Brief Communications

The GLRA1 Missense Mutation W170S Associates Lack of Zn^{2+} Potentiation with Human Hyperekplexia

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Hyperekplexia is a neurological disorder associated primarily with mutations in the α1 subunit of glycine receptors (GlyRs) that lead to dysfunction of glycinergic inhibitory transmission. To date, most of the identified mutations result in disruption of surface expression or altered channel properties of α1-containing GlyRs. Little evidence has emerged to support an involvement of allosteric GlyR modulation in human hyperekplexia. Here, we report that recombinant human GlyRs containing α1 or $\alpha1\beta$ subunits with a missense mutation in the α1 subunit (W170S), previously identified from familial hyperekplexia, caused remarkably reduced potentiation and enhanced inhibition by Zn^{2+}. Interestingly, mutant $\alpha1^{W170S}\beta$ GlyRs displayed no significant changes in potency or maximum response to glycine, taurine, or β-alanine. By temporally separating the potentiating and the inhibitory effects of Zn^{2+}, we found that the enhancement of Zn^{2+} inhibition resulted from a loss of Zn^{2+}-mediated potentiation. The W170S mutation on the background of H107N, which was previously reported to selectively disrupt Zn^{2+} inhibition, showed remarkable attenuation of Zn^{2+}-mediated potentiation and thus indicated that W170 is an important residue for the Zn^{2+}-mediated GlyR potentiation. Moreover, overexpressing the $\alpha1^{W170S}$ subunit in cultured rat neurons confirmed the results from heterologous expression. Together, our results reveal a new zinc potentiation site on α1 GlyRs and a strong link between Zn^{2+} modulation and human disease.

Introduction

Hyperekplexia is an inherited neuronal disorder characterized by hypertonia and exaggerated startle reflex to unexpected sensory stimuli (Bakker et al., 2006). Hyperekplexia is caused by dysfunction of glycinergic transmission that can result from diverse root causes that include loss of function of glycine receptors (GlyRs), reduced receptor clustering of GlyRs at synapses, or reduced glycine release from presynaptic terminals (Harvey et al., 2008). Most cases of identified familial hyperekplexia are associated with dominant or recessive missense mutations of the *glra1* gene, which encodes the α1 subunit of GlyRs (Lynch, 2004). These mutations commonly lead to severe impairments of α1 GlyR-mediated synaptic inhibition either by reducing surface expression of synaptic GlyRs or by disrupting basic channel properties, such as agonist binding affinity, channel gating, and channel conductance (Chung et al., 2010). Complete loss of function mutations of α1 subunit has also been reported, causing hyperekplexia symptoms that are clinically indistinguishable from patients carrying missense *glra1* mutations (Brune et al., 1996). However, in animal models, deletion of functional α1 subunits caused more severe symptoms and juvenile death in mutant mice, suggesting that deficits in human α1 subunit might be more efficiently compensated (Buckwalter et al., 1994).

GlyRs are activated by multiple endogenous agonists, including glycine, β-alanine, and taurine. In addition, GlyRs also exhibit significant allosteric modulation by various effectors, such as Zn^{2+}, endocannabinoids, steroids, and alcohols (Lynch, 2004). Among these allosteric modulators, Zn^{2+} is enriched in presynaptic vesicles in many regions of the CNS and acts as a potent allosteric modulator of inhibitory GlyRs (Sensi et al., 2011). Low concentrations ($<10\ \mu M$) of Zn^{2+} potentiate GlyR currents, whereas high concentrations ($>10\ \mu M$) inhibit GlyR-mediated responses (Bloomenthal et al., 1994; Laube et al., 1995). Before this study, there has been no evidence from reported human mutations that hyperekplexia is caused by impaired allosteric modulation without affecting basic receptor properties of GlyRs. Although selectively removing sensitivity to Zn^{2+}-mediated potentiation produced exaggerated startle reactions in knockin mice (Hirzel et al., 2006), the different phenotype severities between human and murine *glra1* null mutations make it difficult to predict whether simply disrupting Zn^{2+}-mediated modulation of GlyRs also causes hyperekplexia in humans.

