alpha 3$  GlyRs after 15 minutes of whole-cell recording ( $-36 \pm 6\%$  for  $\alpha 3S$ ;  $-56 \pm 7\%$  for  $\alpha 3L$ ) (Fig. 2F), excluding inhibitory effects of the G protein activation on  $\alpha 3$  GlyRs. These data demonstrate that  $\alpha 3$  GlyRs can directly interact with  $G\beta\gamma$ . Nevertheless, these interactions were not translated into a functional modulation of the ion channel, suggesting either the presence of molecular determinants that prevent  $G\beta\gamma$  and ethanol modulation or the absence of key structural elements for alcohol binding and subsequent modulation.

**Role of TM2-TM3 Domains in the Ethanol Sensitivity of  $\alpha 3$  GlyRs.** Compelling evidence has described the presence of critical residues for alcohol binding in the TM2 and TM3 regions of  $\alpha 1$  GlyRs (Mihic et al., 1997; Harris et al., 2008). Residues in loop 2 have also been suggested in ethanol binding and modulation (Perkins et al., 2008, 2009, 2012). Thus, differences in the sequence within the extracellular loop

2 and the TM2-TM3 domains of  $\alpha 3$  GlyRs could be a plausible explanation for their low ethanol sensitivity. However, we found only one nonconserved alanine residue at position A254 of the GlyR  $\alpha 3$  (Fig. 3A). Interestingly, this amino acid in GlyR  $\alpha 1$  and  $\alpha 2$  has been found to be essential for ethanol potentiation and  $G\beta\gamma$  modulation (Yevenes et al., 2010). On the other hand, several other key residues necessary for ethanol potentiation in  $\alpha 1$  GlyRs described by our group and others (e.g., A52 in the extracellular loop 2, S267 in TM2, and A288/S296 in TM3) were 100% conserved (Mihic et al., 1997; Perkins et al., 2008; Yevenes et al., 2010) (Fig. 3A). Thus, it is possible that the single A $\rightarrow$ G substitution at position 254 in the TM2 region of  $\alpha 3$  GlyRs may underlie alcohol insensitivity of this subtype. To examine this possibility, we constructed  $\alpha 3S$  and  $\alpha 3L$  GlyRs with the mutation A254G in the TM2 domain, finding that GlyR  $\alpha 3S^{A254G}$  or  $\alpha 3L^{A254G}$  mutants were not sensitive to ethanol ( $\alpha 3S^{A254G}$ ,  $11 \pm 3\%$ ;  $\alpha 3L^{A254G}$ ,  $1 \pm 3\%$ ) (Fig. 3B). Additionally, the mutations significantly changed the glycine sensitivity but not the maximal current amplitude (Table 1). These results indicate that the A254G substitution did not enhance the ethanol sensitivity of  $\alpha 3$  GlyRs, thus the  $\alpha 1$  phenotype was not rescued, and this is expressed as a statistically significant difference from  $\alpha 1$  (Table 1,  $P < 0.001$  with respect to  $\alpha 1$  wild type). These data indicate that the mechanisms underlying the low ethanol sensitivity of  $\alpha 3$  GlyRs are fundamentally different from those proposed for  $\alpha 2$  GlyRs. Thus, we next aimed to identify the additional molecular elements in  $\alpha 3$  GlyRs that determine their ethanol insensitivity.

**Molecular Elements in the TM3-TM4 Intracellular Domain Influence the Ethanol Sensitivity of  $\alpha 3$  GlyRs.** The previous results showed that modification in the TM2 domain of  $\alpha 3$  GlyR was not sufficient to change the alcohol sensitivity. Therefore, we focused our attention on molecular elements downstream of the TM3 domain, since these showed the highest variability in different GlyR subtypes. To explore these elements, we first analyzed a chimeric GlyR which combines the coding region downstream of the TM3 domain of GlyR  $\alpha 1$  with the region upstream of TM3 of GlyR  $\alpha 3$  (GlyR  $\alpha 3$ - $\alpha 1$ ) (Fig. 4A). This chimeric GlyR showed an increased apparent affinity for glycine (Fig. 4B; Table 1) but did not display significant ethanol potentiation ( $8 \pm 2\%$ ) or G protein modulation ( $10 \pm 8\%$ ) (Fig. 4, C and D). This chimeric construct, however, still had residue A254, which is of known importance for the modulation of GlyR  $\alpha 1$  by ethanol and was necessary to confer ethanol sensitivity to GlyR  $\alpha 2$  (Yevenes et al., 2010). Thus, we also evaluated the ethanol potentiation and G protein modulation of an  $\alpha 3^{A254G}$ - $\alpha 1$  GlyR mutant (Fig. 4, C and D). Interestingly, the glycine-evoked current of GlyR  $\alpha 3^{A254G}$ - $\alpha 1$  displayed a robust potentiation, and  $\alpha 1$ -like phenotype, after G protein activation ( $80 \pm 17\%$ ) (Fig. 4E) and significant ethanol potentiation ( $46 \pm 5\%$ , 100 mM) (Fig. 4D). In agreement with previous studies (Yevenes et al., 2008), these data support the existence of a high correlation between both modulations. These results indicate that the low ethanol sensitivity of GlyR  $\alpha 3$  is determined by TM2 and other molecular elements downstream of the TM3 domain, which include the ICD, the TM4 domain, and the extracellular C terminus. Interestingly, because the low alcohol sensitivity of  $\alpha 2$  GlyRs has been fully linked to extracellular loop 2 and TM2-TM3 domain residues (Yevenes et al., 2010), these so far unrecognized elements downstream from the TM3 domain of  $\alpha 3$  GlyRs appear to be

