Asymmetric contribution of α and β subunits to the activation of αβ heteromeric glycine receptors

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Abstract

This study investigated the role of β subunits in the activation of αβ heteromeric glycine receptor (GlyR) chloride channels recombinantly expressed in HEK293 cells. The approach involved incorporating mutations into corresponding positions in α and β subunits and comparing their effects on receptor function. Although cysteine-substitution mutations to residues in the N-terminal half of the α subunit M2–M3 loop dramatically impaired the gating efficacy, the same mutations exerted little effect when incorporated into corresponding positions of the β subunit. Furthermore, although the α subunit M2–M3 loop cysteines were modified by a cysteine-specific reagent, the corresponding β subunit cysteines showed no evidence of reactivity. These observations suggest structural or functional differences between α and β subunit M2–M3 loops. In addi-

tion, a threonine → leucine mutation at the 9′ position in the β subunit M2 pore-lining domain dramatically increased the glycine sensitivity. By analogy with the effects of the same mutation in other ligand-gated ion channels, it was concluded that the mutation affected the GlyR activation mechanism. This supports the idea that the GlyR β subunit is involved in receptor gating. In conclusion, this study demonstrates that β subunits contribute to the activation of the GlyR, but that their involvement in this process is significantly different to that of the α subunit.

Keywords: allosteric interactions, gating, glycine receptor chloride channel, receptor structure and function, substituted cysteine accessibility method.

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Glycine receptor chloride channels (GlyRs) mediate inhibitory neurotransmission in the spinal cord and brainstem. It is a member of the pentameric ligand-gated ion channel gene family which also includes the nicotinic acetylcholine receptor cation channel (nAChR), the serotonin type 3 receptor, the γ-aminobutyric acid type A receptor (GABA$_A$R), as well as invertebrate glutamate and histidine receptors (Gisselmann *et al.* 2002), GlyRs in adult rats comprise heteromers of α and β subunits expressed in a 3α:2β stoichiometry (Langosch *et al.* 1988). Since α subunits efficiently form homomeric receptors in heterologous expression systems, they provide a convenient model system for investigating the relationship between receptor structure and function (reviewed in Rajendra *et al.* 1997). The role of the β subunit, which has a 47% amino acid homology with the α1 subunit, has received considerably less scrutiny. The aim of the present study is to determine whether GlyR α and β subunits have symmetrical or near symmetrical roles in the activation of the receptor.

GlyR function is affected by β subunits in a variety of ways. For example, although the GlyR β subunit does not

express as a homomer, it increases the expression efficiency of functional GlyRs when coexpressed with the α subunit (Grenningloh *et al.* 1990; Bormann *et al.* 1993). A specific 20-amino-acid motif in the β subunit M3-M4 intracellular loop is proposed to be the gephyrin-binding domain (Meyer *et al.* 1995). Gephyrin anchors GlyRs to the subsynaptic cytoskeleton thereby facilitating GlyR clustering at the postsynaptic membrane (Kuhse *et al.* 1995). Apart from its clustering role, GlyR β subunits have also been shown to affect other aspects of receptor function. For example, they

influence ion permeation (Bormann *et al.* 1993) and affect the sensitivity of the receptor to glycine (Rees *et al.* 2002), tropisetron (Supplisson and Chesnoy-Marchais 2000), neurosteroids (Maksay *et al.* 2001), cyanotriphenylborate (Rundstrom *et al.* 1994) and picrotoxin analogs (Steinbach *et al.* 2000; Shan *et al.* 2001). The effects observed could have resulted from β subunit-mediated changes in binding affinities of these ligands. To date, there is no evidence that β subunits participate in the allosteric conformational changes that lead to the activated state.

This study compared the contributions of α and β subunits to GlyR activation using three different assays. First, the GlyR α subunit M2–M3 loop is a critical element of the signal transduction pathway linking the agonist binding site to the channel activation gate. Consistent with this role, mutations to residues in this domain have large effects on the agonist EC_{50} values (Lynch *et al.* 1997). In addition, a substituted cysteine accessibility scan demonstrated that the surface exposure of residues in this domain varied between the channel closed and open states (Lynch *et al.* 2001). The present study investigated whether this method could identify a similar role for the β subunit M2–M3 loop in the agonist transduction process. Second, Shan *et al.* (2001; 2002) recently showed that a cysteine introduced into the 6′ position of the α subunit M2 domain reacted with the cysteine-modifying agent, methanethiosulfonate ethyltrimethylammonium (MTSET), in the open state only. The present study sought to determine whether the corresponding cysteine substitution in the β subunit exhibited a similar state-dependent MTSET reactivity. Finally, it has been shown in recombinantly expressed ααβδγ nAChRs (Filatov and White 1995; Labarca *et al.* 1995) and ααββγ GABA$_A$Rs (Chang and Weiss 1999; Thompson *et al.* 1999), that mutations to 9′ leucines have a similar effect on agonist sensitivity regardless of whether they are introduced into agonist-binding or nonagonist-binding subunits. We employed a similar approach in the GlyR to investigate a role for the β subunit in receptor activation. Together, the results show that the β subunit is involved in receptor activation but that its contribution is significantly different to that of the α subunit.

Experimental procedures

Mutagenesis and expression of GlyR cDNAs

The human GlyR α1 and β subunit cDNAs were subcloned into the pCIS2 and pIRES2-EGFP plasmid vectors (Clontech, Palo Alto, CA, USA), respectively. Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA, USA) and the successful incorporation of mutations was confirmed by sequencing the clones. Adenovirus-transformed human embryonic kidney 293 cells (ATCC CRL 1573) were passaged in a 50 : 50 mixture of minimum essential medium and Dulbecco's modified Eagle's medium supplemented with 2 mM glutamate, 10% fetal calf serum and the antibiotics, penicillin 50 IU/mL and streptomycin 50 μg/mL (GibcoBRL, Grand Island, NY, USA). Cells were transfected using a calcium phosphate precipitation protocol. When cotransfecting the GlyR α and β subunits, their respective cDNAs were combined in a ratio of 1 : 10. After exposure to transfection solution for 24 h, cells were washed twice using the culture medium and used for recording over the following 24–72 h.