In this study, we report that the missense mutation W170S in the GlyR α1 subunit, which was recently identified from Omani families with hyperekplexia and mild mental retardation (Al-Futaisi et al., 2012), caused almost complete loss of Zn^{2+}-mediated potentiation and enhancement of Zn^{2+}-mediated

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inhibition. Unlike previously reported missense α1 mutations from human cases of hyperekplexia, $\alpha1^{W170S}$-containing GlyRs displayed no significant alterations in agonist sensitivities, maximal current responses, or current–voltage (I-V) relations. The major alteration of $\alpha1^{W170S}$ was the ablated sensitivity to Zn^{2+}-mediated potentiation, which was determined in both a recombinant expression system and in cultured neurons. Collectively, our findings demonstrate that impaired allosteric modulation of α1 GlyRs by Zn^{2+} may directly lead to human hyperekplexia.

Materials and Methods

cDNA constructs and transfection. Wild-type (WT) human GlyR α1 (hGlra1) and the human GlyR β subunit (hGlrb) were subcloned into the pBK-CMV NB-200 expression vector (Liu et al., 2010). $\alpha1^{W170S}$, $\alpha1^{H107N}$, and $\alpha1^{W170S/H107N}$ plasmids were constructed by site-directed mutagenesis of hGlra1. The sequences of all the plasmids were confirmed by automated DNA sequencing. Purified plasmids encoding the WT or mutant GlyR α1 alone or with β subunits (1:10; total plasmid amount 3–4.5 μg) were transfected into HEK293T cells by electroporation (NEPA21, NEPA GENE). The α1 and α1β compositions of GlyRs were confirmed by their shifted sensitivity to picrotoxin using whole-cell patch-clamp recordings. A small amount (~0.5 μg) of pcDNA3-GFP was cotransfected along with GlyR subunits to act as a transfection marker and facilitate the visualization of transfected cells during electrophysiological experiments. Cells were replated on glass coverslips after transfection and cultured for an additional 15–24 h before patch-clamp recordings.

Neuronal culture and overexpression. Cultured cortical neurons were prepared from the neocortex of day 18 fetal rats (from embryos of either sex) as described previously (Liu et al., 2010) and were transiently transfected with α1 subunits (1 μg) and GFP (0.3 μg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Electrophysiological recordings were performed 3–7 d after plating.

Whole-cell patch-clamp recordings. Whole-cell recordings were performed under voltage-clamp mode using an Axopatch 200B (Molecular Devices). Whole-cell currents were recorded at a holding potential of −60 mV, and signals were low-pass filtered at 2 kHz and digitized at 10 kHz (Digidata, 1440A). Recording pipettes (3–5 MΩ) were filled with intracellular solution that contained the following (in mM): 140 CsCl, 10 HEPES, 4 Mg-ATP, and 0.5 BAPTA (pH 7.20, osmolarity, 290–295 mOsm). The coverslips were continuously superfused with the extracellular solution containing the following (in mM): 140 NaCl, 5.4 KCl, 10 HEPES, 1.0 $MgCl_2$, 1.3 $CaCl_2$, and 20 glucose (pH 7.4, 305–315 mOsm). To evoke glycine currents, we used fast perfusion of glycine and other agonists with a computer-controlled multibarrel fast perfusion system (Warner Instruments). Maximum currents (I_{max}) were evoked by the highest concentration of the agonist as determined by the dose–response curves. Zn^{2+} was applied both in the bath and together with the agonist-containing solutions. All experiments were performed at 23–25°C.

Homology modeling. The mature hGlyR α1 subunit was modeled on the crystal structure of the glutamate-gated chloride channel α (GluCl) (Hibbs and Gouaux, 2011) using I-TASSER server (Zhang, 2008; Roy et al., 2010, 2012). All 3D images were subsequently rendered using the UCSF Chimera package (Pettersen et al., 2004).

Data analysis. Values are expressed as mean ± SEM. One-way ANOVA or a two-tailed Student's *t* test was used for statistical analysis, and *p* values <0.05 were considered to be statistically significant. Dose–response curves were created by fitting data to the Hill equation: $I = I_{max}/[1 + (EC_{50}/[A])^{nH}]$, where I is the current, I_{max} is the maximum current, [A] is a given concentration of agonist, and n_H is the Hill coefficient.