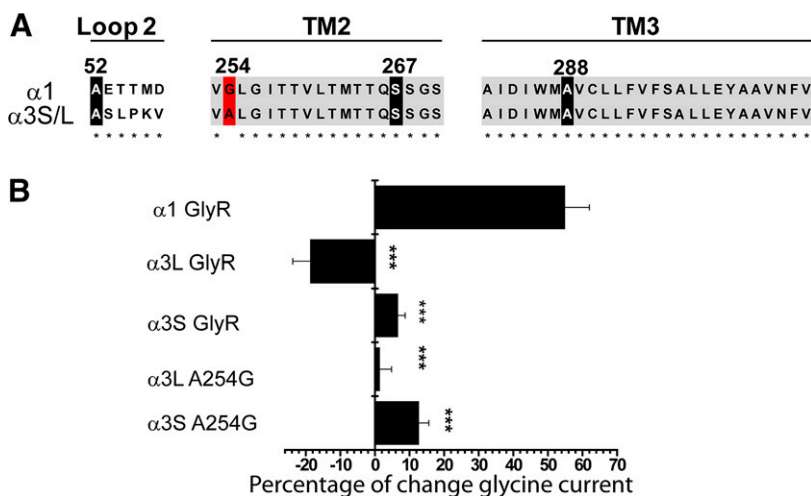


**Fig. 2.**  $\alpha 3$  GlyRs and G protein  $\beta\gamma$  interaction and functional modulation. (A) (Top) GST pull-down assays for GlyR  $\alpha 1$  and  $\alpha 3$  TM3-TM4 ICDs and  $G\beta\gamma$ . (Bottom) Quantification of relative amount of bound  $G\beta\gamma$ . (B) Transfected HEK293 cells stained with antibodies against GlyR  $\alpha 1$  and  $\alpha 3$  subunits and FLAG-M2 epitope (red) that recognizes tagged  $G\beta 1$ . (C) The graph summarizes the correlation coefficients between GlyR subunits and  $G\beta\gamma$ . (D) Representative glycine-evoked current traces at the initial time and after 15 minutes of intracellular dialysis of nonhydrolyzable analog  $GTP\gamma S$  for GlyRs  $\alpha 1$  and  $\alpha 3$ . The segmented line shows the control current amplitude value. (E) Time course of  $GTP\gamma S$  dialysis on GlyR  $\alpha 1$  and  $\alpha 3$  subunits. (F) The graph summarizes the effects of normal internal solution and internal solution containing  $GTP\gamma S$  after 15 minutes on the glycine-evoked current. For all panels, the results are the mean  $\pm$  S.E.M. For (E), all points were significant after 4 minutes (\*\*\*)  $P < 0.001$ , analysis of variance with respect to  $\alpha 1$ ). \*\*\*Represents statistical differences between normal internal solution (control) and  $GTP\gamma S$  internal solution in GlyR  $\alpha 1$ . ns, lack of significance in control (GTP) and presence of  $GTP\gamma S$  for  $\alpha 3S$  and  $\alpha 3L$ .

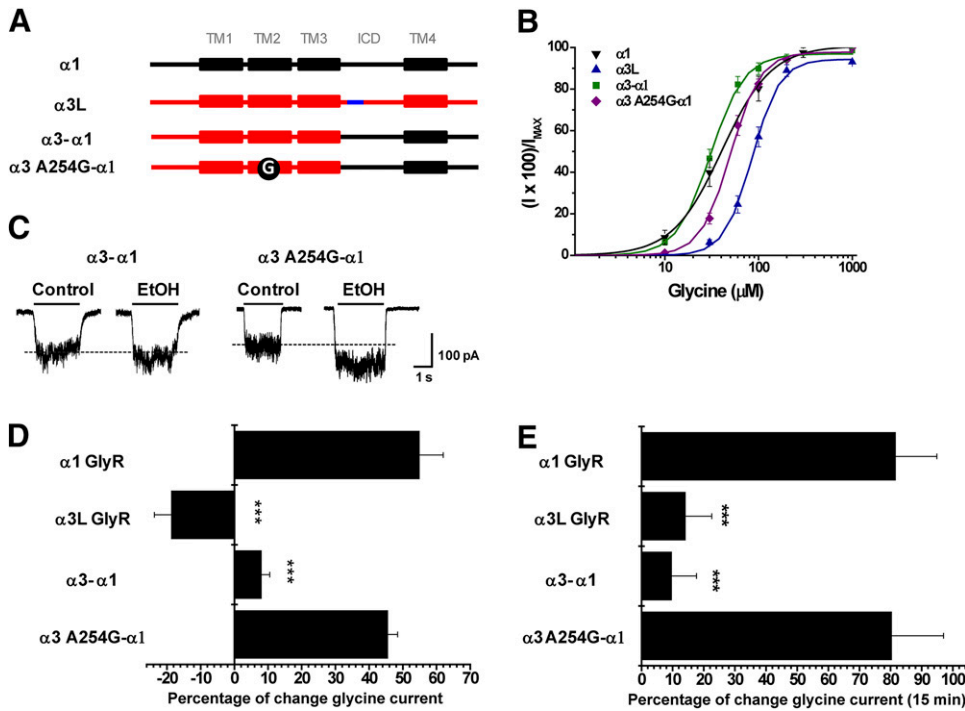
unique. Therefore, we next aimed to identify these elements using additional chimeric and mutant GlyRs.