Electrophysiology

The cells were observed using a fluorescent microscope and currents were measured using the whole cell patch-clamp configuration. Cells were perfused by a control solution that contained (in mM): 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, with the pH adjusted to 7.4 with NaOH. Patch pipettes were fabricated from borosilicate hematocrit tubing (Vitrex, Modulohm, Denmark) and heat polished. Pipettes had a tip resistance of 1.5–3 MΩ when filled with the standard pipette solution which contained (in mM): 145 CsCl, 2 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, 10 EGTA, with the pH adjusted to 7.4 with NaOH. After establishment of the whole cell configuration, cells were voltage-clamped at −40 mV and membrane currents were recorded using Axopatch 1D amplifiers and pCLAMP6 or 7 software (Axon Instruments, Foster City, CA, USA). Cells were perfused by solutions which were gravity-induced through a parallel array of microtubular barrels.

Because the α subunit can efficiently assemble into functional GlyRs as either α homomers or αβ heteromers, green fluorescent protein expression was used to identify cells expressing GlyR β subunits. The incorporation of β subunits into heteromeric GlyRs was functionally confirmed by picrotoxin sensitivity (Pribilla *et al.* 1992). When coexpressed with α subunits in HEK293 cells, incorporation of β subunits increased the picrotoxin IC50 from around 25 to 500 μM in the presence of an EC_{50} glycine concentration (Handford *et al.* 1996). In the present study, we measured the effect of 1 mM picrotoxin on the magnitude of currents activated by an EC_{30} glycine concentration and cells were assumed to express αβ heteromeric GlyRs if 1 mM picrotoxin inhibited the current by less than 50%. Note that β subunit expression results confers an invariant 3α:2β stoichiometry onto all expressed GlyRs (Kuhse *et al.* 1993).

Dithiothreitol (DTT) was prepared daily as a 1-mM concentration in the standard bathing solution. MTSET (from Toronto Research Chemicals, Toronto, Canada) was applied to cells immediately after being dissolved into room temperature bathing solution. The following procedures were used to test its effects. After establishment of the recording configuration, cells were bathed in 1 mM DTT for 1 min to ensure that exposed sulfhydryl groups were fully reduced. Then the glycine dose–response was measured by applying increasing glycine concentrations at 1 min intervals. Following this, three consecutive brief applications of an EC_{30} and an EC_{100} glycine concentration were applied at 1 min intervals to establish that the current magnitude was invariant. Provided current amplitude remained constant (± 5%), the averaged EC_{30} and an EC_{100} current amplitudes were used as the controls. Following MTSET application, cells were washed in control solution for at least 2 min before the current magnitudes were measured again. In long-term patch recordings, MTSET-induced changes were found to be invariant for periods as long as 40 min although they were rapidly and completely reversed by a 1-min exposure to 1 mM DTT (Lynch

et al. 2001). This strongly suggests that the changes were due to covalent modification of introduced cysteines. It is estimated that a 10% irreversible change in current over 1 min would have been reliably detected. The percentage current change following MTSET exposure was calculated as

$$(I_{glycine,\ after}/I_{glycine,\ before} - 1) \times 100\%$$

If an irreversible effect was observed, MTSET was applied at a concentration of 100 μM to measure its reaction rate. As MTSET caused only potentiation in this study, the following single exponential function was fitted to the current response of individual cells:

$$I_{glycine,\ after} = I_{glycine,\ before} + ae^{-t/\tau}$$

where a is the scaling factor and τ is the reaction time constant. The reaction rate was then calculated as $1/(\tau * [MTSET])$. The receptor desensitization rate was low ($< 0.005\ s^{-1}$) for all mutant GlyRs used in this study and as such did not impact significantly on the measurement of MTSET reactivity rates.

Data analysis

Results are expressed as mean ± standard error of the mean (SEM) of three or more independent experiments. All the data were analyzed using Origin 4.0 (Northampton, MA, USA). The empirical Hill equation, fitted by a non-linear least squares algorithm, was used to calculate the EC_{50} and Hill coefficient (n_H) values for glycine activation. Statistical significance was determined by one-way ANOVA using the Students–Newmans–Keul *post hoc* test for unpaired data, with $p < 0.05$ representing significance.

Results

GlyR β subunit M2–M3 loop

We previously identified six consecutive cysteine-substituted residues in the α homomeric GlyR M2–M3 loop that displayed an irreversible current change following exposure to MTSET (Lynch *et al.* 2001). These residues are shown in bold in Fig. 1. In each of these mutant GlyRs, MTSET caused a shift in the glycine EC_{50} without affecting the saturating glycine-activated current. Furthermore, the MTSET reaction rate with each of the six cysteines was increased in the channel open state (Lynch *et al.* 2001). Taken together with previous results indicating that M2–M3 loop mutations dramatically changed the taurine agonist

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GlyR α1    270  S  R  A  S  L  P  K  V  S  Y  V  K  A  I  D

GlyR β     294  L  A  A  E  L  P  K  V  S  Y  V  K  A  L  D

                   C  C  C  C  C  C
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Fig. 1 Sequence alignment of the human GlyR α1 and β subunit M2–M3 loops. Residues in bold indicate the residues in the α1 subunit which react with MTSET when mutated individually to cysteines (Lynch *et al.* 2001). The letters *C* indicate the corresponding residues in the β subunit that were individually mutated to cysteines in the present study.

efficacy (Lynch *et al.* 1997), it was concluded that MTSET modification primarily affected the isomerization rate between the receptor closed and open states. A single channel kinetic study on α homomeric GlyRs incorporating the K276E mutation has reinforced this interpretation (Lewis *et al.* 1998).