Results

The startle disease mutation W170S did not alter heteromeric GlyR agonist sensitivities or channel permeability

Glycine receptor-mediated currents were tested by whole-cell voltage-clamp recordings in HEK293T cells expressing WT or mutant GlyRs. Because the extracellular N terminus of α1 contains the GlyR agonist binding site and previously reported missense mutations at this region typically exhibit a feature of impaired sensitivity to agonist activation (Chung et al., 2010), we first examined whether agonist binding affinities were altered in $\alpha1^{W170S}$ GlyRs. The dose–response curve of glycine-activated, homomeric $\alpha1^{W170S}$ GlyRs currents was shifted toward the right compared with homomeric $\alpha1^{WT}$ GlyRs, with the EC_{50} increased from 62.9 ± 5.0 μM to 127.6 ± 6.9 μM (n_H = 3.38 ± 0.49 and 1.69 ± 0.14; $n = 5$ and $n = 10$, respectively; $p < 0.05$; Fig. 1*A*). The agonist sensitivity of $\alpha1^{W170S}$ was also examined with the other two endogenous GlyR partial agonists, β-alanine and taurine. Homomeric $\alpha1^{W170S}$ showed increased sensitivity to activation by β-alanine (EC_{50} = 226.9 ± 14.3 μM and 99.4 ± 8.0 μM, n_H = 2.40 ± 0.03 and 1.56 ± 0.03, $n = 9$ and $n = 9$ in $\alpha1^{WT}$ and $\alpha1^{W170S}$, respectively; $p < 0.01$; Fig. 1*B*) and no change in sensitivity to activation by taurine (EC_{50} = 256.6 ± 29.9 and 213.7 ± 19.1 μM, n_H = 1.55 ± 0.05 and 1.57 ± 0.04, $n = 8$ and $n = 8$, respectively; $p > 0.05$; Fig. 1*C*). As the majority of α1-containing GlyRs in the adult CNS consist of heteromeric α1β GlyRs that may exhibit different current kinetics than homomeric receptors (Lynch, 2004), we also investigated the potential for altered agonist sensitivities in heteromeric $\alpha1^{W170S}\beta$ GlyRs. Interestingly, the $\alpha1^{W170S}\beta$ receptors did not show significant impairment of the sensitivity to glycine (EC_{50} = 116.4 ± 5.1 and 80.0 ± 5.1 μM, n_H = 2.45 ± 0.02 and 1.96 ± 0.03, $n = 5$ and $n = 6$ in $\alpha1^{WT}\beta$ and $\alpha1^{W170S}\beta$, respectively; $p > 0.05$; Fig. 1*D*), β-alanine (EC_{50} = 91.0 ± 7.6 and 95.5 ± 8.0 μM, n_H = 1.55 ± 0.03 and 1.83 ± 0.04, $n = 9$ and $n = 8$, respectively; $p > 0.05$; Fig. 1*E*), or taurine (EC_{50} = 371.1 ± 25.6 and 194.4 ± 18.9 μM, n_H = 1.60 ± 0.03 and 1.45 ± 0.04, $n = 8$ and $n = 8$, respectively; $p < 0.05$; Fig. 1*F*). These data suggest that the hyperekplexia phenotype observed in humans carrying W170S alleles is not likely to be mediated by impaired agonist responsiveness of mutant GlyRs.