**The Alternative Spliced Region of 15 Amino Acids within the TM3-TM4 Intracellular Domain of  $\alpha 3L$  GlyRs Negatively Influences Alcohol Effects and G Protein Modulation.** To identify novel residues downstream from the

TM3 domain of  $\alpha 3$  GlyRs that may exert negative effects on alcohol sensitivity, we studied two chimeric GlyRs in which only the ICDs of  $\alpha 3S$  and  $\alpha 3L$  isoforms were incorporated into the  $\alpha 1$  GlyR background ( $\alpha 1$ - $\alpha 3S$  ICD and  $\alpha 1$ - $\alpha 3L$  ICD) (Fig. 5A). The glycine-activated current elicited by the  $\alpha 1$ - $\alpha 3L$  ICD GlyR still displayed low sensitivity to ethanol ( $10 \pm 3\%$ ) and to G protein



**Fig. 3.** Effects of the TM2 mutation A254G on the ethanol sensitivity of  $\alpha 3$  GlyRs. (A) Multiple sequence alignment between GlyR  $\alpha 1$  and  $\alpha 3$  subunits, including important residues in loop 2 (A52), TM2 (G254 or A254, S267), and TM3 (A288) that are essential for potentiation of GlyR  $\alpha 1$  by ethanol. (B) The graph summarizes the effects of 100 mM ethanol on GlyRs  $\alpha 1$ ,  $\alpha 3$ , and the  $\alpha 3S^{A254G}$  and  $\alpha 3L^{A254G}$  mutants. The results are the mean  $\pm$  S.E.M., and all data were significant (\*\*\*)  $P < 0.001$ , analysis of variance with respect to GlyR  $\alpha 1$ .



**Fig. 4.** Molecular elements downstream of the TM3-TM4 intracellular domain negatively regulate the ethanol sensitivity of  $\alpha 3$  GlyRs. (A) Schematic representations of the chimeras GlyR  $\alpha 3-\alpha 1$  and  $\alpha 3-\alpha 1^{A254G}$  created from  $\alpha 1$  (black) and  $\alpha 3$  (red) subunits. The 15 additional amino acids present in  $\alpha 3L$  are displayed in blue, and the A254G mutation in TM2 is indicated by a black circle. (B) The graph shows the concentration-response relationship for wild-type and both chimeric GlyRs. (C) Representative current traces showing the effect of 100 mM ethanol on chimeras GlyR  $\alpha 3-\alpha 1$  and  $\alpha 3-\alpha 1^{A254G}$ . (D) The graph summarizes the effects of 100 mM ethanol on wild-type  $\alpha 1$ ,  $\alpha 3$ , and chimeric GlyRs. (E) Modulation of chimeric GlyRs after 15 minutes of intracellular dialysis with GTP $\gamma$ S. For all panels, the results are the mean  $\pm$  S.E.M. from normalized glycine-activated currents. Differences were significant for  $\alpha 1$  ( $***P < 0.001$ , analysis of variance).

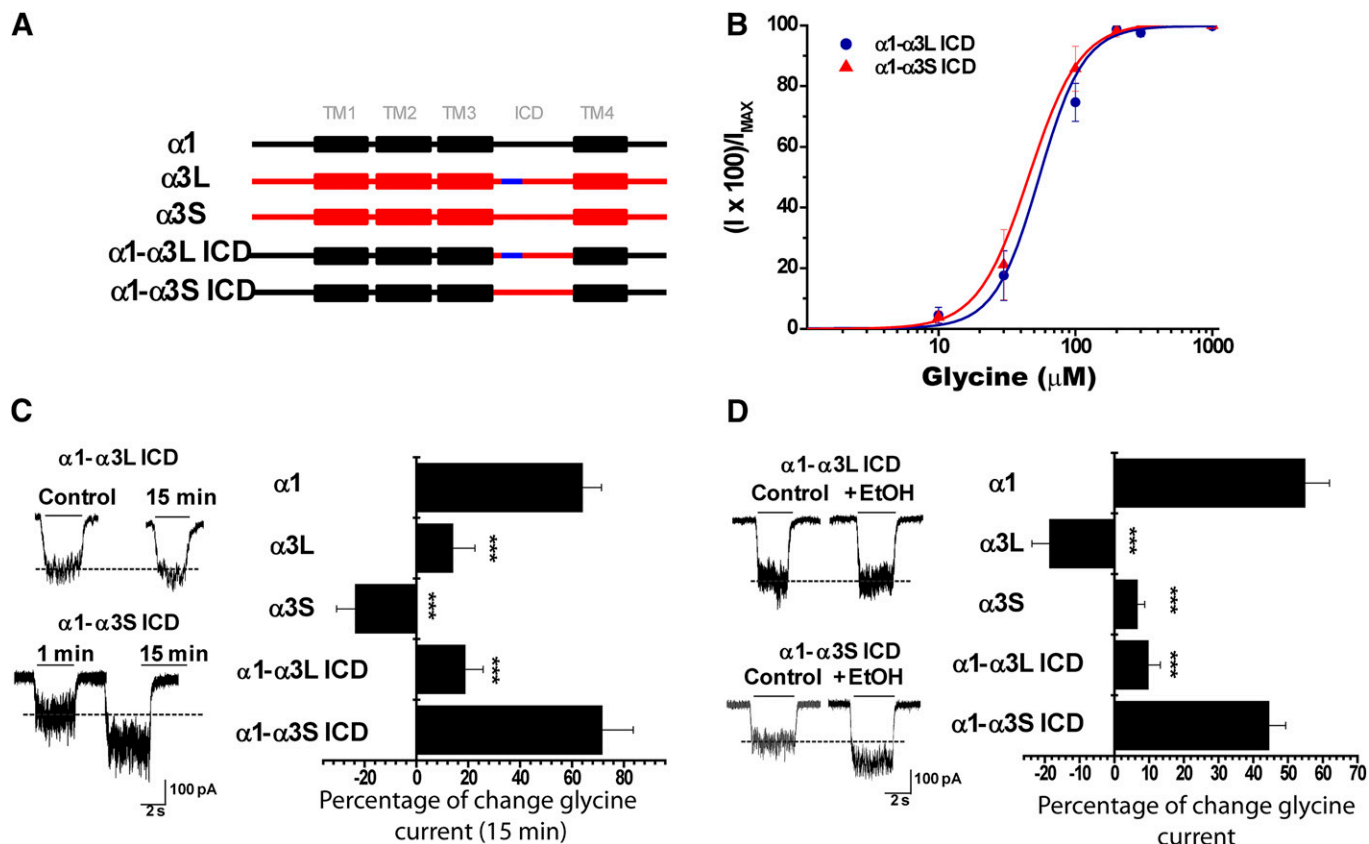
activation ( $18 \pm 3\%$ ), despite having glycine sensitivity closer to the values of wild-type  $\alpha 1$  subunit GlyRs (Fig. 5, B–D; Table 1). Interestingly, the  $\alpha 1-\alpha 3S$  ICD GlyR construct showed a significant potentiation of the glycine-evoked currents after 15 minutes of dialysis with GTP $\gamma$ S ( $72 \pm 17\%$ ) and following the application of ethanol ( $45 \pm 5\%$ ) (Fig. 5, C and D). Since the only difference between these two constructs is the presence of the alternative spliced 15-amino-acid cassette within the TM3-TM4 domain in  $\alpha 3L$  GlyRs (Nikolic et al., 1998), these data suggest a negative regulatory role of the  $\alpha 3L$  cassette in ethanol sensitivity and G $\beta\gamma$  modulation. To further study the role of the  $\alpha 3L$  cassette on the GlyR pharmacology, we inserted it into a homologous position in the  $\alpha 1$  GlyR ICD ( $\alpha 1+\alpha 3L$  INS) (Fig. 6A). Notably, the presence of the  $\alpha 3L$  cassette in  $\alpha 1$  GlyRs significantly reduced the enhancement of the glycine-activated current by activation of G proteins ( $-1 \pm 7\%$ ) and ethanol ( $5 \pm 6\%$ ) (Fig. 6, C and D). Collectively, these results described an unrecognized intracellular element involved in the low ethanol sensitivity displayed by  $\alpha 3L$  GlyRs and demonstrate the critical influence of intracellular elements on alcohol pharmacology.