In the present study, we mutated the corresponding β subunit M2–M3 loop residues individually to cysteines (as denoted by a *C* in Fig. 1) and determined their MTSET reactivity. To avoid possible non-specific effects of MTSET, it is preferable to remove the extracellular non cross-linked external cysteines in both subunits. This was achieved in the α subunit by incorporating the C41A mutation (Lynch *et al.* 2001) and α subunits incorporating this mutation are henceforth designated $α^\Delta$. The β subunit contains endogenous, presumably non cross-linked, external cysteines at positions 17, 115 and 291. Although both the C115A and C291A mutations were well tolerated and had no effect on the glycine EC_{50} (not shown), the C17A mutation precluded the functional expression of the β subunit. Thus, it was not possible to eliminate C17, and β subunits incorporating both the C115A and C291A mutations are henceforth designated as $β^\Delta$.

Although α subunits form functional homomeric GlyRs, β subunits are expressed only as heteromers with the α subunit in a 3α : 2β stoichiometry (Langosch *et al.* 1988). The EC_{50}, n_H and peak current magnitude (I_{max}) values for $α^\Delta$ GlyRs, $α^\Delta β^\Delta$ GlyRs and heteromers incorporating $β^\Delta$ subunit cysteine substitution mutations are shown in Table 1. These results indicate that the $β^\Delta$ subunit M2–M3 loop cysteine mutations had minimal effects on the glycine EC_{50} values. The MTSET reactivity of each mutant GlyR was assessed by applying 1 mM MTSET in the presence of 30 μM glycine. This glycine concentration corresponded to the $EC_{30} - EC_{50}$ value, depending on the location of the mutation. MTSET was applied for 30–60 s which is sufficient time for the reaction to reach steady-state in $α^\Delta$ GlyRs incorporating mutations at the homologous positions (Lynch *et al.* 2001). As shown in the examples in Fig. 2(a) and summarized in Fig. 2(b), MTSET had no measurable effect on GlyRs incorporating cysteines at any of the six tested $β^\Delta$ subunit M2–M3 loop positions. This contrasts dramatically with the large irreversible effects of MTSET observed in $α^\Delta$ homomeric GlyRs incorporating cysteines in the corresponding positions (Lynch *et al.* 2001).

The difference may have been due to a 'cysteine dosage' effect: the αβ heteromers contain only two introduced cysteines whereas the α homomers contain five. To investigate the relationship between the magnitude of the MTSET effect and the number of cysteines per receptor, we varied the number of introduced cysteines at the S273 position in the $α^\Delta$ homomeric GlyR and at the corresponding position (E297) of the $β^\Delta$ subunit. As previously demonstrated (Lynch *et al.* 2001), the $α^\Delta_{S273C}$ GlyR responded to MTSET exposure via an

Table 1 Summary of the functional properties of WT and mutant GlyRs

Receptor	I_{max} (nA)	EC_{50} (μM)	n_H	n
α^Δ	2.1 ± 1.0	22 ± 5	3.1 ± 0.3	7
$\alpha^\Delta\beta^\Delta$	2.6 ± 0.7	25 ± 4	2.7 ± 0.3	8
$\alpha^\Delta\beta^\Delta_{A295C}$	2.0 ± 0.3	40 ± 4	2.2 ± 0.4	5
$\alpha^\Delta\beta^\Delta_{A296C}$	2.6 ± 0.8	35 ± 2	2.5 ± 0.3	4
$\alpha^\Delta\beta^\Delta_{E297C}$	2.7 ± 1.1	76 ± 7	1.8 ± 0.3	4
$\alpha^\Delta\beta^\Delta_{L298C}$	4.5 ± 1.6	51 ± 2	2.3 ± 0.2	4
$\alpha^\Delta\beta^\Delta_{P299C}$	1.9 ± 0.6	51 ± 1	1.7 ± 0.5	5
$\alpha^\Delta\beta^\Delta_{K300C}$	2.0 ± 0.7	27 ± 3	2.2 ± 0.5	4
$\alpha^\Delta_{T6'C}$	1.4 ± 0.3	52 ± 1	1.6 ± 0.1	4
$\alpha^\Delta_{T6'C}\,\beta^\Delta$	2.4 ± 0.6	38 ± 8	2.1 ± 0.2	4
$\alpha^\Delta\beta^\Delta_{F6'C}$	1.7 ± 0.2	57 ± 9	2.6 ± 0.3	3
$\alpha^\Delta_{T6'C}\,\beta^\Delta_{F6'C}$	1.9 ± 0.6	34 ± 5	2.2 ± 0.3	4
α_{T204A}	3.1 ± 0.5	6400 ± 2900	2.5 ± 0.8	4
$\alpha_{WT}\,\beta_{L9'T}$	1.4 ± 0.4	4.1 ± 1.2	1.2 ± 0.2	5
$\alpha_{T204A}\,\beta_{WT}$	5.0 ± 1.5	3600 ± 1500	1.8 ± 0.2	3
$\alpha_{T204A}\,\beta_{L9'T}$	1.1 ± 0.1	264 ± 77	1.2 ± 0.2	3

irreversible increase current activated by a 30-μM (EC_{30}) glycine concentration but with no effect in the presence of a 600-μM (EC_{100}) glycine concentration (Fig. 3a, right panel). This effect was reversed by a 1-min application of 1 mM DTT (Fig. 3a, right panel). The averaged effects of MTSET and DTT on the glycine EC_{30} and EC_{100} current magnitudes are summarized in Fig. 3(d). The MTSET reaction rate with the α^Δ_{S273C} GlyR at the glycine EC_{30} concentration is shown in Table 2.