Missense mutations in other regions of α1 have been previously characterized that result in only moderate changes in GlyR agonist affinity yet also a remarkable reduction of maximal whole-cell currents (Saul et al., 1999). We found that, in $\alpha1^{W170S}\beta$ receptors, the maximal currents (I_{max}) evoked by saturating agonist concentrations, including 3 mM glycine ($\alpha1^{WT}\beta$, I_{max} = 9.4 ± 1.2 nA, $n = 7$; $\alpha1^{W170S}\beta$, I_{max} = 7.5 ± 0.9 nA, $n = 11$; $p > 0.05$), β-alanine ($\alpha1^{WT}\beta$, I_{max} = 8.4 ± 1.2 nA, $n = 7$; $\alpha1^{W170S}\beta$, I_{max} = 7.3 ± 1.0 nA, $n = 11$; $p > 0.05$), or 5 mM taurine ($\alpha1^{WT}\beta$, I_{max} = 7.3 ± 1.4 nA, $n = 7$; $\alpha1^{W170S}\beta$, I_{max} = 6.6 ± 0.9 nA, $n = 11$; $p > 0.05$) were not significantly changed compared with the $\alpha1^{WT}\beta$ receptors (Fig. 1*G*). The I_{max} induced by β-alanine ($\alpha1^{WT}\beta$, I_{ala}/I_{gly} = 90.7 ± 8.6%, $n = 7$; $\alpha1^{W170S}\beta$, I_{ala}/I_{gly} = 95.7 ± 4.5%, $n = 11$; $p > 0.05$) or taurine ($\alpha1^{WT}\beta$, I_{tau}/I_{gly} = 90.8 ± 17.5%, $n = 7$; $\alpha1^{W170S}\beta$, I_{tau}/I_{gly} = 85.8 ± 5.2%, $n = 11$; $p > 0.05$) as a percentage of the glycine-induced I_{max} from the same cell was also indistinguishable between $\alpha1^{WT}\beta$ and $\alpha1^{W170S}\beta$ GlyRs (Fig. 1*H*). In addition, the I-V relationship of 100 μM glycine-induced responses in $\alpha1^{W170S}\beta$ receptors showed no difference compared with $\alpha1^{WT}\beta$ receptors, indicating that there were no changes in Cl^- ion permeability (Fig. 1*I*,*J*). Together, these results suggest that the basic channel properties of recombinant $\alpha1^{W170S}\beta$ GlyRs were not significantly different from that of $\alpha1^{WT}\beta$ GlyRs.

Impaired Zn^{2+}-mediated modulation in $\alpha1^{W170S}$-containing GlyRs

To investigate how the W170S mutation affects glycine α1 receptors, we generated a homology model of α1 GlyR based upon the