These results, however, still do not explain the low ethanol potentiation displayed by the  $\alpha 3S$  and the  $\alpha 3S^{A254G}$  mutants (Fig. 3), which lack the  $\alpha 3L$  splice cassette. Therefore, additional molecular determinants within the TM4 or the C-terminal region could be negatively influencing the alcohol pharmacology of  $\alpha 3$  subunit GlyRs.

**Importance of the C-Terminal Region on the Ethanol Sensitivity and G $\beta\gamma$  Modulation of  $\alpha 3$  GlyRs.** Several lines of evidence have demonstrated the importance of the TM4 domain in the Cys-loop family. Early studies on nAChRs suggested regulatory roles of the TM4 domain for their structure and function (Ortiz-Miranda et al., 1997). For example, mutations in the  $\alpha$ - and  $\beta$ -subunit TM4 domains, which are postulated to be in close proximity to the lipid-protein interface, significantly altered ion channel gating (Ortiz-Miranda et al., 1997). More recent studies demonstrated additional critical

roles of aromatic residues within the TM4 in the pentameric assembly and function of GlyRs (Haeger et al., 2010). Interestingly, other reports have detected residues in TM4 involved in the ethanol potentiation of  $\alpha 1$  GlyRs (Lobo et al., 2006). Thus, it is plausible that the TM4 domain may play a role in the low alcohol sensitivity of  $\alpha 3$  GlyRs. Evaluation of the primary sequences of  $\alpha 1$  and  $\alpha 3$  GlyRs revealed  $\sim 70\%$  amino acid identity, although most of the critical residues described for  $\alpha 1$  GlyRs (Lobo et al., 2008) were fully conserved. To test for a possible role of TM4 in the low alcohol sensitivity of  $\alpha 3$  GlyRs, we incorporated the TM4 domain into the construct  $\alpha 1-\alpha 3S$  ICD ( $\alpha 1-\alpha 3S$  ICD TM4) (Fig. 7A). The data show that incorporation of the TM4 domain of GlyR  $\alpha 3$  did not affect the ethanol sensitivity ( $49 \pm 5\%$ ) or G protein modulation ( $73 \pm 10\%$ ) (Fig. 7, C and D), excluding a negative influence of  $\alpha 3$  TM4 on the alcohol potentiation. We therefore focused our attention toward the putative extracellular C terminus.

