Function of hyperekplexia-causing α_1R271Q/L glycine receptors is restored by shifting the affected residue out of the allosteric signaling pathway

Running title: restoration of mutant glycine receptor function

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Abbreviations: GlyR, glycine receptor; ECD, extracellular domain; TMD, transmembrane domain; GABAAR, type A γ-aminobutyric acid receptor; WT, wild-type; VCF, voltage-clamp fluorometry; MTSR, sulforhodamine methanethiosulfonate; TMRM, tetramethylrhodamine methyl ester; PPF, propofol; nAChR, nicotinic acetylcholine receptor.

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Summary

Background and purpose: Glycine receptor (GlyR) α_1 subunit R271Q and R271L (α_1R271Q/L) mutations cause the neuromotor disorder, hereditary hyperekplexia. Studies suggest that the 271 residue is located within the allosteric signaling pathway linking the agonist binding site to the channel gate. The present study aims to investigate a possible mechanism for restoring the function of the α_1R271Q/L GlyR.

Experimental approach: A 12-amino-acid segment incorporating the 271 residue on the GlyR $\alpha_1$271Q/L subunit was replaced by the homologous segment from the GlyR β subunit (α_{1Ch}271Q/L GlyR). The function of the α_{1Ch}271Q/L GlyR was examined by whole-cell patch-clamp recording and voltage-clamp fluorometry techniques.

Key results: The function of the α_{1Ch}271Q/L GlyR was restored to the level of the WT α_1 GlyR. Moreover, in the α_{1Ch} GlyR, in contrast to the α_1 GlyR, the channel function was not sensitive to various substitutions of the 271 residue, and the conformational change in the vicinity of the 271 residue was uncoupled from the channel gating.

Conclusions and implications: The 271 residue is shifted out of the allosteric signaling pathway in the α_{1Ch} GlyR. We propose that this mechanism provides a novel drug design strategy not only for GlyR α_1R271Q/L-caused hereditary hyperekplexia, but also for any pathological condition that is caused by missense mutation- or covalent modification-induced disorders involving residues in allosteric signaling pathways. Such a strategy makes it possible to design an ideal drug, which only corrects the function of the mutant or modified protein without affecting the wild-type or naive protein.

Introduction

Missense mutations and abnormal covalent modifications of certain residues in proteins are causes of a huge body of pathological conditions. Hereditary hyperekplexia (startle disease), which is a neuromotor disorder characterized by exaggerated startle reflexes and hypertonia in response to sudden unexpected auditory or tactile stimuli, is mainly caused by hereditary mutations to the inhibitory postsynaptic neurotransmitter receptor, the glycine receptor (GlyR) chloride channel (Chung *et al.*, 2010; Harvey *et al.*, 2008).

The GlyR exists as a pentamer. Each subunit is composed of an N-terminal extracellular domain (ECD) and four transmembrane domains (TMD) M1-4. Agonist binding to the ECDs (Brejc *et al.*, 2001; Hibbs and Gouaux, 2011; Unwin, 2005), via an allosteric signaling pathway (channel gating pathway), leads to the opening of the channel pore, which is lined by the M2 TMDs (Figure 1A) (Bocquet *et al.*, 2009; Bouzat *et al.*, 2004; Hibbs and Gouaux, 2011; Hilf and Dutzler, 2009; Hilf and Dutzler, 2008; Lee *et al.*, 2009; Lummis *et al.*, 2005; Unwin, 2005).

The most commonly occurring hyperekplexia-causing mutations are R271Q and R271L (R271Q/L) in the GlyR α_1 subunit (Zhou *et al.*, 2002). This residue lies at the extracellular mouth of the channel pore, physically located between the agonist binding sites and channel gate (Hibbs and Gouaux, 2011; Hilf and Dutzler, 2009; Hilf and Dutzler, 2008; Unwin, 2005) (Figure 1A). The R271Q/L mutations exert their pathological effects by reducing agonist glycine sensitivity (Figure 2A and B) (Lynch, 2004). Many other residue substitutions at this site, such as R271A, also reduce glycine sensitivity (Figure 2B) (Langosch *et al.*, 1994; Lynch *et al.*, 2001; Lynch *et al.*, 1997; Rajendra *et al.*, 1994). Furthermore, taurine, which is a low-efficacy GlyR agonist,

completely fails to activate the α_1R271Q/L/A GlyR channel opening (Fig 3A and B) (Rajendra *et al.*, 1995). Moreover, this residue and those in its vicinity also experience a conformational change during channel gating and more importantly this change is coupled to the channel gating process (Pless *et al.*, 2007). Taken together, these results suggest that the 271 residue is located within the channel gating pathway that functionally links the agonist binding site to the channel gate in the GlyR (Figure 1A).

Hereditary hyperekplexia, including those resulting from the R271Q/L GlyR mutations, are currently treated by using benzodiazepines, such as clonazepam, which act presumably by potentiating another inhibitory postsynaptic receptor, the type A γ-aminobutyric acid receptor (GABAAR) (Bakker *et al.*, 2009; Thomas *et al.*, 2010; Zhou *et al.*, 2002). However, the treatment is non-specific and symptomatic. Although there are barely any case reports, due to the limited literature, on the side effects of using clonazepam to treat hyperekplexia, drowsiness, ataxia and behavior problems have been often listed as side effects when using clonazepam to treat other more common neurological disorders, such as epilepsy (Browne, 1976). Moreover, in contrast to the majority of hyperekplexia-causing mutations, which are recessive and do not require life-long treatment, the R271L/Q GlyR mutations are dominant, present life-long symptoms and require long-term treatment (Chung *et al.*, 2010; Harvey *et al.*, 2008; Rees *et al.*, 2006). This posits a high chance of potential serious side-effects if the benzodiazepine clonazepam is used. To minimize the occurrence of side effects, the ideal treatment would be one that specifically corrects the structural or functional defect imposed by the disease mutation.

Here we report that the replacement of a 12-amino-acid (12-AA) segment incorporating the 271 residue on the GlyR α_1 subunit with the homologous segment from the GlyR β subunit restores the function of the α_1R271Q/L GlyR. Further experiments suggest that such a restoration is achieved by altering the local microenvironment in the vicinity of the 271 residue and in consequence shifting this residue out of the dominant channel gating pathway.