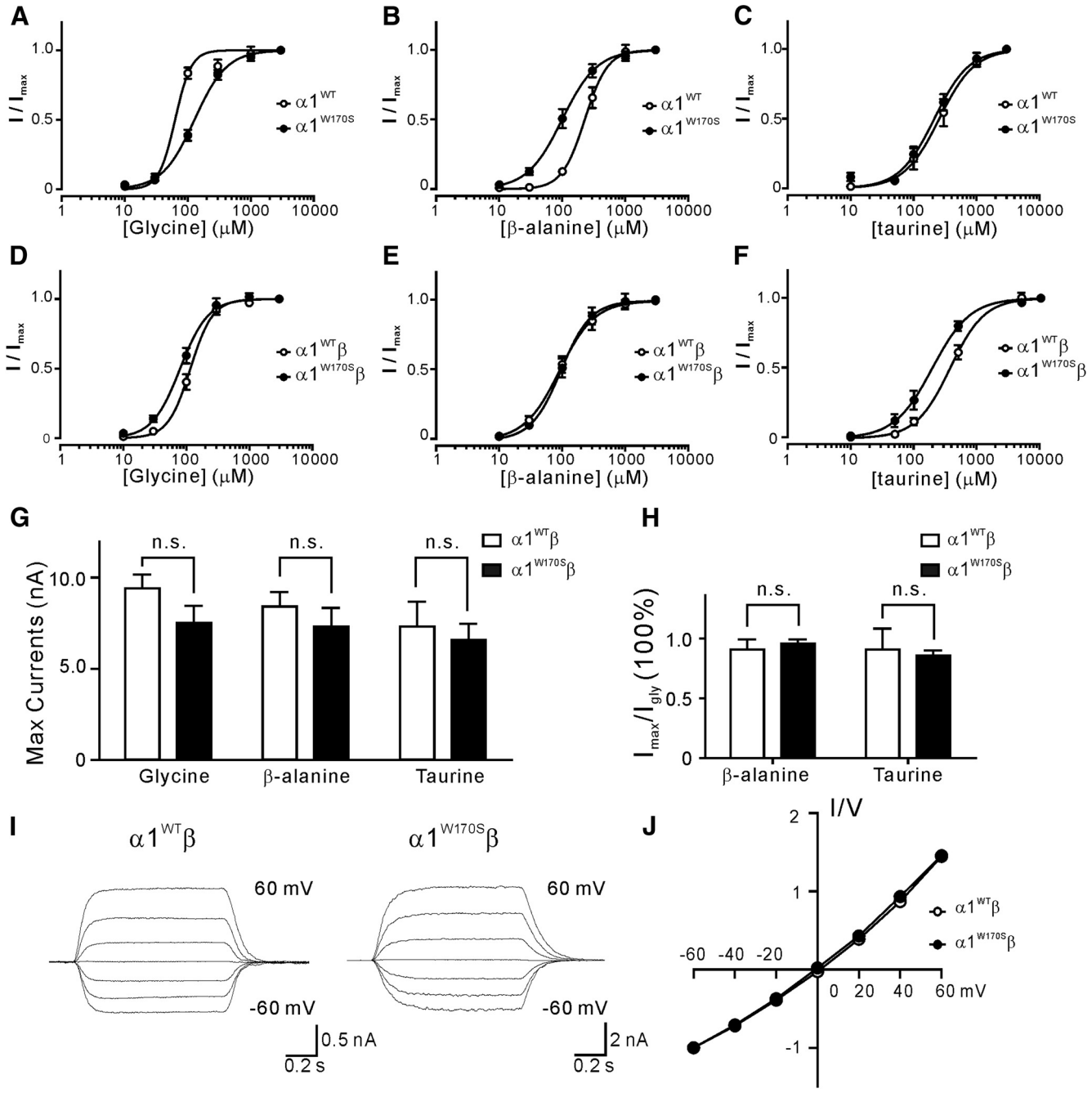

Figure 1. The W170S mutation in $\alpha1\beta$ GlyRs showed no significant changes in agonist sensitivities, maximal current responses, or I-V relationship. ***A–C***, Dose–response curves for $\alpha1^{WT}$ and $\alpha1^{W170S}$ homomeric GlyRs normalized to maximal currents induced by glycine (***A***), β-alanine (***B***), and taurine (***C***). ***D–F***, Dose–response curves for $\alpha1^{WT}\beta$ and $\alpha1^{W170S}\beta$ heteromeric GlyRs normalized to maximal currents induced by glycine (***D***), β-alanine (***E***), and taurine (***F***). ***G***, Maximal responses (I_{max}) of glycine-, β-alanine-, and taurine-induced currents in $\alpha1^{WT}\beta$ and $\alpha1^{W170S}\beta$ GlyRs. ***H***, The β-alanine- and taurine-mediated I_{max} were normalized to glycine-mediated I_{max} from the same cell. ***I***, Representative traces of 100 μM glycine-induced currents at different holding potentials from −60 mV to 60 mV. ***J***, Peak I-V relations (normalized to the current recorded at −60 mV) of $\alpha1^{WT}\beta$ and $\alpha1^{W170S}\beta$ GlyRs. n.s., Not significant.

template of the crystal structure of glutamate-gated chloride channels, which share 45% identical and 62% positive sequences with the GlyR α1 subunit (Hibbs and Gouaux, 2011). The homology model showed that W170 was located at loop F in the outer face of the N terminus domain. It is structurally close to the previously reported Zn^{2+} potentiation site (Miller et al., 2005b), with particular proximity (<4 Å) to D194, a key residue for Zn^{2+} binding (Fig. 2*A*). Therefore, we investigated the possibility that the W170S mutation might affect the sensitivity of GlyRs to Zn^{2+} modulation. Low concentrations (0.1–1 μM) of Zn^{2+} remarkably enhanced homomeric $\alpha1^{WT}$ currents evoked by 30 μM glycine (EC_{10}), whereas high concentrations (>10 μM) of Zn^{2+} inhibited responses of $\alpha1^{WT}$ GlyRs (Fig. 2*B* top, *C*). Interestingly, Zn^{2+}-mediated potentiation of GlyRs was substantially attenuated in $\alpha1^{W170S}$ GlyRs (Fig. 2*B*, bottom; *C*, top). Coexpression of the β subunit did not rescue the deficit of Zn^{2+} potentiation in $\alpha1^{W170S}$, suggesting that the ablated Zn^{2+} potentiation was preserved in $\alpha1^{W170S}\beta$ GlyRs (Fig. 2*C*, bottom).