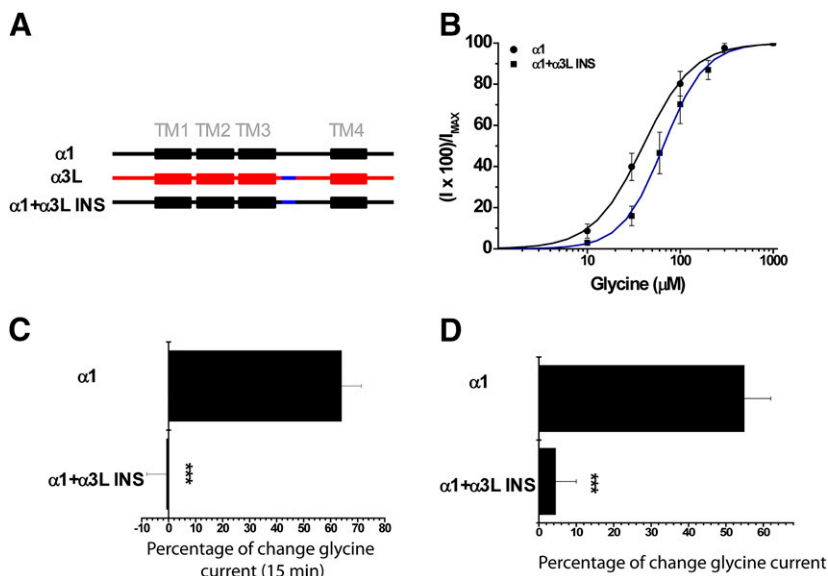
The exact role of the extracellular C terminus in the Cys-loop physiology and pharmacology has remained elusive. Nevertheless, studies on nAChRs have suggested that residues at the external end of TM4 may interact with the ligand binding domain via either an electrostatic interaction (Xiu et al., 2005) or protein-sugar interactions (Dellisanti et al., 2007), contributing to the gating of the ion channel. However, the influence of the C-terminal region on GlyR pharmacology is unknown. The amino acid sequences of the putative C terminus of GlyR  $\alpha 1$  and  $\alpha 3$  are not highly conserved (64% amino acid identity), raising the question of whether these differences may play a role in GlyR alcohol pharmacology. Therefore, we incorporated the C terminus (CT) of GlyR  $\alpha 1$  into the template of the ethanol-resistant construct  $\alpha 3S^{A254G}$  ( $\alpha 3S^{A254G}-\alpha 1_{CT}$ ) (Fig. 7A). Notably, this GlyR mutant displayed a potentiation of the glycine-activated currents with 100 mM ethanol ( $40 \pm 6\%$ ) together with a current enhancement after G protein activation ( $68 \pm 25\%$ ) without changes in apparent affinity for glycine (Fig. 7, B–D). Finally, to analyze the structural relationship of



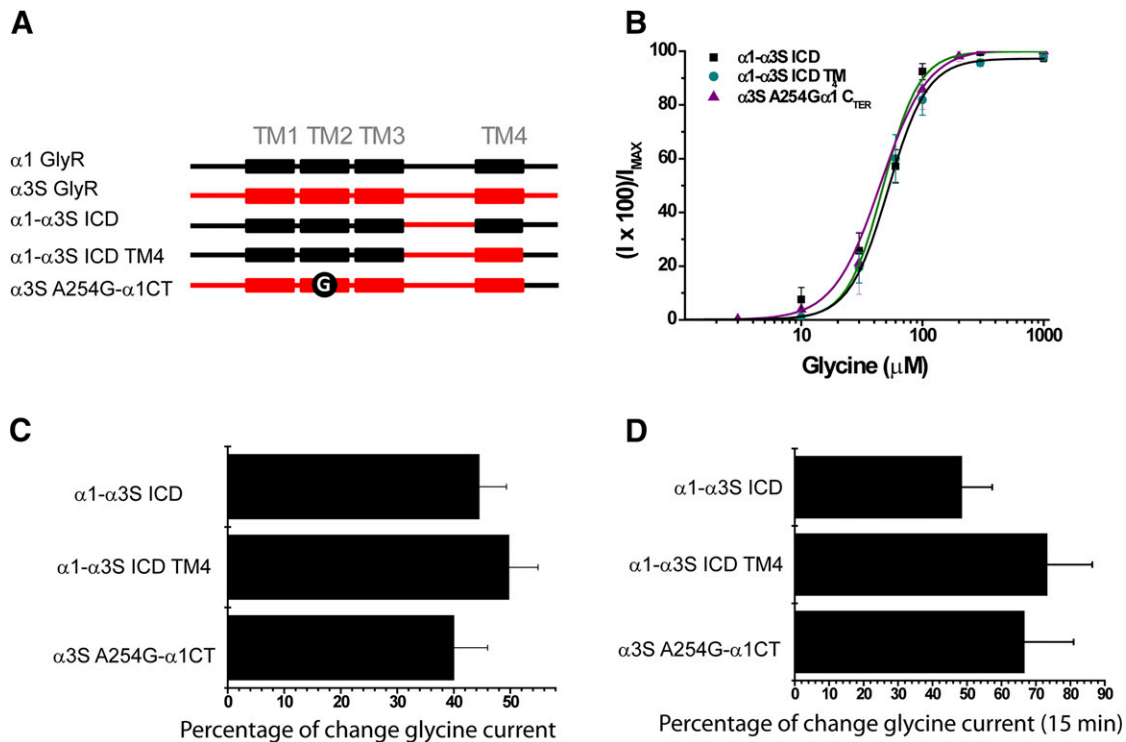
**Fig. 5.** The alternatively spliced 15 amino acid cassette within the TM3-TM4 ICD of  $\alpha 3L$  GlyRs is a negative regulator of ethanol sensitivity and G protein modulation. (A) Schematic representations of chimeric receptors made replacing the TM3-TM4 ICD of GlyR  $\alpha 1$  (black) by the corresponding region in GlyR  $\alpha 3$  (red). The blue region represents the 15-amino-acid insert present in  $\alpha 3L$ . (B) Concentration-response curves for  $\alpha 1$ - $\alpha 3L$  ICD using  $\alpha 3S$  and  $\alpha 3L$  isoforms. (C) Representative glycine-evoked current traces at the initial time and after 15 minutes of intracellular dialysis using GTP $\gamma$ S on chimeric GlyR  $\alpha 1$ - $\alpha 3S$  or  $\alpha 1$ - $\alpha 3L$  ICDs. The graph summarizes the effects of GTP $\gamma$ S on wild-type  $\alpha 1$ ,  $\alpha 3$  subunits and chimeric receptors GlyR  $\alpha 1$ - $\alpha 3S$  or  $\alpha 1$ - $\alpha 3L$  ICDs. (D) Representative current traces in the absence and presence of 100 mM ethanol. The graph summarizes the modulation of wild-type GlyR  $\alpha 1$ ,  $\alpha 3$  subunits and chimeric GlyR  $\alpha 1$ - $\alpha 3S$  or  $\alpha 1$ - $\alpha 3L$  ICDs by ethanol. For all panels, the results are the mean  $\pm$  S.E.M. from normalized glycine-activated currents. Differences were significant for  $\alpha 1$  (\*\*\*)  $P < 0.001$ , analysis of variance).