Like residue replacement, the binding of a small molecule could also alter local conformation (del Sol *et al.*, 2009; Kar *et al.*, 2010; Kumar *et al.*, 2000; Todd and Freire, 1999), and therefore our proposal could form the basis for a universal mutant or modified residue-specific drug design strategy: an allosteric drug (Kar *et al.*, 2010) can be designed to alter the microenvironment in the vicinity of the affected residue and thereby eliminate the residue from the dominant allosteric signaling pathway. Such a strategy may make it possible to design an "ideal" drug that simply corrects the function of the mutant or modified protein without affecting the wild-type (WT) or naive protein.

Methods

Mutagenesis and chimera construction of the GlyR cDNAs

Nomenclature used in this article conforms to the *Guide to Receptors and Channels* published in the British Journal of Pharmacology (Alexander *et al.*, 2009).

The human GlyR α_1 cDNAs were subcloned into the pcDNA3.1zeo+ (Invitrogen) or pGEMHE (Liman *et al.*, 1992) plasmid vectors for expression in HEK293 cells or *Xenopus* oocytes,

respectively. Site-directed mutagenesis and chimera construction were performed using the QuickChange (Stratagene, La Jolla, CA, USA) mutagenesis and multiple-template-based sequential PCR protocols, respectively.

The multiple-template-based sequential PCR protocol for chimera construction was developed in our laboratory and has recently been described in detail elsewhere (Shan and Lynch, 2010). This procedure does not require the existence of restriction sites, or the purification of intermediate PCR products, and needs only two or three simple PCRs followed by general subcloning steps. Most importantly, the chimera joining sites are seamless and the success rate for construction is nearly 100% (Shan and Lynch, 2010).

In the VCF experiments, to eliminate non-essential background cysteines, the C41A mutation was introduced into the GlyR α_1 cDNAs in the pGEMHE vector (Shan *et al.*, 2003), and a further C267S mutation was introduced into the 12-AA region of the GlyR α_{1Ch} cDNA. This manipulation did not alter channel function.

HEK293 cell culture, expression and electrophysiological recording

The effects of various substitutions of the 271 residue on the glycine and taurine sensitivity of the α_1 and α_{1Ch} GlyRs were determined by experiments on HEK293 cells. Details of the HEK293 cell culture, GlyR expression and electrophysiological recording of the HEK293 cells are described elsewhere (Shan *et al.*, 2001b). Briefly, HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were transfected using a calcium phosphate precipitation

protocol. In addition, the pEGFP-N1 (Clontech) was co-transfected to facilitate identifying the transfected cells.

Glycine and taurine-induced currents were measured using the whole cell patch-clamp configuration. Cells were treated with external Ringer's solution and internal CsCl solution (Shan *et al.*, 2001b). Cells were voltage-clamped at -40 mV.

Xenopus oocyte preparation, expression and VCF recording

VCF experiments were performed on GlyRs expressed in *Xenopus* oocytes. Details of oocyte preparation, GlyR expression and VCF recording are described elsewhere (Pless *et al.*, 2007). Briefly, the mMessage mMachine kit (Ambion, Austin, TX) was used to generate capped mRNA. The mRNA was injected into oocytes of the female *Xenopus laevis* frog with 10 ng per oocyte. After injection, the oocytes were incubated in ND96 solution (Pless *et al.*, 2007) for 3–4 days at 18 °C before recording.

The sulfhydryl-reactive reagents, sulforhodamine methanethiosulfonate (MTSR, Toronto Research Chemicals, North York, Ontario, Canada) and tetramethylrhodamine methyl ester (TMRM, Invitrogen), were used to label the 271C residues. On the day of recording, the oocytes were labeled with 10μM MTSR for 25s or 10 μM TMRM for 60 min, either in the absence or presence of glycine. The oocytes were then transferred to the recording chamber and perfused with ND96 solution. The current was recorded by the two-electrode voltage clamp configuration and the recording electrode was filled with 3 M KCl. Cells were voltage-clamped at -40 mV. The

fluorescence was recorded using the PhotoMax 200 photodiode detection system (Dagan Corp., Minneapolis, MN).

Data analysis

Results are expressed as mean ± standard error of the mean (SEM) of three or more independent experiments. The empirical Hill equation, fitted by a non-linear least squares algorithm (SigmaPlot 9.0, Systat Software, Point Richmond, CA), was used to calculate the EC_{50} values for glycine- or taurine-induced current and fluorescence changes. Statistical significance was determined using the Student's t-test.

Results

Replacement of the 12-AA segment incorporating the 271 residue restores the function of α_1R271Q/L GlyR

The GlyR and the GABAAR, two major chloride-permeable postsynaptic neurotransmitter receptors, share common structural and functional characteristics and possibly even the same evolutionary origin (Lynch, 2004; Miller and Smart, 2010; Thompson *et al.*, 2010). It has long been recognized that, with few exceptions, an Arg at sites corresponding to the 271 position of the GlyR α_1 subunit is a signature of both the GlyR and GABAAR subunit members (including the GlyR α_1 subunit) (Figure S1). One of the exceptions is the GlyR β subunit, where an Ala exists at this position (Figure 1B). The heteromeric GlyR that incorporates three Ala-carrying β subunits together with two α_1 subunits exhibits a glycine sensitivity similar to that of the homomeric α_1 GlyR (Grudzinska *et al.*, 2005; Shan *et al.*, 2001b). On the other hand, replacing

the Arg in the α_1 GlyR with Ala compromises channel function and mimics the phenotype of the α_1R271Q/L GlyR (Figure 2A and B) (Lynch *et al.*, 1997).

Supposing that this paradox might be due to a local effect, we replaced the 12-AA segment (262-273 residues) incorporating the 271 residue in the GlyR α_1 subunit with the homologous segment from the GlyR β subunit (Figure 1B). The modified subunit was named the GlyR α_{1Ch} subunit (Ch is short for chimera) (Figure 1B). Surprisingly, the α_{1Ch} GlyR, which has an Ala at the 271 position, showed a glycine sensitivity 2600 times higher than the α_1R271A GlyR and even an order of magnitude higher than the α_1WT GlyR (Figure 2B and D, Table 1). Since the α_1R271A GlyR mimics the phenotype of α_1R271Q/L GlyRs, we wondered whether this 12-AA segment replacement also restored the function of α_1R271Q/L GlyRs. We next introduced either Gln or Leu to the 271 position of the α_{1Ch} GlyR. Both constructs demonstrated glycine sensitivities 20000 (Gln) and 2500 (Leu) times higher than their corresponding substitutions in the α_1 GlyR (Figure 2B, C and D, Table 1). We concluded that the 12-AA segment replacement restored the function of the α_1R271Q/L GlyRs.