We then coexpressed the α^Δ_{S273C} subunit with either the β^Δ or the β^Δ_{E297C} subunit to produce the $\alpha^\Delta_{S273C}\beta^\Delta$ and $\alpha^\Delta_{S273C}\beta^\Delta_{E297C}$ GlyRs. The glycine EC_{50}, n_H, and I_{max} values for each of these receptors are summarized in Table 2. The 10-fold current reduction induced by the E297C mutation could have been due to reduced surface expression, unitary conductance or open probability. Since we were primarily interested in the effects of MTSET, the origin of this difference was not considered further. Examples of the effects of MTSET on the $\alpha^\Delta_{S273C}\beta^\Delta$ and $\alpha^\Delta_{S273C}\beta^\Delta_{E297C}$ GlyRs are shown in Fig. 3(b,c), respectively. The steady-state effects of MTSET on the EC_{30} and saturating current magnitude for both receptors are summarized in Fig. 3(d) and their MTSET reaction rates are summarized in Table 2.

The results indicate that the effects of MTSET on the $\alpha^\Delta_{S273C}\beta^\Delta$ and the $\alpha^\Delta_{S273C}\beta^\Delta_{E297C}$ GlyRs are indistinguishable (Table 2). In each case, MTSET increased the glycine EC_{30} current amplitude by approximately 100%, which was significantly reduced relative to that observed in the α^Δ_{S273C} homomeric GlyR (Fig. 3d). In addition, there were no significant differences among the MTSET reaction rates (Table 2). Since the magnitude of the MTSET-induced current increase in the $\alpha^\Delta_{S273C}\beta^\Delta_{WT}$ GlyR was significantly less than observed in the α^Δ_{S273C} GlyR, it is likely that the number of α subunit cysteines is a determinant of the magnitude of the MTSET effect. However, since the magnitude of the MTSET-mediated current increase in the

$\alpha^\Delta_{S273C}\beta^\Delta_{E297C}$ GlyR is not significantly different to that seen in the $\alpha^\Delta_{S273C}\beta^\Delta$ GlyR, it can be concluded that introducing cysteines into the β^Δ subunit does not replicate the effect of introducing them into the corresponding location in the α^Δ subunit. Thus, a cysteine dosage effect alone can explain the absence of a measurable MTSET effect on GlyR β^Δ subunit M2–M3 loop cysteines. We therefore conclude that MTSET either does not modify any of the β^Δ subunit M2–M3 loop cysteines, or if it does, then the modified cysteines have no effect on the energetics of receptor activation.

GlyR β^Δ subunit F6′C mutation

We recently demonstrated that the conformational equilibrium of the homomeric $\alpha^\Delta_{T6'C}$ GlyR was biased strongly towards the open state by 1 mM MTSET applied in the presence of saturating concentration of glycine (see Fig. 2 of Shan *et al*. 2002). This effect was not observed when MTSET was applied in the closed state (Shan *et al*. 2001). The modification was reversed by the reducing agent, DTT, but was not replicated by oxidizing agents (Shan *et al*. 2002), implying that it was due to the covalent attachment of ethyltrimethylammonium groups to one or more 6′ cysteines. In an attempt to determine whether the β subunit contributes to the channel activation process, the present study sought to investigate the MTSET reactivity of β subunit 6′ cysteines. The α^Δ subunit contains a threonine at the 6′ position, whereas the β^Δ subunit contains a phenylalanine.