the C terminus to ethanol sensitivity, in silico prediction showed differences in the C-terminal region for GlyR  $\alpha 1$  and  $\alpha 3$ . The structural alignment, including TM4 to the C terminus, showed a deviation in the helical structure of  $\alpha 3$  with respect to

$\alpha 1$  (Fig. 8A), which combined with the greater length of the C-terminal segment generated a “less packed” structure for  $\alpha 3$ , associated with an increase in the accessible surface area compared with  $\alpha 1$  (Fig. 8B). Additionally, in the model of  $\alpha 3$ ,



**Fig. 6.** The presence of  $\alpha 3L$  INS sequence blunted the ethanol potentiation and G protein modulation of  $\alpha 1$  GlyRs. (A) Schematic representation of chimera  $\alpha 1$ - $\alpha 3L$  INS created by insertion of 15 amino acids (blue) of  $\alpha 3L$  (red) into the  $\alpha 1$  GlyR subunit (black). (B) Concentration-response curves for wild-type GlyR  $\alpha 1$  and  $\alpha 1$  +  $\alpha 3L$ . (C) Modulation of chimera  $\alpha 1$  +  $\alpha 3L$  by intracellular dialysis of GTP $\gamma$ S. (D) Effects of 100 mM ethanol on activity of chimera  $\alpha 1$  +  $\alpha 3L$ . For all panels, the results are the mean  $\pm$  S.E.M. from normalized glycine-activated currents. Differences were significant with respect to  $\alpha 1$  (\*\*\*)  $P < 0.001$ , analysis of variance).



**Fig. 7.** The C terminus, but not the TM4 domain, influences the ethanol sensitivity and G protein modulation of GlyR  $\alpha 3$ . (A) Schematic representations of chimeric receptors created using  $\alpha 1$  (black) and  $\alpha 3S$  (red) subunits,  $\alpha 1$ - $\alpha 3S$  ICD generated by replacing only the ICD,  $\alpha 1$ - $\alpha 3S$  ICD TM4 which includes the TM4 of  $\alpha 3S$  and the C-terminal domain of  $\alpha 1$ , and  $\alpha 3S$ - $\alpha 1CT$  A254G. (B) Concentration-response curves for all chimeric GlyRs described previously. (C) The graph summarizes the potentiation of glycine-evoked currents by 100 mM ethanol on chimeric GlyRs. (D) Modulation of chimeric receptors by GTP $\gamma$ S after intracellular dialysis for 15 minutes. For all panels, the results are the mean  $\pm$  S.E.M. from normalized glycine-activated currents. No differences were detected.

alanine 254 is found at a similar position to G254 on  $\alpha 1$ , and both are facing the inner side of TM2, explaining its critical role in the modulation of glycine receptors by ethanol and G protein (Fig. 8B). Finally, we tested the impact of the C terminus on the  $\alpha 1$  subunit by truncating this region in  $\alpha 1\Delta CT$ . Interestingly, the data showed that this receptor expressed a functional ion channel that was equally sensitive to ethanol as the  $\alpha 1$  wild type ( $51 \pm 8\%$ ) (Table 1). Taken together, our results demonstrate that the C terminus in GlyR  $\alpha 3$ , but not in  $\alpha 1$ , is another structural element that determines the low ethanol sensitivity of  $\alpha 3$  glycine receptors, and suggest a pivotal role of C-terminal domains in alcohol pharmacology of some GlyRs.