As noted above, the α_1R271Q/L GlyRs are completely insensitive to activation by the low-efficacy agonist, taurine (Figure 3A and B) (Rajendra *et al.*, 1995). We thus investigated whether the 12-AA replacement also restored the taurine sensitivity of α_1R271Q/L GlyRs to WT levels. As shown in Figure 3C and D and Table 1, taurine behaved as a full-agonist in activating the α_{1Ch}271Q/L/A GlyRs with a sensitivity even higher than in the WT α_1 GlyR. We therefore concluded that the 12-AA replacement also restored taurine sensitivity of the α_1R271Q/L GlyRs.

Replacement of the 12-AA segment incorporating the 271 residue diminishes the residue's contribution to channel gating.

To further characterize the 271 residue in the α_{1Ch} GlyR, we replaced the 271 Ala with Arg, which is the residue at the 271 position of the α_1WT GlyR. Surprisingly, the α_{1Ch}271R GlyR indicated a glycine sensitivity similar to that of the α_{1Ch}271Q/L/A GlyRs (Figure 2C and D, Table 1), in sharp contrast to the case of the α_1 GlyR, where the $\alpha_1$271R(WT) GlyR showed a glycine sensitivity 180-400 times higher than the α_1R271Q/L/A GlyRs (Figure 2A and B, Table 1). Consistently, the α_{1Ch}271R GlyR also demonstrated taurine sensitivity and maximal response similar to those of the α_{1Ch}271Q/L/A GlyRs (Figure 3C and D, Table 1). Such insensitivity of the α_{1Ch} GlyR to various residue substitutions at the 271 position implies that this residue might have a diminished contribution to channel gating in the chimeric receptor.

However, it is also possibly because the energy barrier of the channel gating pathway, which is reflected by the glycine EC_{50}s in this case (Colquhoun, 1998), has reached its lowest limit ("ceiling effect") in the α_{1Ch}271Q/L/A GlyRs, as these constructs had very low glycine EC_{50}s, around 1 μM (Table 1). In this scenario, the energy barrier (glycine EC_{50}) would not reduce further when a more gating-favorable Arg is in place. If that is the case, we argue that the channel function should not be enhanced by a potentiator. To test this possibility, we applied the GlyR potentiator, propofol (PPF), to the α_{1Ch}271Q GlyR, which exhibited the lowest glycine EC_{50} among the Q/L/A substitutions (Table 1). As shown in Figure 4A, PPF enhanced the sub-saturating glycine induced current by 93 ± 10 % (n = 4). Moreover, PPF left-shifted the glycine dose-response curve of the α_{1Ch}271Q GlyR (EC_{50} < 0.3 μM, n = 4 in the presence of PPF versus EC_{50} = 0.65 ± 0.06 μM, n = 4 in the absence of PPF, Figure 4B and C). Note that it is not possible

to quantitate glycine concentrations less than 0.3 μM due to a variable contribution from the glycine that inevitably contaminates salt solutions (0.01-0.1 μM). These data imply that the energy barrier of the channel gating pathway of the α_{1Ch}271Q GlyR has not reached the lowest limit, confirming that the insensitivity of the α_{1Ch} GlyR to various residue substitutions at the 271 position is due to this residue's diminished contribution to channel gating.

Replacement of the 12-AA segment incorporating the 271 residue alters its local microenvironment

We next sought to determine the underlying mechanism for the different contributions of the 271 residue to channel gating in the α_1 and α_{1Ch} GlyRs. To achieve this, we turned to the voltage-clamp fluorometry (VCF) technique. VCF detects local conformational changes in the vicinity of a residue when the residue is labeled with a fluorescent dye (Gandhi and Isacoff, 2005; Pless and Lynch, 2008). Rhodamine fluorescent dyes are usually used, because rhodamine fluorescence exhibits an increase in quantum efficiency as the hydrophobicity of its environment is increased. Thus, rhodamine fluorescence intensity reports the change of hydrophobicity of its immediate microenvironment, which is often caused by local conformational changes. The VCF experiments were carried out in *Xenopus* oocytes as fluorescence detection is not routinely possible in HEK293 cell-expressed GlyRs (Pless and Lynch, 2008).

To label the 271 position with a rhodamine fluorescent dye, a cysteine was introduced to this position so that the dye can be attached through a disulfide bond (Gandhi and Isacoff, 2005; Pless and Lynch, 2008). Interestingly, the $\alpha_1$271C and α_{1Ch}271C GlyRs exhibited glycine EC_{50} values of 4300 ± 200 μM (n = 4) and 2.1 ± 0.4 μM (n = 5), respectively. It is thus evident that the 271C

residue behaves in the same manner as the Q/L substitutions, in both the α_{1Ch} and α_1 GlyRs. The result of the VCF investigation is therefore expected to reflect the behavior of the 271Q/L substitutions.

As previously reported (Pless *et al.*, 2007), we confirmed that the rhodamine fluorescent dye MTSR, when attached to the 271C residue in the α_1 GlyR, exhibited an increase in fluorescence intensity (reflected by the upwards step of the fluorescence trace) upon glycine application (Figure 5A). This implies that MTSR detected an increase of hydrophobicity in the vicinal microenvironment due to a local conformational change during channel gating. Moreover, as the fluorescence and current glycine dose-response relationships overlapped, we concluded that the local conformational change is coupled with channel gating process. This conclusion is consistent with the suggestion that the 271 residue in the α_1 GlyR lies within the dominant channel gating pathway, as previously proposed (Langosch *et al.*, 1994; Lynch *et al.*, 2001; Lynch *et al.*, 1997; Rajendra *et al.*, 1995; Rajendra *et al.*, 1994).

Following the same protocol, the α_{1Ch}271C GlyR was labeled with MTSR and subjected to VCF investigation. Surprisingly, no fluorescence change was detected upon glycine application (Figure 5A). The 271C residue was possibly not labeled by the MTSR due to structural inaccessibility. Alternatively, this residue was labeled, but during channel gating, either no conformational change occurred in the vicinity of the 271 residue, or the microenvironment hydrophobicity detected by the MTSR fluorophore was not altered even though a local conformational change took place. Nevertheless, such different behaviors of the 271 residue between the α_1 and α_{1Ch} GlyRs suggest that either the static microenvironment or the dynamic

microenvironment change during channel gating, or both, in the vicinity of the 271 residue in the α_{1Ch} GlyR are altered by the 12-AA segment replacement from those in the α_1 GlyR.