Zn^{2+} potentiation of GlyR current responses evoked from partial agonists, such as taurine, might behave differently from the po-

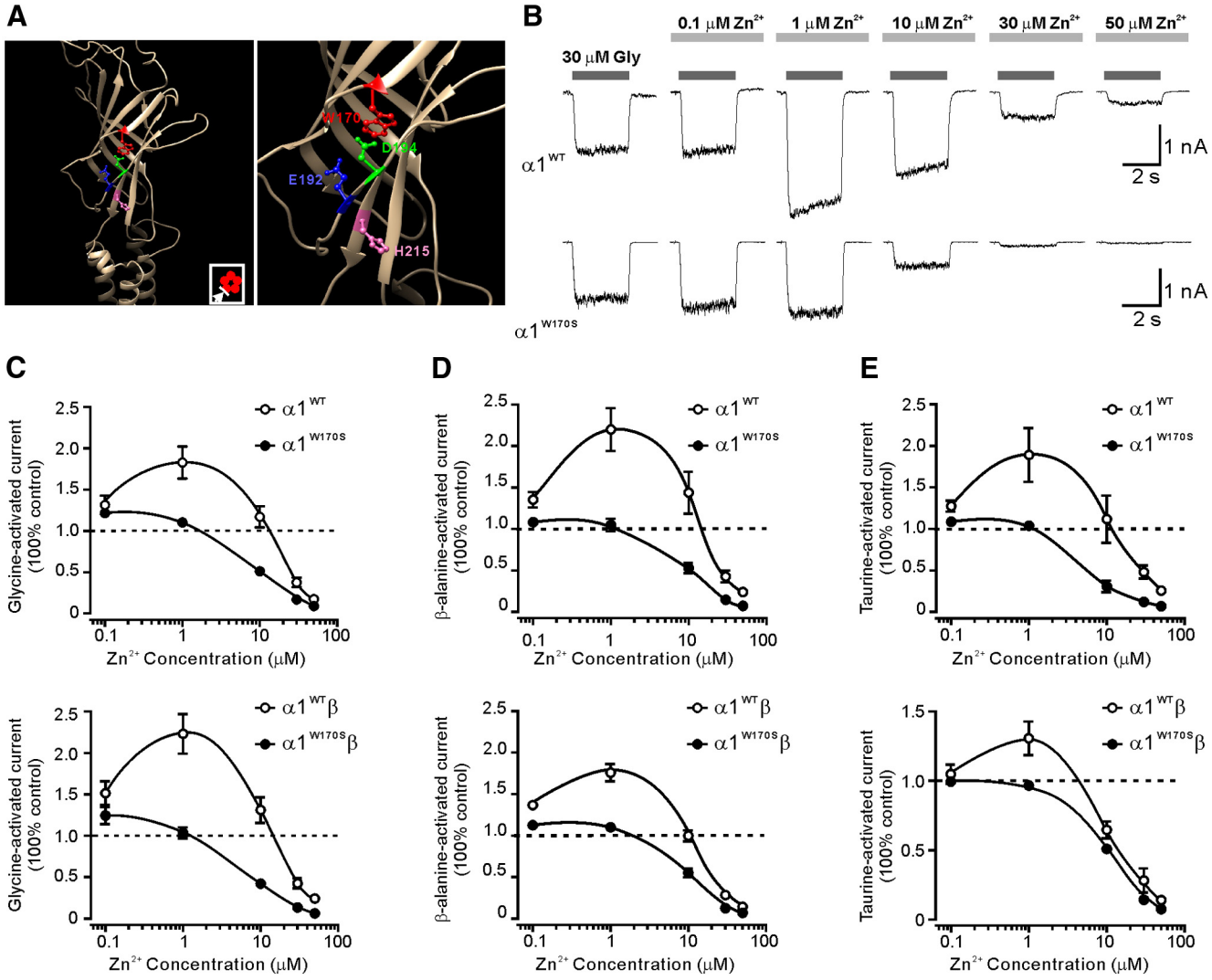

Figure 2. W170S mutation impaired Zn^{2+}-mediated potentiation and enhanced Zn^{2+}-mediated inhibition of GlyR currents activated by different agonists. ***A***, Left, The homology model of the GlyR $\alpha1$ subunit based on the GluCl viewed from the outer face. Inset, Plan view of the GlyR pentamer. The arrow indicates the viewing angle. Right, The expanded illustration of the amino acid residues that affect Zn^{2+} potentiation. ***B***, Representative traces represent biphasic modulation of Zn^{2+} on homomeric $\alpha1^{WT}$ (top) or $\alpha1^{W170S}$ (bottom) GlyR currents activated by glycine (EC_{10}). ***C***, Averaged Zn^{2+} concentration–response curves for the modulation of EC_{10} responses to glycine-activated currents in homomeric (top; $\alpha1^{WT}$, $n = 7$; $\alpha1^{W170S}$, $n = 10$) and heteromeric $\alpha1^{W170S}\beta$ GlyRs (bottom; $\alpha1^{WT}$, $n = 7$; $\alpha1^{W170S}$, $n = 10$). ***D***, Averaged Zn^{2+} concentration–response curves for the modulation of EC_{10} responses to β-alanine-activated currents in homomeric (top; $\alpha1^{WT}$, $n = 5$; $\alpha1^{W170S}$, $n = 5$) and heteromeric $\alpha1^{W170S}\beta$ GlyRs (bottom; $\alpha1^{WT}$, $n = 6$; $\alpha1^{W170S}$, $n = 5$). ***E***, Averaged Zn^{2+} concentration–response curves for the modulation of EC_{10} responses to taurine-activated currents in homomeric (top; $\alpha1^{WT}$, $n = 5$; $\alpha1^{W170S}$, $n = 8$) and heteromeric $\alpha1^{W170S}\beta$ GlyRs (bottom; $\alpha1^{WT}$, $n = 7$; $\alpha1^{W170S}$, $n = 5$).

tentiation effect of glycine-evoked responses (Lynch et al., 1998). Therefore, we tested the effects of Zn^{2+} on $\alpha1^{W170S}$ and $\alpha1^{W170S}\beta$ GlyRs-mediated currents induced by β-alanine or taurine at EC_{10}, respectively. We found that W170S similarly abolished Zn^{2+}-mediated potentiation