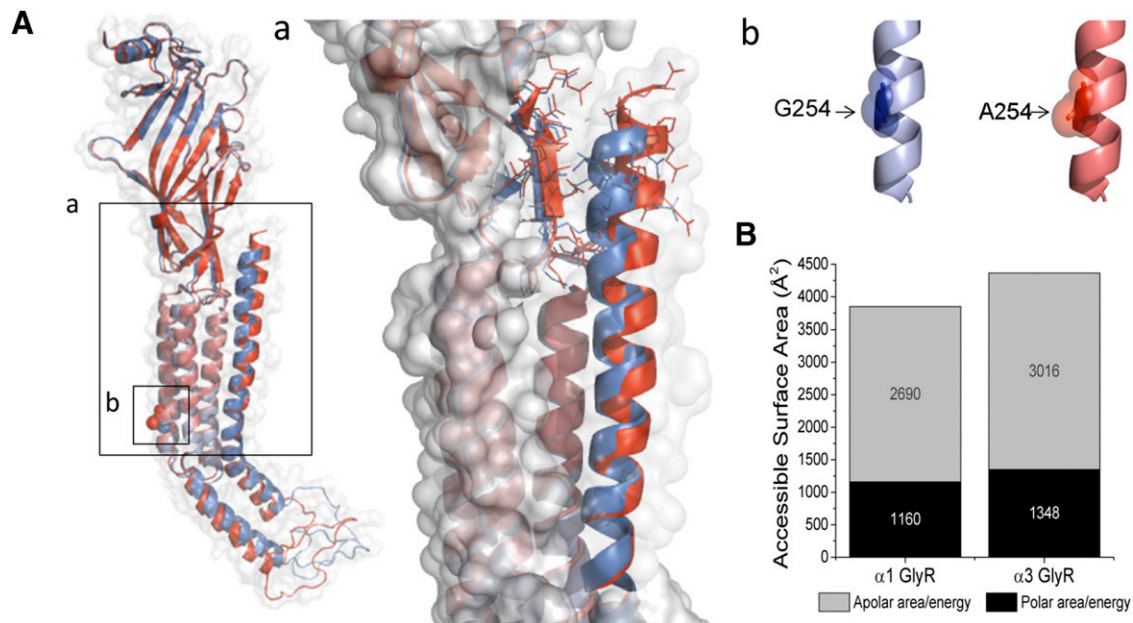
## Discussion

The wide range of ethanol effects on GlyRs have been explained by two hypotheses: 1) binding of alcohol within the ion channel (Mihic et al., 1997) or 2) indirect regulation of the channel caused by alcohol modulation of signal transduction pathways (Yevenes et al., 2006, 2008, 2010). A binding pocket formed by transmembrane residues I229 in TM1 (Lobo et al., 2008), S267 in TM2 (Mihic et al., 1997), A288 in TM3 (Mihic et al., 1997), and I409, Y410, and K411 in TM4 (Lobo et al., 2006) has been proposed to play a key role in alcohol binding to GlyRs. Residue A52 is another relevant amino acid, transferring binding energy into channel opening. Nevertheless, the lack of effect of ethanol on GlyR  $\alpha 3$  cannot be explained by these elements, since all of these residues are conserved within the GlyR  $\alpha 3$  subunit.

The second possibility postulates that ethanol modulates GlyR by an indirect mechanism through the activation or inhibition of intracellular signals. The most widely studied and recognized pathways involved in intracellular regulation of ligand-gated ion channel function involve phosphorylation through protein kinases. Indeed, the function of GlyR and other members of the ligand-gated ion channel superfamily is affected by activation of cAMP-dependent kinases and protein kinase C (Smart, 1997; Harvey et al., 2004). In this context, GlyR  $\alpha 3$  is known to be negatively modulated by phosphorylation of S346 by protein kinase A (PKA) (Harvey et al., 2004). Interestingly, the GlyR  $\alpha 1$  subunit does not have this PKA phosphorylation site, and the addition of the  $\alpha 3L$  cassette (S346 lies outside this region) prevented the functional modulation of GlyR  $\alpha 1$  by alcohol. Furthermore, an equivalent PKA phosphorylation site is present in GlyR  $\alpha 2$ , where mutations upstream of TM3 confer ethanol and  $G\beta\gamma$  modulation (Yevenes et al., 2010). In summary, phosphorylation by PKA does not seem to be important for ethanol action on GlyRs.

Besides the differences in protein sequence, the GlyR  $\alpha 3$  subunit exhibits variants generated by alternative splicing that confer differential properties in the regulation by protein kinases (Lynch, 2004), extent and time course of desensitization (Breitinger et al., 2002), structural properties (Breitinger et al., 2009), ion channel gating (Breitinger et al., 2009), and receptor clustering and diffusion (Notelaers et al., 2012). In this framework, we analyzed the involvement of the intracellular domain and the GlyR  $\alpha 3L$  cassette in ethanol and  $G\beta\gamma$  modulation. We found that the  $\alpha 3L$  cassette conferred loss of





**Fig. 8.** Molecular modeling of GlyR  $\alpha 1$  and  $\alpha 3$ . (A) Structural alignment between  $\alpha 1$  (blue) and  $\alpha 3$  (red) including the magnification of the segment TM4–C terminus and the complementary region of extracellular domain showing the residues involved in structural stabilization (a). The TM2 residues described in this study are also highlighted (b). (B) Calculation of accessible solvent surface for both GlyR subunits supporting the idea of a “less packed” conformation for GlyR  $\alpha 3$ .

ethanol and  $G\beta\gamma$  modulation on the GlyR  $\alpha 1$  subunit (Fig. 6). These results highlight a new role for the  $\alpha 3L$  cassette as a negative regulatory domain for this allosteric modulator. This negative regulation can be explained by differences in intracellular domain structural flexibility, since the presence of the L-insert facilitates an  $\alpha$ -helix structure to this region, not present in the  $\alpha 3S$  isoform (Breitinger et al., 2009). These results suggest that the lack of sensitivity of  $\alpha 3$  is dependent on the presence of the  $\alpha 3L$  cassette and molecular requirements downstream of the ICD. We also found that both GlyR  $\alpha 1$  and  $\alpha 3$  bind  $G\beta\gamma$  (Fig. 2), but only the GlyR  $\alpha 1$  conformation allowed an effective conversion of  $G\beta\gamma$  binding into functional allosteric modulation (Yevenes et al., 2006, 2008, 2010).