The glycine EC_{50}, n_H, and I_{max} values of the $\alpha^\Delta_{T6'C}$, the $\alpha^\Delta\beta^\Delta_{F6'C}$, the $\alpha^\Delta_{T6'C}\beta^\Delta$ and the $\alpha^\Delta_{T6'C}\beta^\Delta_{F6'C}$ GlyRs are summarized in Table 1. In particular, it can be seen that incorporation of the F6′C mutation into the β^Δ subunit had little effect on the glycine EC_{50} (Table 1). In the $\alpha^\Delta\beta^\Delta_{F6'C}$ GlyR, 1 mM MTSET had no effect regardless of whether it was applied in the absence of glycine, or in the presence of a saturating (0.6 mM) glycine concentration (Fig. 4a). The effect of
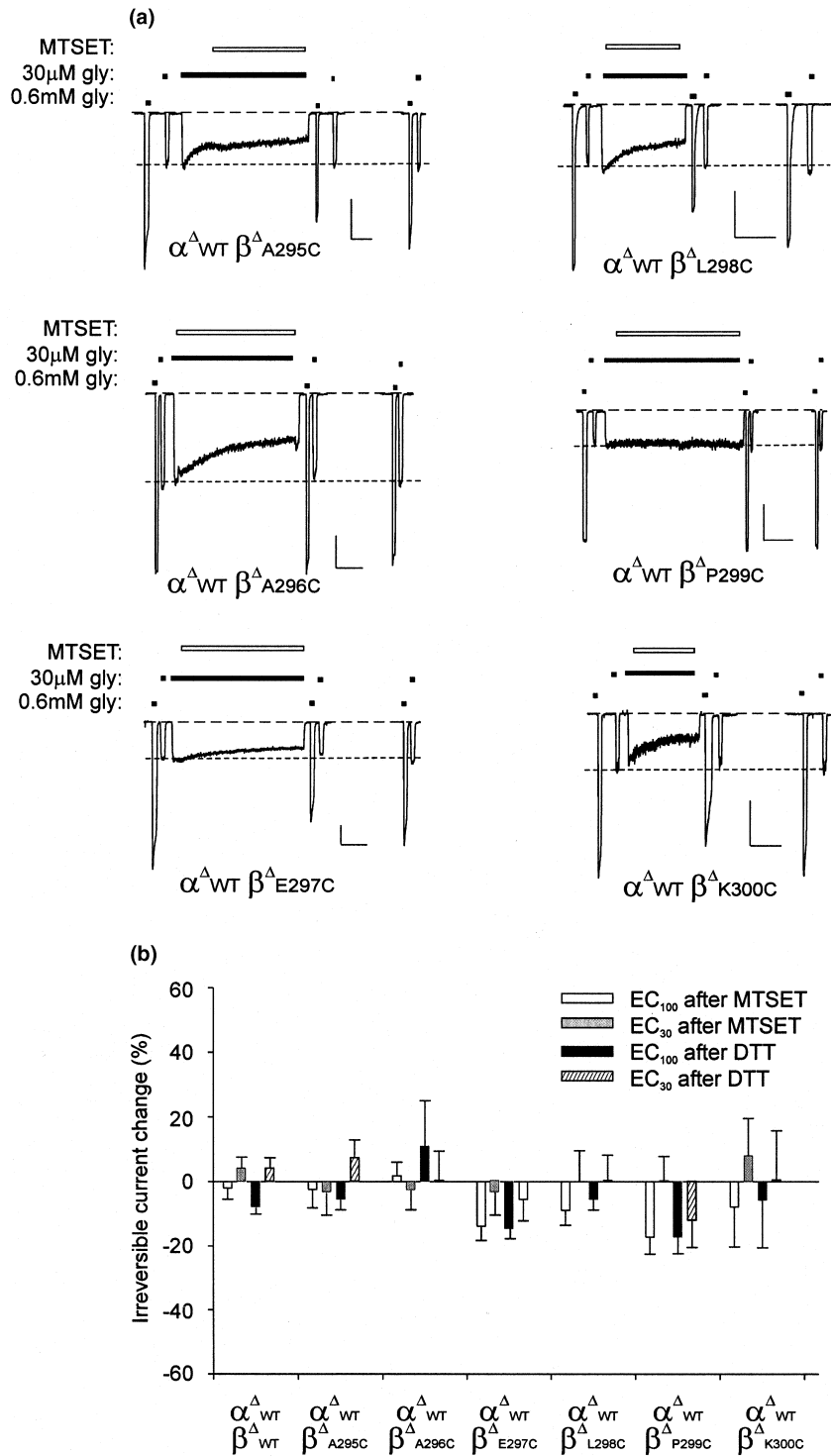
Fig. 2 No effect of MTSET on GlyRs incorporating cysteine-substitution mutations in the M2–M3 loop. (a) Currents in each panel were recorded from cells expressing the indicated subunits. In all panels, the horizontal scale bars represent 10 s and the vertical scale bars represent 1 nA. MTSET was applied at 1 mM for the period indicated by the white bar. Glycine applications are indicated by the black bars. In each panel, a 30-μM glycine concentration activated the smaller magnitude currents and a 600-μM (saturating) glycine application activated the larger currents. All currents in each panel were recorded from the same cell and the break in recording represents a delay of 2 min. The long dashed lines represent the zero current level and short dashed lines represent the peak magnitude of the current activated by 30 μM glycine. (b) Summary of the effects of MTSET and DTT on glycine-gated currents. The percentage change was calculated as $(I_{glycine, after}/I_{glycine, before} - 1) \times 100$. It can be seen that MTSET had no significant effect on currents activated by EC_{30} or EC_{100} glycine in any of the indicated GlyRs. A subsequent 2-min exposure to 1 mM DTT was also without significant effect on currents activated by either glycine concentration.

MTSET is quantitated in Table 3 by measuring the percentage of the original saturating current magnitude that is locked in the open state by a 1-min application of 1 mM MTSET. The corresponding results obtained for the α^Δ, $\alpha^\Delta\beta^\Delta$ and $\alpha^\Delta_{T6'C}$ GlyRs are included for comparison. The saturating glycine concentration used in each of these determinations is also included. It is apparent that the $\alpha^\Delta\beta^\Delta_{F6'C}$ GlyR is not functionally modified by MTSET. Since this GlyR contains only two 6′ cysteines, this negative result may have arisen from a cysteine dosage effect. The influence of cysteine number was investigated by comparing the MTSET reactivity of the $\alpha^\Delta_{T6'C}\beta^\Delta_{F6'C}$ GlyR and the $\alpha^\Delta_{T6'C}\beta^\Delta$ GlyR. As
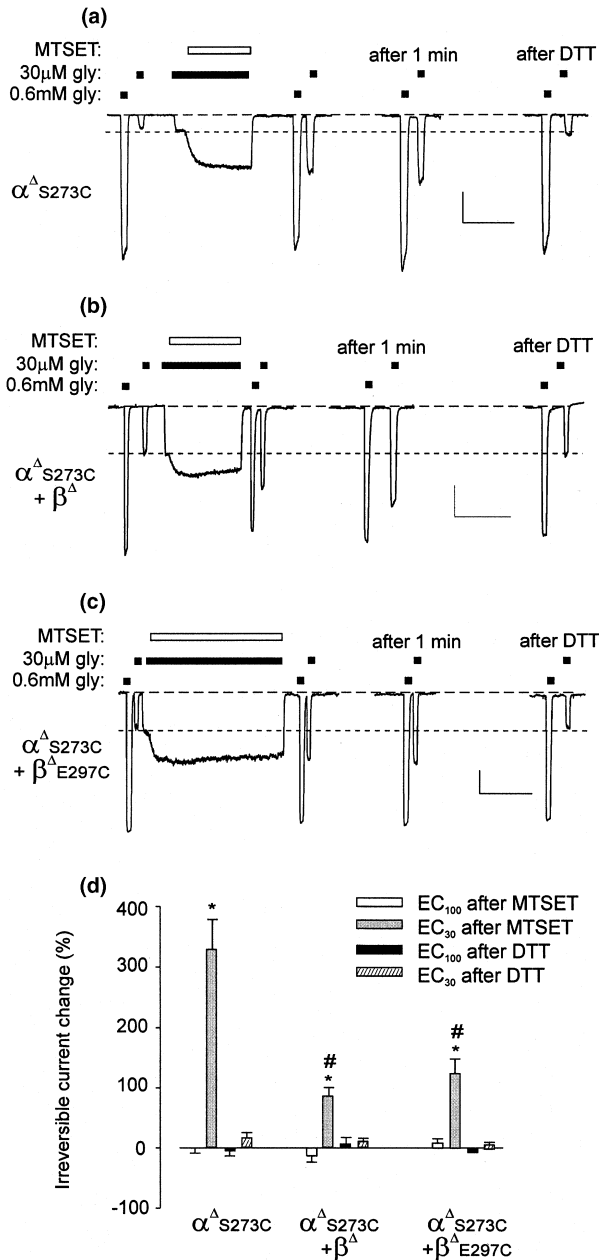
(a)