Considering that rhodamine fluorophores are structurally different and may thus respond differently to a given conformational change when attached to the $\alpha_1$271C GlyR (Pless *et al.*, 2007), we next investigated the response of another rhodamine fluorescent dye TMRM in the $\alpha_1$271C and α_{1Ch}271C GlyRs. In the TMRM-labeled $\alpha_1$271C GlyR, the fluorescence intensity was increased upon glycine application (reflected by the upwards step of the fluorescence trace, Figure 5B). In contrast, in the TMRM-labeled α_{1Ch}271C GlyR, the fluorescence intensity was decreased upon glycine application (reflected by the downwards step of the fluorescence trace, Figure 5D). Such different direction of fluorescence intensity change provides a more direct indication that either the static microenvironment or the dynamic microenvironment change, or both, during channel gating, in the vicinity of the 271 residue in the α_{1Ch}GlyR, are distinct from those in the α_1 GlyR.

More interestingly, the dose-response curve of fluorescence was right-shifted from that of current in the α_{1Ch}271C GlyR when TMRM was used (fluorescence EC_{50} = 36 ± 8 μM, n = 4 versus current EC_{50} = 2.0 ± 0.2 μM, n = 5, $p < 0.01$, Figure 5E). This is in contrast with the $\alpha_1$271C GlyR, where the dose-response curves of fluorescence and current overlapped (fluorescence EC_{50} = 770 ± 150 μM, n = 5 versus current EC_{50} = 960 ± 120 μM, n = 5, $p > 0.05$, Figure 5C), consistent with what was observed when MTSR was used (Pless *et al.*, 2007). These data suggest that the conformational change in the vicinity of the 271 residue in the α_{1Ch}271C GlyR, unlike in the $\alpha_1$271C GlyR, is uncoupled from the channel gating process. We hence propose that, in the

α_{1Ch} GlyR, the 271 residue is not essential for channel gating and might not reside within the dominant channel gating pathway. Such a proposal is also supported by the fact that the α_{1Ch} GlyR channel function is not sensitive to various residue substitutions at the 271 position, as described above.

Discussion

The function of α_1R271Q/L GlyRs is restored by shifting the affected residue out of the dominant channel gating pathway.

Here we report that replacement of a 12-AA segment incorporating the 271 residue of the GlyR α_1 subunit with the homologous segment of the GlyR β subunit restores channel function of the hereditary hyperekplexia-causing α_1R271Q/L GlyRs. More interestingly, through residue substitution and VCF investigation, we concluded that this rescue effect is achieved by adjusting the local microenvironment and in consequence diminishing the 271 residue's contribution to channel gating. It has been proposed that multiple allosteric signaling pathways exist in proteins, and which pathways dominate is determined by protein topologies, specific binding events, covalent modifications and cellular conditions (del Sol *et al.*, 2009). Residue replacement, which potentially changes the protein topology (Sinha and Nussinov, 2001), can shift the dominant signaling pathway from one pathway to another. In our experiment, the 271 residue lies within the dominant channel gating pathway in the α_1 GlyR. However, the 12-AA segment replacement induces a local conformational change and, in consequence, shifts the dominant channel gating pathway to an alternative one, where the 271 residue does not reside (Figure 6A). The hypothesis that the 271 residue does not reside within the dominant channel gating pathway is reminiscent of ivermectin induced GlyR channel activation. Ivermectin is a GlyR agonist, which binds to the

GlyR and gates the channel opening in a manner distinct from the physiological agonist glycine (Hibbs and Gouaux, 2011; Pless *et al.*, 2007; Shan *et al.*, 2001a). For example, the α_1 GlyR function activated by ivermectin is almost conserved when the R271Q mutation is introduced (Shan *et al.*, 2001a). Moreover, the MTSR-labeled $\alpha_1$271C GlyR does not show any fluorescence change upon ivermectin application (Pless *et al.*, 2007). Both observations imply that the 271 residue does not reside within the ivermectin mediated channel gating pathway.

Implications for a residue-specific drug design strategy

Many pathophysiological conditions are caused by residues being either missense mutated or abnormally covalently modified (for example, by phosphorylation). The relevant treatment strategy is usually symptomatic. For example, to treat GlyR mutation-caused hereditary hyperekplexia, benzodiazepines, such as clonazepam, are used (Thomas *et al.*, 2010; Zhou *et al.*, 2002). The benzodiazepines, which are GABAAR potentiators, can counter the over-excitation symptoms due to the compromised GlyR function. However, such an "off-target" treatment strategy is the source of a wide range of side effects.

A more specific treatment strategy is to directly target the affected protein. A drug is usually designed either to enhance (in loss-of-function) or to inhibit (in gain-of-function) the function of the affected protein. However, these effects are usually global rather than mutation- or modification-specific, as the drug affects the WT or naïve protein as well as the mutant or modified protein (Joerger and Fersht, 2007; Wang *et al.*, 2003). This will lead to a lack of specificity as proteins usually have multiple subtypes (e.g α_1, α_2 and α_3 GlyRs) of different genomic origins, which share a high degree of homology and, in consequence, similar structure

and function relationships. Any drug acting on one subtype (e.g. the mutant protein, GlyR α_1R271Q/L) has a very high chance of affecting other subtypes (e.g. other WT subtypes of the mutant protein such as GlyR α_2 and α_3) as well. As protein subtypes are usually distributed in various tissues and thus have different physiological or pathological roles from each other, a drug that is supposed to only act on the specific target subtype in the ideal state but affects multiple other subtypes in reality, will cause undesirable side effects. Another consideration is that abnormal residue covalent modification of a given protein under a certain pathological condition usually only occurs in a localized region of the human body. A drug that affects the naïve as well as the modified proteins may correct the modifications in the localized region, but would also interfere with processes in other regions where the target protein expresses but without any modification. This is another source of undesirable side-effects.

One way to circumvent this "global effect" is to design a residue (mutant or modified)-specific drug. This ideal drug should affect the mutant or modified protein but not the WT or naïve protein. Despite many attempts, this goal has been successfully achieved in only a few cases. One successful case is the mutant p53-targeting drug, PRIMA-1. PRIMA-1 affects the function of mutant p53 but not the WT p53 (Bykov *et al.*, 2002a; Bykov *et al.*, 2002b), through a mechanism of modifying thiol groups within the protein (Lambert *et al.*, 2009). However, such a mechanism apparently cannot become a universal strategy for mutant or modified protein-specific drug design.

We proposed in this article that the affected residue could be shifted out of the dominant allosteric signaling pathway by the local conformational change induced by residue substitutions.