Three residues were described as necessary for  $G\beta\gamma$  modulation and ethanol sensitivity in GlyR  $\alpha 1$  and  $\alpha 2$  subunits (Yevenes et al., 2010): residue A52 in loop 2 of the extracellular domain (Yevenes et al., 2010; Perkins et al., 2012), G254 in TM2 (Mihic et al., 1997; Yevenes et al., 2010), and S296 in TM3 (Yevenes et al., 2010) (using GlyR  $\alpha 1$  numbering). We proposed that they allow a molecular transition between a resting closed state and a preopened closed state (denominated “flipped” state) of the glycine-bound GlyR (Plested et al., 2007) facilitating ion-channel opening after  $G\beta\gamma$  binding (Yevenes et al., 2010). Here, we found that G254 in  $\alpha 3$  was required, but not sufficient, for ethanol sensitivity and  $G\beta\gamma$  modulation of homomeric  $\alpha 3$  GlyRs (Figs. 3 and 4). Because G254 is important for gating properties, the presence of an alanine residue in  $\alpha 3$  possibly interferes with the structural rearrangements associated with the binding of  $G\beta\gamma$ , similar to that in GlyR  $\alpha 2$  (Yevenes et al., 2010). The replacement of alanine by glycine in residue 254 contributes to a partial recovery of ethanol sensitivity (Fig. 3B), but only additional modifications allow complete recovery.

Participation of the TM4 and extracellular C terminus in GlyR physiology and pharmacology is largely unknown. It was

recently proposed that TM4 and the C-terminal segment are involved in some physiologic and structural properties of GlyRs (Villmann et al., 2009). Furthermore, electrophysiological studies have shown that mutations in TM4 residues can influence the gating of nAChRs and  $\alpha 1$  GABA<sub>A</sub> receptors (Jenkins et al., 2002), and even produce nonfunctional channels in AChR and GlyR (Haeger et al., 2010). Additionally, it was suggested that residues at the C terminus of TM4 in nAChR may interact with the ligand binding domain via either an electrostatic interaction (Xiu et al., 2005) or protein-sugar interactions (Dellisanti et al., 2007) contributing to channel gating (Xiu et al., 2005). Interestingly, the alignment of TM4 in GlyR  $\alpha 1$  and  $\alpha 3$  subunits showed nine nonconserved residues, in addition to the two residues present at the C terminus, which could be responsible for the insensitivity to modulation by  $G\beta\gamma$  and ethanol. The present results discard TM4 as a negative influence on GlyR ethanol and  $G\beta\gamma$  modulation (Fig. 7C). This result differs from other studies that have suggested that this region is important for closed and glycine-activated states (Han et al., 2013), indicating that these findings might be specific for GlyR  $\alpha 1$  (Chen et al., 2009; Han et al., 2013). Furthermore, no significant differences in  $EC_{50}$  and  $I_{max}$  parameters were found in the chimeric receptors with changed TM4 or C-terminal domains ( $\alpha 1$ - $\alpha 3S$  ICD TM4,  $\alpha 3S$  A254G- $\alpha 1_{CT}$ ), and they were modulated by ethanol and  $G\beta\gamma$  (Fig. 7). Thus, we propose that the difference in ethanol and  $G\beta\gamma$  modulation is associated with a different degree of proximity or structural complementarity between TM4 and the C terminus with extracellular domains. Through predictive molecular modeling studies and comparative analysis between GlyR  $\alpha 1$  and  $\alpha 3$ , we determined that  $\alpha 3$  presents a shift in its helical structure (TM4 and C terminus) (Fig. 8A) in agreement with a previous study (Han et al., 2013). This structural difference in  $\alpha 3$  would produce a conformation less favorable for  $G\beta\gamma$  and ethanol modulation, and it is related to an increase

in the volume of the  $\alpha 3$  C-terminal region caused by the presence of larger side chains of the amino acids HQQD located at the sequence end of  $\alpha 3$ , accompanied by an increase in the accessible surface area of  $\alpha 3$  compared with  $\alpha 1$  (Fig. 8B). Along with this, replacement of the  $\alpha 3$  C terminus by its corresponding segment of  $\alpha 1$  in the chimeras  $\alpha 3$ - $\alpha 1$  A254G and  $\alpha 3$ S- $\alpha 1$ <sub>CT</sub> A254G returns the complementarity between the C-terminal and extracellular domains being modulated by ethanol and G protein (Figs. 4 and 7). The situation is quite different for  $\alpha 1$  since the elimination of its C terminal ( $\alpha 1$ ACT) did not affect its sensitivity to ethanol. This finding suggests that the C terminal of  $\alpha 1$  affects the overall properties of  $\alpha 3$ , and that ethanol modulation depends on diverse regions along this protein.

In a physiologic context, GlyRs containing  $\alpha 3$  participate in the spinal component of inflammatory hyperalgesia (Ahmadi et al., 2002; Harvey et al., 2004). They are also found in Botzinger complex, Pre-Botzinger complex, and spinal trigeminal nucleus in the brainstem where they appear to control rhythmic activity of respiratory networks (Manzke et al., 2010). Overall, our data support the conclusion that GlyRs containing  $\alpha 3$  in these locations are relatively insensitive to ethanol and G $\beta\gamma$  modulation. We also conclude that residue G254, a key element for ethanol sensitivity and G $\beta\gamma$  modulation in GlyR  $\alpha 1$  and  $\alpha 2$  (Yevenes et al., 2010), is necessary but not sufficient for ethanol sensitivity and G $\beta\gamma$  modulation of  $\alpha 3$  GlyRs. Rather, we found that the  $\alpha 3$ L cassette and the extracellular C terminus are novel determinants for ethanol sensitivity and G $\beta\gamma$  modulation for GlyRs containing  $\alpha 3$ .

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#### Authorship Contributions

Participated in research design: Sánchez, Burgos, Yévenes, Aguayo.

Conducted experiments: Sánchez, San Martín, Yévenes, Moraga-Cid.

Contributed new reagents or analytic tools: Harvey.

Performed data analysis: Sánchez, San Martín, Moraga-Cid, Burgos.

Wrote or contributed to the writing of the manuscript: Sánchez, Burgos, Yévenes, Harvey, Aguayo.

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