MTSET:
30 μM gly:
0.6 mM gly:

after 1 min   after DTT

α^{Δ}_{S273C}

(b)

MTSET:
30 μM gly:
0.6 mM gly:

after 1 min   after DTT

$\alpha^{\Delta}_{S273C} + \beta^{\Delta}$

(c)

MTSET:
30 μM gly:
0.6 mM gly:

after 1 min   after DTT

$\alpha^{\Delta}_{S273C} + \beta^{\Delta}_{E297C}$

(d)

Fig. 3 Effects of MTSET on GlyRs incorporating the α^{Δ} subunit S273C mutation. In all panels, the horizontal scale bars represent 10 s and the vertical scale bars represent 1 nA. (a) Effect of 100 μM MTSET on currents activated by 30 μM glycine in the α^{Δ}_{S273C} GlyR. Note the large irreversible increase in current magnitude. In this and subsequent panels, the currents labeled 'after DTT' were recorded following a 2-min wash in 1 mM DTT. The long dashed lines represent the zero current level and short dashed lines represent the peak magnitude of the current activated by 30 μM glycine. (b) Irreversible effect of 100 μM MTSET on currents activated by 30 μM glycine in the $\alpha^{\Delta}_{S273C}\beta^{\Delta}$ GlyR. (c) Irreversible effect of 100 μM MTSET on currents activated by 30 μM glycine in the $\alpha^{\Delta}_{S273C}\beta^{\Delta}_{E297C}$ GlyR. (d) Summary of the effects of MTSET and DTT on currents activated by EC_{30} or EC_{100} glycine concentrations. The percentage current change induced by MTSET was calculated as $(I_{glycine, after}/I_{glycine, before} - 1) \times 100$. Asterisks indicate those effects of MTSET that were significantly different to their effects on the $\alpha_{WT}\beta_{WT}$ GlyR. MTSET-induced current increases were invariably reversed by a 2-min application of 1 mM DTT. The # symbol indicates the current magnitudes that were significantly reduced relative to those of the α^{Δ}_{S273C} GlyR.

cannot explain the lack of effect of MTSET on the $\alpha^{\Delta}\beta^{\Delta}_{F6'C}$ GlyR.

GlyR β subunit L9′T mutation

In the recombinantly expressed muscle nAChR, mutating the 9′ leucines to serines or threonines had an equal effect on the ACh dose–response regardless of which subunit was mutated (Filatov and White 1995; Labarca *et al.* 1995). It was concluded that all five 9′ leucine residues contribute equally to receptor gating despite the presence of binding sites at only two subunit interfaces. This approach was repeated on the GlyR in an attempt to obtain evidence for an allosteric contribution of the β subunit to receptor gating. GlyR α and β subunits also contain leucines at the 9′ position. Accordingly, the L9′T mutation was incorporated into the β subunit to determine its effect on the glycine sensitivity of the αβ heteromeric GlyR. Averaged glycine dose–responses for the α GlyR, the αβ GlyR and the $\alpha\beta_{L9'T}$ GlyR are shown in Fig. 5(b) (filled symbols), with the parameters of best fit summarized in Table 1. This shows that the β subunit L9′T mutation causes a significant (fivefold) decrease in the mean glycine EC_{50} value. We then created a low affinity glycine binding site by incorporating the T204A mutation into the α subunit (Rajendra *et al.* 1995) and repeated the above experimental protocol. Sample glycine dose–responses for

summarized in Table 3, the effects of 1 mM MTSET on both receptors were indistinguishable from those on the homomeric $\alpha^{\Delta}_{T6'C}$ GlyR. Thus, the reduced number of cysteines

Table 2 Summary of the functional properties of GlyRs incorporating α^{Δ}_{S273C} subunits

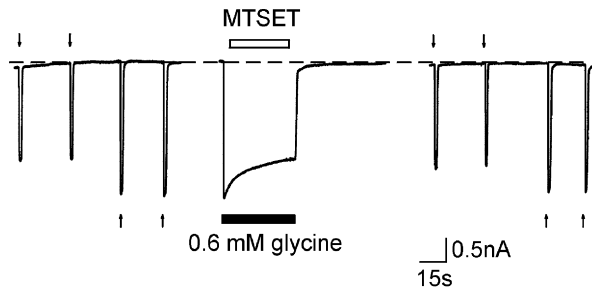| Receptor	I_{max} (nA)	EC_{50} (μM)	n_H	MTSET reaction rate	n $(10^3\ M^{-1}.s^{-1})$
α^{Δ}_{S273C}	5.4 ± 0.5	44 ± 9	1.3 ± 0.2	12.5 ± 2.0	8
$\alpha^{\Delta}_{S273C} + \beta^{\Delta}$	5.9 ± 1.0	67 ± 3	1.5 ± 0.1	8.1 ± 2.3	4
$\alpha^{\Delta}_{S273C} + \beta^{\Delta}_{E297C}$	0.6 ± 0.2	45 ± 9	1.0 ± 0.2	9.9 ± 2.1	3

MTSET reaction rate was measured in the presence of 30 μM glycine.

Fig. 4 MTSET does not functionally modify the heteromeric $\alpha^{\Delta}\beta^{\Delta}_{F6'C}$ GlyR. Sub-saturating (60 μM) glycine applications are indicated by downward arrows or the unfilled bar and saturating (600 μM) glycine applications are indicated by upward arrows. MTSET was applied at 1 mM for the period indicated by the white bar. Breaks in the recording indicate time delays of 1–2 min.