Since binding of a small molecule, like residue substitutions, can also induce conformational change and redistribute the dominant signaling pathway (del Sol *et al.*, 2009; Kar *et al.*, 2010; Kumar *et al.*, 2000; Todd and Freire, 1999), our proposal could form the basis for a universal mutant or modified residue-specific drug design strategy: an allosteric drug (Kar *et al.*, 2010) can be designed to alter the microenvironment in the vicinity of the affected residue and to activate an alternative allosteric signaling pathway that excludes the affected residue (Figure 6A). This drug action can be realized to have a neutral effect on the WT or naïve protein through activating the alternative allosteric signaling pathway with a strength equivalent to the original one (Figure 6A). However, the drug should restore the function of the mutant or modified proteins to the WT level, since the affected residue is no longer within the dominant allosteric signaling pathway and hence does not affect the protein function (Figure 6A). This missense mutation- or covalent modification-specific drug design strategy would help tackle one of the most serious problems existing among the drugs clinically used today: lack of specificity.

Possible drug design strategy for the GlyR R271Q/L hereditary hyperekplexia

The 12-AA segment that restores the function of the α_1R271Q/L GlyR is located along the extracellular half of the M2 segment and the M2-M3 domain (Figure 1A and 6B). Both domains, together with the extracellular halves of the M1 and M3 segments, form a cavity, which contains the binding site of many clinically related drugs or substances including alcohol (Mihic *et al.*, 1997) , neurosteroids (Hosie *et al.*, 2006), general anesthetics (Nury *et al.*, 2011) and ivermectin (Collins and Millar, 2010; Hibbs and Gouaux, 2011; Lynagh and Lynch, 2010) and therefore can be used as the potential docking site for drugs that specifically correct the GlyR R271Q/L mutations. Interestingly, the general anesthetic, PPF, which binds into this cavity and potentiates

the GlyR function, restores the WT phenotype of the hyperekplexic GlyR R271Q transgenic mice (O'Shea *et al.*, 2004), although a wide range of side effects would be expected, since PPF also potentiates the GABAAR and inhibits the nicotinic acetylcholine receptor (nAChR) (Franks, 2008). Nevertheless, PPF could possibly serve as the seeding backbone for designing a drug specifically correcting the GlyR R271Q/L mutations. The final ideal GlyR R271Q/L mutation corrector, by exploiting the novel drug design strategy proposed in the article, could be achieved to affect the function of the α_1R271Q/L GlyR but not any other protein including the α_1WT, α_2 α_3 GlyRs and closely related GABAAR and nAChR.

It should be noted though that this mutation corrector is only effective in treating hereditary hyperekplexia caused by α1R271Q/L mutations, but not by any mutation arising from other sites of the GlyR α_1 subunit, from the GlyR β subunit or from the SLC6A5 glycine transporter. Considering that the absolute number of patients diagnosed with hyperekplexia caused by α1R271Q/L mutations is low, it might not be commercially feasible to develop a specific α1R271Q/L mutation corrector. Instead, the target-specific drug design strategy we propose here provides a general principle for developing drugs that correct mutations or abnormal residue-modifications in proteins.

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Table 1, Properties of glycine- and taurine- induced currents of GlyRs.

GlyR	glycine		taurine		
	EC_{50} (μM)	n	EC_{50} (μM)	Imax,tau/Imax,gly (%)	n
α_1					
271R(WT)	33 ± 2	4	261 ± 32	99 ± 1	3
271A	5700 ± 1600	4	N.D.	0	3
271Q	13200 ± 2000	4	N.D.	0	3
271L	8000 ± 490	3	N.D.	0	3
α_{1Ch}					
271R	0.87 ± 0.19	4	3.7 ± 0.3	92 ± 5	4
271A(WT)	2.2 ± 0.2	4	8.7 ± 0.9	99 ± 1	4
271Q	0.65 ± 0.06	4	7.3 ± 0.7	95 ± 2	4
271L	3.4 ± 1.1	4	16 ± 3	100 ± 0	4

N.D., not determined because taurine exhibited no agonist efficacy

Figure legends

Figure 1: Location of the 271 residue on the GlyR α_1 subunit. (A) In structural models of the pentameric GlyR (top view, left panel) and single α_1 subunit (side-view, right panel) (Chung *et al.*, 2010), the 271 residue (red) is physically located between the agonist binding site and the channel gate. The 12-amino-acid segment incorporating the 271 residue is highlighted in blue in the model of single α_1 subunit. (B) Sequences of the M2 and M2-M3 domains of the GlyR α_1, β, and α_{1Ch} subunits are shown. The 271 residues are underlined.

Figure 2: Effects of various substitutions of the 271 residue on the glycine sensitivity of the α_1 and α_{1Ch} GlyRs. Example traces of currents induced by increasing glycine concentrations in the indicated constructs of the α_1 and α_{1Ch} GlyRs are shown in (A) and (C), respectively. Averaged normalized glycine dose–response curves for various substitutions of the 271 residue of the α_1 and α_{1Ch} GlyRs are shown in (B) and (D), respectively (n = 3 or 4).

Figure 3: Effects of various substitutions of the 271 residue on the taurine sensitivity of the α_1 and α_{1Ch} GlyRs. Example traces of currents induced by increasing taurine concentrations in the indicated constructs of the α_1 and α_{1Ch} GlyRs are shown in (A) and (C), respectively. Averaged normalized taurine dose–response curves for various substitutions of the 271 residue of the α_1 and α_{1Ch} GlyRs are shown in (B) and (D), respectively (n = 3 or 4).

Figure 4: Propofol potentiation of α_{1Ch}271Q GlyR function. (A) Example of propofol potentiating sub-saturating glycine-induced α_{1Ch}271Q GlyR currents. (B) Example traces of α_{1Ch}271Q GlyR currents induced by increasing glycine concentrations in the absence and

presence of propofol. (C) Averaged normalized glycine dose–response curves of the α_{1Ch}271Q GlyR in the absence and presence of propofol (n = 4).

Figure 5: VCF of the α_1 and α_{1Ch} GlyRs. Example current (I) and fluorescence (F) traces of the $\alpha_1$271C and α_{1Ch}271C GlyRs labeled with MTSR or TMRM are shown in (A), (B) and (D). Averaged normalized glycine dose-response curves of current (I) and fluorescence (F) of the $\alpha_1$271C and α_{1Ch}271C GlyRs labeled with TMRM are shown in (C) and (E), respectively (n = 4 or 5).

Figure 6: Model of the residue-specific drug design strategy. (A) In a protein with a certain residue, either mutant or modified (red circle), the protein function is compromised because the affected residue blocks the dominant allosteric signaling pathway (green strip). The protein function can be restored by activating an alternative allosteric signaling pathway that does not include the affected residue. This restoration can be achieved through adjusting the local microenvironment, either internally, by substituting the amino acids in the vicinity of the affected residue (blue line), or externally, by applying a drug (blue triangle) that has an equivalent effect as the vicinal amino acid substitution. If the newly activated alternative allosteric signaling pathway has equivalent strength as the original one in the WT or naïve protein, neither the vicinal amino acid substitution nor external drug application apparently affects the WT or naïve protein function. (B) When designing a drug (blue triangle) that specifically corrects the GlyR 271Q/L mutations (red residue), a possible docking site for this drug is the cavity formed by the extracellular halves of the M1, M2 and M3 segments and the M2-M3 domain, which is the binding site of many clinically related drugs and substances. The location of the 271 residue and

the potential drug molecule are indicated in the structural models of the pentameric GlyR (top view, top panel) and single α_1 subunit (side view, bottom panel) (Chung *et al.*, 2010).

Statement of conflicts of interest

None

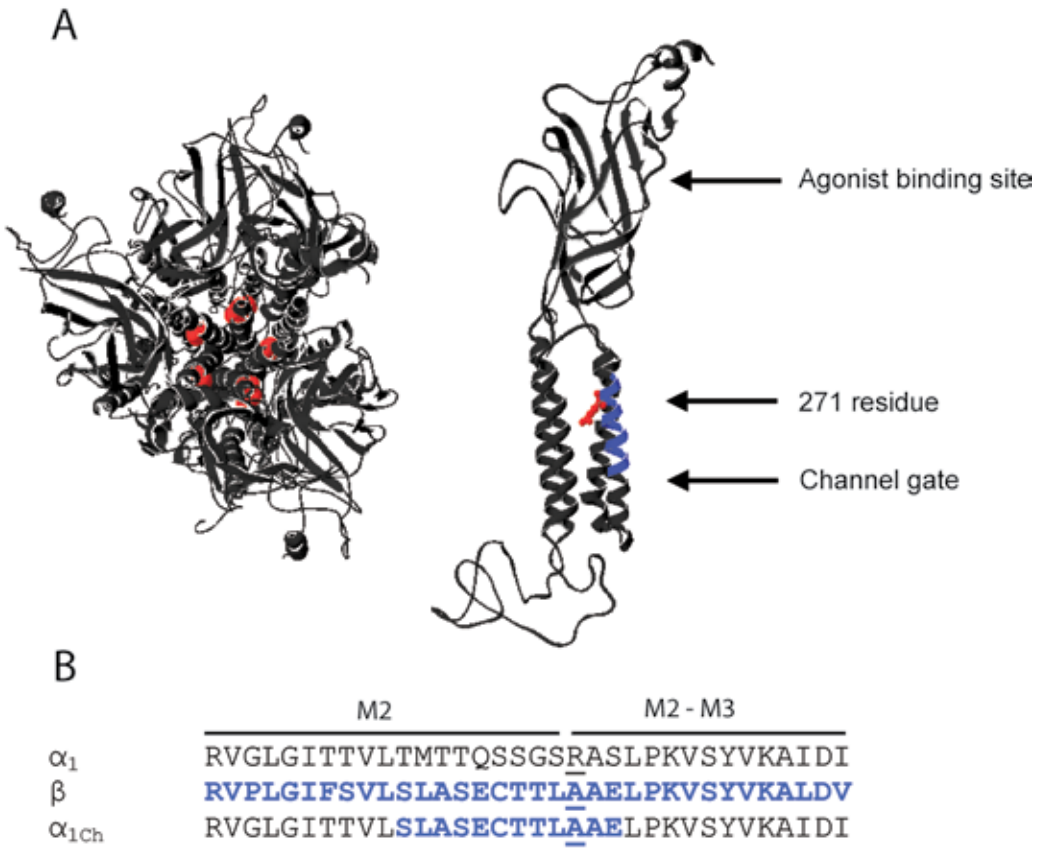
A
Agonist binding site
271 residue
Channel gate
B
M2
M2 - M3
α1 RVGLGITTVLTMTTQSSGSRASLPKVSYVKAIDI
β RVPLGIFSVLSLASECTTLAAELPKVSYVKALDV
α1Ch RVGLGITTVLSLASECTTLAAELPKVSYVKAIDI

Figure1

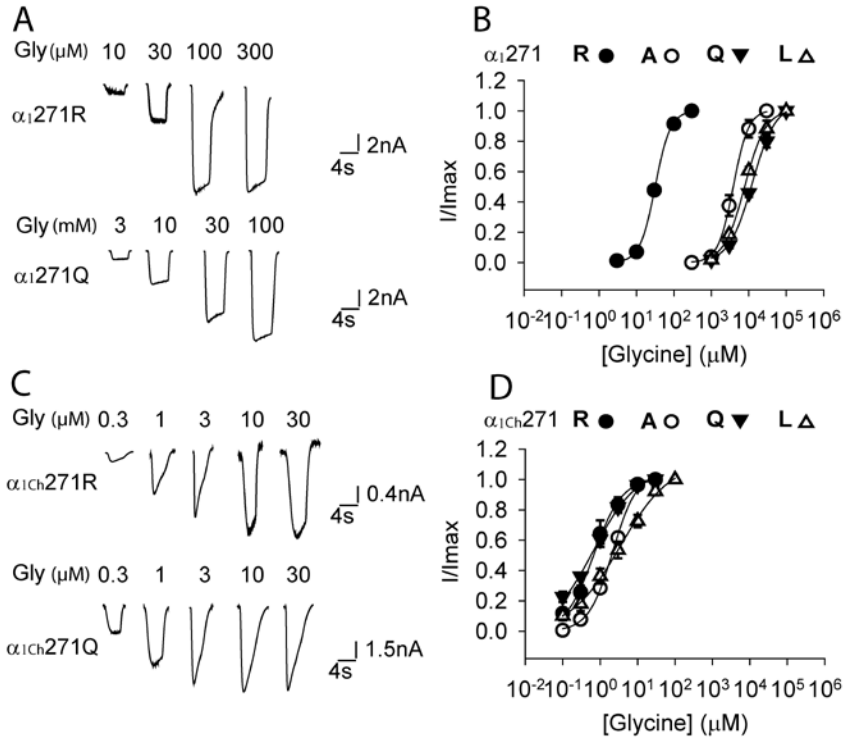
A
Gly (μM) 10 30 100 300
α1271R
2nA
4s
Gly (mM) 3 10 30 100
α1271Q
2nA
4s
B
α1271 R● A○ Q▼ L△
I/Imax
[Glycine] (μM)
C
Gly (μM) 0.3 1 3 10 30
α1Ch271R
0.4nA
4s
Gly (μM) 0.3 1 3 10 30
α1Ch271Q
1.5nA
4s
D
α1Ch271 R● A○ Q▼ L△
I/Imax
[Glycine] (μM)