Table 3 MTSET effects on GlyRs

Receptor	[Glycine] (mM)	% of peak current locked open	n
α^{Δ}	0.25	3.7 ± 1.2	3
$\alpha^{\Delta}_{T6'C}$	0.5	86 ± 2.4*	3
$\alpha^{\Delta}\beta^{\Delta}$	0.4	−0.3 ± 2.6	3
$\alpha^{\Delta}_{T6'C}\ \beta^{\Delta}$	0.35	84 ± 1.3*	3
$\alpha^{\Delta}\beta^{\Delta}_{F6'C}$	0.6	6.0 ± 2.7	3
$\alpha^{\Delta}_{T6'C}\ \beta^{\Delta}_{F6'C}$	0.35	85 ± 7.6*	3

*Significantly different from α_{WT} homomeric GlyR.

the $\alpha_{T204A}\beta$ and $\alpha_{T204A}\beta_{L9'T}$ GlyRs are shown in Fig. 5(a) and averaged glycine dose–responses for the α_{T204A}, the $\alpha_{T204A}\beta$ and $\alpha_{T204A}\beta_{L9'T}$ GlyRs are shown in Fig. 5(b) (unfilled symbols) with parameters of best fit given in Table 1. In this case, the β subunit L9′T mutation caused a larger 12.6-fold decrease in the mean glycine EC$_{50}$ value. Thus, the effect of the L9′T mutation in the GlyR β subunit is similar to the effect of the same mutation in either ligand-binding or non-ligand-binding subunits of the nAChR (Filatov and White 1995; Labarca *et al.* 1995).

Discussion

Criteria for defining β subunit involvement in activation

The muscle nAChR is composed of two α subunits that form the principal agonist binding domains, one δ and one γ (or ε) subunit that form the complementary binding domains and one non-ligand binding β subunit (Corringer *et al.* 2000; Karlin 2002). Upon agonist binding to one or two sites, the five subunits undergo an apparently uniform conformational change to the activated state (Unwin 1995). However, due to structural differences between the subunits, these conformational changes will not necessarily be perfectly symmetrical.

Indeed, subunit-specific effects of mutations on nAChR gating have frequently been observed (Galzi *et al.* 1991; Labarca *et al.* 1995; Blanton *et al.* 1998; Chen and Auerbach 1998; Grosman and Auerbach 2000). Similarly, if conformational changes occur in the GlyR β subunit, it is expected that they should be different from those of the α subunit.

This study compared GlyR α and β subunit conformational changes using different three assays. Since the first two of these assays probed state-dependent changes in the surface accessibility of introduced cysteines, they provide a reasonably direct assay of receptor conformational change. However, the third assay, involving the β subunit L9′T mutation, sought to draw conclusions about receptor gating from changes in glycine EC$_{50}$ values. Since the glycine EC$_{50}$ is a function of both the ligand-binding affinity the gating equilibrium constant (Colquhoun 1998), our results are difficult to interpret when taken in isolation. This is particularly true since mutations in the M1 domain of the nAChR, a long way from the binding site, have been shown to predominantly affect binding (Wang *et al.* 1997; Hatton *et al.* 2003). As discussed in detail below, our conclusions concerning the effect of the L9′T mutation are drawn by analogy with the effects of the same mutation in other ligand-gated ion channel members.

Effects of mutations to the M2–M3 loop

Mutations to the GlyR α subunit M2–M3 loop have dramatic effects on the glycine EC$_{50}$ value. This was first demonstrated in α homomeric GlyRs incorporating one of the following human startle disease mutations: R271L, R271Q, K276E or Y279C (Rajendra *et al.* 1994; Langosch *et al.* 1994; Lynch *et al.* 1997; Lewis *et al.* 1998). It was subsequently shown that alanine- and cysteine-substitution mutants in the N-terminal portion of this loop (i.e. from R271 – K276) caused similarly large glycine EC$_{50}$ changes (Lynch *et al.* 1997, 2001). Such effects are expected if this loop participates in the agonist signal transduction process. However, the present study shows that none of the six corresponding β subunit mutations had a significant effect on these values. Two lines of evidence argue against the possibility that this was due only to the fewer number of mutated M2–M3 loop residues per receptor. First, Langosch *et al.* (1994) showed that progressively increasing the number of R271L and R271Q mutant α subunits per receptor from one to four caused a stepwise increase in the glycine EC$_{50}$, indicating a cumulative effect of the number of α subunit mutations per receptor. Second, the present study showed that introducing cysteines into β subunits does not compensate for the effect of removing them from the corresponding position in the α subunit. It is therefore concluded that the β subunit M2–M3 loop is functionally different to that of the α subunit.

Given that the β subunit has a different primary structure to the α subunit, one would not necessarily expect all β subunit M2–M3 loop mutations to affect the
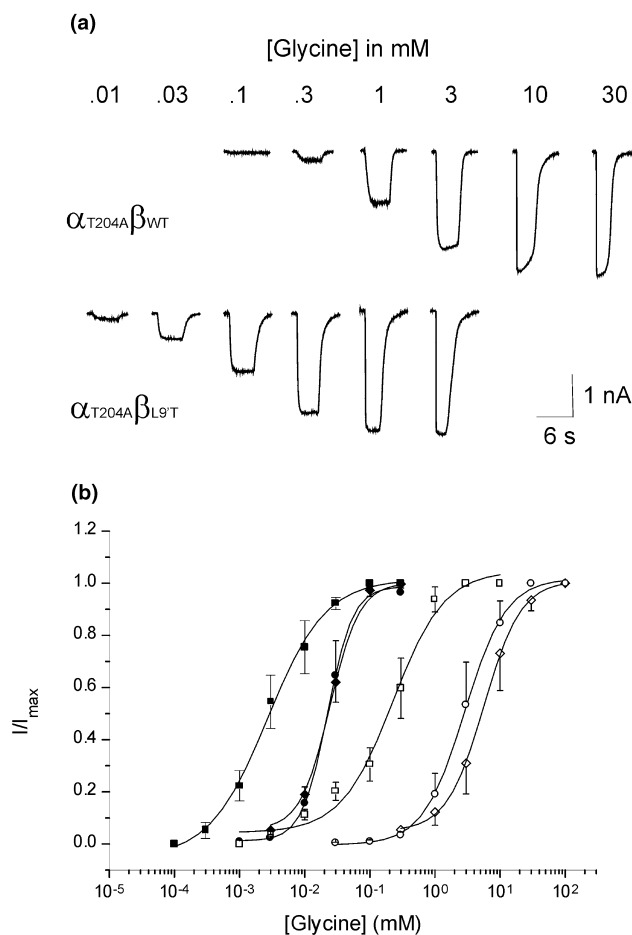
(a)