Figure2

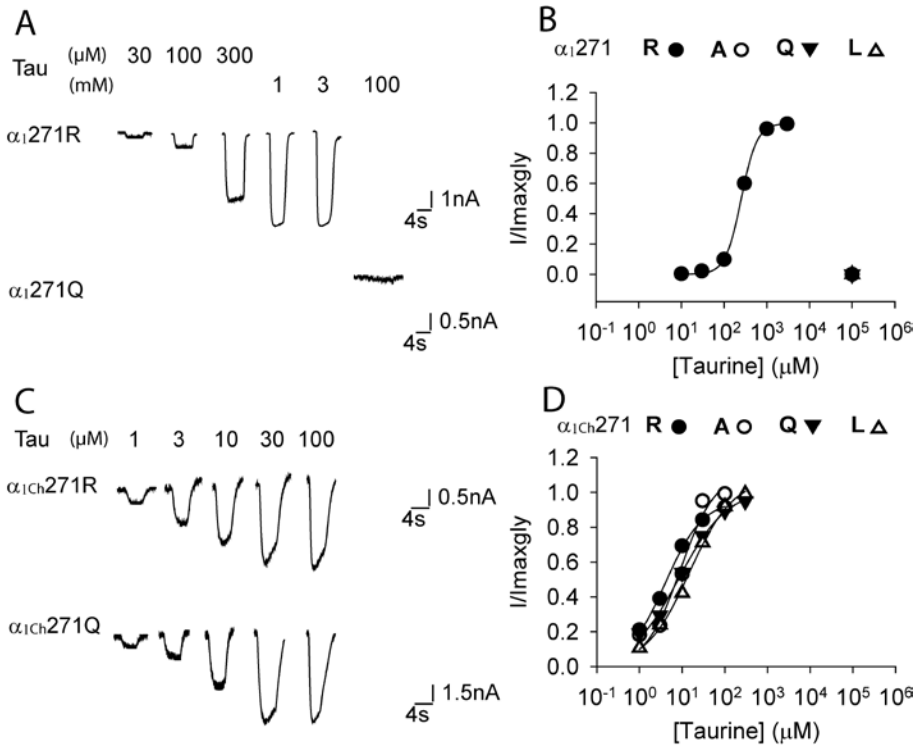
A
Tau (μM) 30 100 300
(mM) 1 3 100
α1271R
1nA
4s
α1271Q
0.5nA
4s
B
α1271 R● A○ Q▼ L△
I/Imaxgly
[Taurine] (μM)
C
Tau (μM) 1 3 10 30 100
α1Ch271R
0.5nA
4s
α1Ch271Q
1.5nA
4s
D
α1Ch271 R● A○ Q▼ L△
I/Imaxgly
[Taurine] (μM)

Figure3

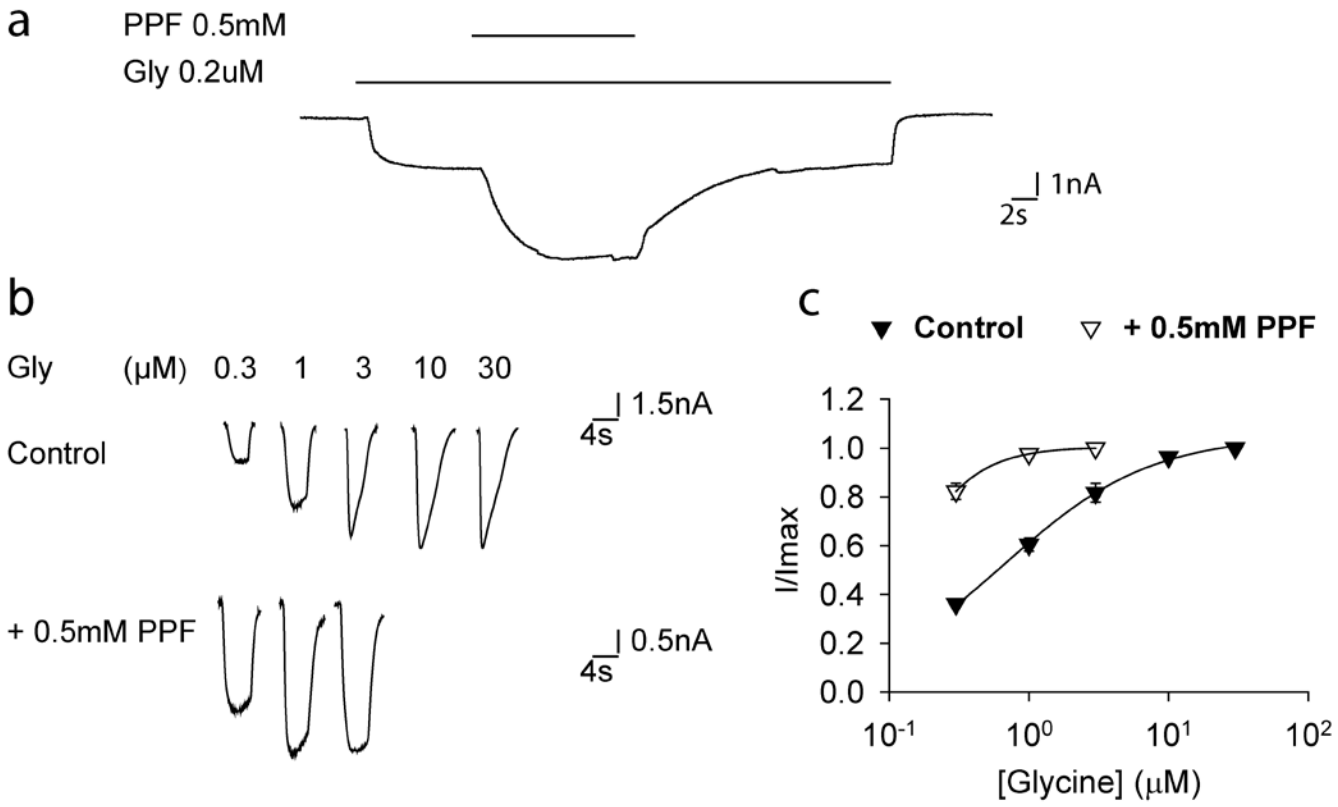
a
PPF 0.5mM
Gly 0.2uM
1nA
2s
b
Gly (μM) 0.3 1 3 10 30
Control
1.5nA
4s
+ 0.5mM PPF
0.5nA
4s
c
Control
+ 0.5mM PPF
I/Imax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-1
100
101
102
[Glycine] (μM)

Figure4

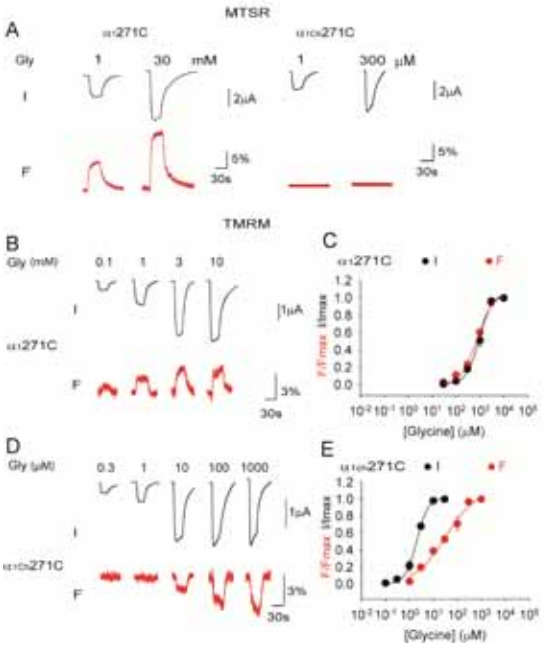
MTSR
A
α1271C
α1Gly271C
Gly
1
30
mM
1
300
μM
I
2μA
2μA
F
5%
30s
5%
30s
TMRM
B
Gly (mM)
0.1
1
3
10
I
1μA
α1271C
F
3%
30s
C
α1271C
I
F
F/Fmax I/Imax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2 10-1 100 101 102 103 104 105
[Glycine] (μM)
D
Gly (μM)
0.3
1
10
100
1000
I
1μA
α1Gly271C
F
3%
30s
E
α1Gly271C
I
F
F/Fmax I/Imax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2 10-1 100 101 102 103 104 105
[Glycine] (μM)

Figure5

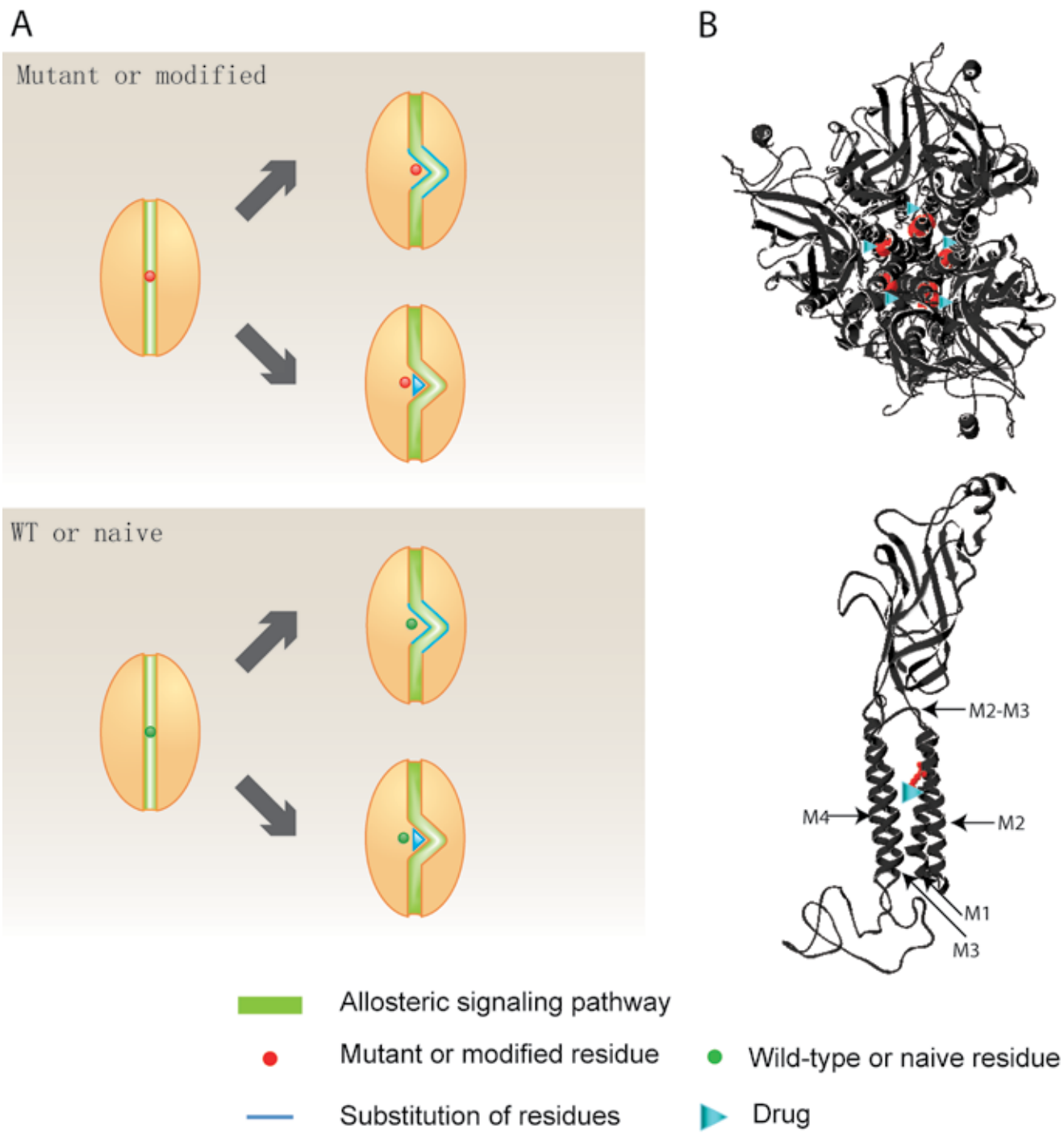
A
Mutant or modified
WT or naive
B
M2-M3
M4
M2
M1
M3
Allosteric signaling pathway
Mutant or modified residue
Wild-type or naive residue
Substitution of residues
Drug

Figure6