[Glycine] in mM

.01   .03   .1   .3   1   3   10   30

$\alpha_{T204A}\beta_{WT}$

$\alpha_{T204A}\beta_{L9'T}$

1 nA

6 s

(b)

Fig. 5 Effect of β subunit L9′T mutation on glycine sensitivity. (a) Sample glycine dose–responses from cells expressing the $\alpha_{T204A}\beta_{WT}$ GlyR (upper panel) and the $\alpha_{T204A}\beta_{L9'T}$ GlyR (lower panel). (b) Averaged normalized glycine dose–responses for the $\alpha_{WT}\beta_{L9'T}$ GlyR (filled squares), the $\alpha_{WT}\beta_{WT}$ GlyR (filled circles), the α_{WT} GlyR (filled diamonds), the $\alpha_{T204A}\beta_{L9'T}$ GlyR (unfilled squares), the $\alpha_{T204A}\beta_{WT}$ GlyR (unfilled circles) and the α_{T204A} GlyR (unfilled diamonds). The curves represent fits to mean data and the averaged parameters of best fit to individual dose–responses are given in Table 1.

glycine EC_{50} value in the same way as they do in the α subunit. However, the fact that the entire string of six mutated residues had no effect is surprising. The results suggest that either the β subunit M2–M3 loop is not involved in gating or that its structure differs dramatically from that of the α subunit.

This view is supported by the results of experiments using MTSET. The present study found that MTSET did not functionally modify GlyRs incorporating β subunit M2–M3 loop cysteines at any of the six tested positions. One possibility is that all residues were buried in both the closed and open states. Another is that one or more of the residues may have been modified by MTSET but that no functional change resulted. Both alternatives imply either that β subunit structure in the region of the M2–M3 loop is different to that of the α subunit or that the domain is not involved in either ligand binding or channel gating.

Effects of mutations at the 6′ and 9′ positions

MTSET did not functionally modify GlyRs incorporating 6′ cysteines in the β subunit. One possibility is that the β subunit 6′ cysteines may be buried in both the closed and open conformations. Such a structural difference from the α

subunit would not be surprising given the unusually low sequence homology (31%) between the M2 domains of the α and β subunits. A second possibility is that MTSET may have covalently modified the β subunit 6′ cysteines without causing a functional modification. Such a situation could arise if the 6′ cysteine is exposed in the pore in both the closed and open states. The lack of functional modification by a single mutation at a single position does not necessarily eliminate a role for this residue in receptor gating.

On the other hand, the β subunit L9′T mutation did cause a significant leftward shift in the glycine EC_{50} value (Fig. 5). As discussed above, this does not necessarily imply an effect on GlyR gating. However, in other members of the ligand-gated receptor family, there is abundant evidence that the primary effect of the same mutation is indeed on channel gating. For example, the nAChR L9′T mutation dramatically effects the desensitization rate, spontaneous leakage currents and the effects of allosteric modulators (Revah *et al.* 1991; Bertrand *et al.* 1992; Devillers-Thiery *et al.* 1992; Bertrand *et al.* 1997). Generally similar effects of the same mutation have also been observed in the $GABA_A R$ and $GABA_C R$ (Chang and Weiss 1998, 1999; Thompson *et al.* 1999; Dalziel *et al.* 2000; Bianchi and Macdonald 2001). The

mutation is invariably considered to affect the channel gating mechanism. Since the effect of the L9′T mutation on GlyR agonist sensitivity strongly resembles its effects on agonist sensitivity of the $\alpha\alpha\beta\delta\gamma$ nAChR (Labarca *et al.* 1995; Filatov and White 1995) and the $\alpha\alpha\beta\beta\gamma$ GABA$_A$R (Chang and Weiss 1999; Thompson *et al.* 1999), it is highly likely that the GlyR L9′T mutation primarily affects receptor gating.

It is not known whether the β subunit contains a high affinity glycine-binding site when expressed as a heteromer with α subunits. However, because the glycine sensitivities of the $\alpha_{T204A}\beta_{WT}$ and α_{T204A} GlyRs are similar, it can be concluded that the α subunits dominate glycine sensitivity in heteromeric GlyRs. Therefore, since the β subunit L9′C mutation dramatically increases the glycine sensitivity of the $\alpha_{T204A}\beta_{L9'C}$ GlyR, it must be doing so by inducing a conformational change in the α subunit. Thus, the results of this study support the idea of cooperative allosteric interactions between α and β subunits. This idea is not surprising since cooperativity between α subunits has been demonstrated in α homomeric GlyRs by single channel kinetic analysis (Beato *et al.* 2002).

Conclusion

The results of the present study indicate the β subunit 9′ leucines contribute to a key step in the structural transition between the closed and open states, and that this step is likely to involve allosteric interactions with the α subunit. This is the first demonstration of β subunit involvement in this role. However, the observation that cysteine substitution mutations have little effect when incorporated into the β subunit M2–M3 loop is difficult to reconcile with the dramatic effects of the same mutations in the α subunit. This result provides evidence for significant structural or functional differences between the α and β subunits. Together, these findings suggest significant asymmetries in the contributions of α and β subunits to GlyR gating.

Acknowledgements

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