of responses induced by β-alanine or taurine in both homomeric and heteromeric receptors (Fig. 2*D*,*E*). The Zn^{2+} inhibition was also increased under these conditions, suggesting that W170S mutations cause either attenuated potentiation or enhanced inhibition of Zn^{2+} in $\alpha1$-containing GlyRs.

Altered Zn^{2+} modulation in W170S mutation was mainly the result of abolished potentiation

At Zn^{2+} concentrations ranging from 0.1 to 1000 μM, the biphasic Zn^{2+}-mediated modulation of $\alpha1^{WT}$ likely results from the superimposition of both positive and negative modulatory effects. Prior studies have shown that, if the GlyR has already been activated by the agonist, the positive and negative modulations of Zn^{2+} can be temporally separated by showing an initial potentiation followed by prolonged inhibition of GlyR currents (Lynch et al., 1998). By using this strategy, we further elucidated whether the altered responses of $\alpha1^{W170S}$ to Zn^{2+} were the result of an ablation of Zn^{2+} potentiation or an increase of Zn^{2+} inhibition. In homomeric $\alpha1^{WT}$ receptors, after 30 μM glycine was applied to induce a GlyR-mediated current, subsequent Zn^{2+} application caused an initial potentiation at concentrations of 0.1 to 50 μM (Fig. 3*A*). The Zn^{2+}-mediated inhibition became more apparent when Zn^{2+} was washed out but glycine remained present (Lynch et al., 1998). In $\alpha1^{W170S}$ GlyRs, however, the initial potentiation was completely absent (Fig. 3*B*,*C*), and the inhibition by Zn^{2+} was consequently enhanced compared with $\alpha1^{WT}$ GlyRs (Fig. 3*D*). These data demonstrated that Zn^{2+}-mediated positive allosteric modulation was ablated in the $\alpha1^{W170S}$.

We further examined the effects of W170S on Zn^{2+}-mediated potentiation by diminishing the influence of the inhibitory effects of Zn^{2+}. The $\alpha1$ mutation, H107N, has been reported to be at least 150-fold less sensitive than the $\alpha1^{WT}$ to Zn^{2+}-mediated

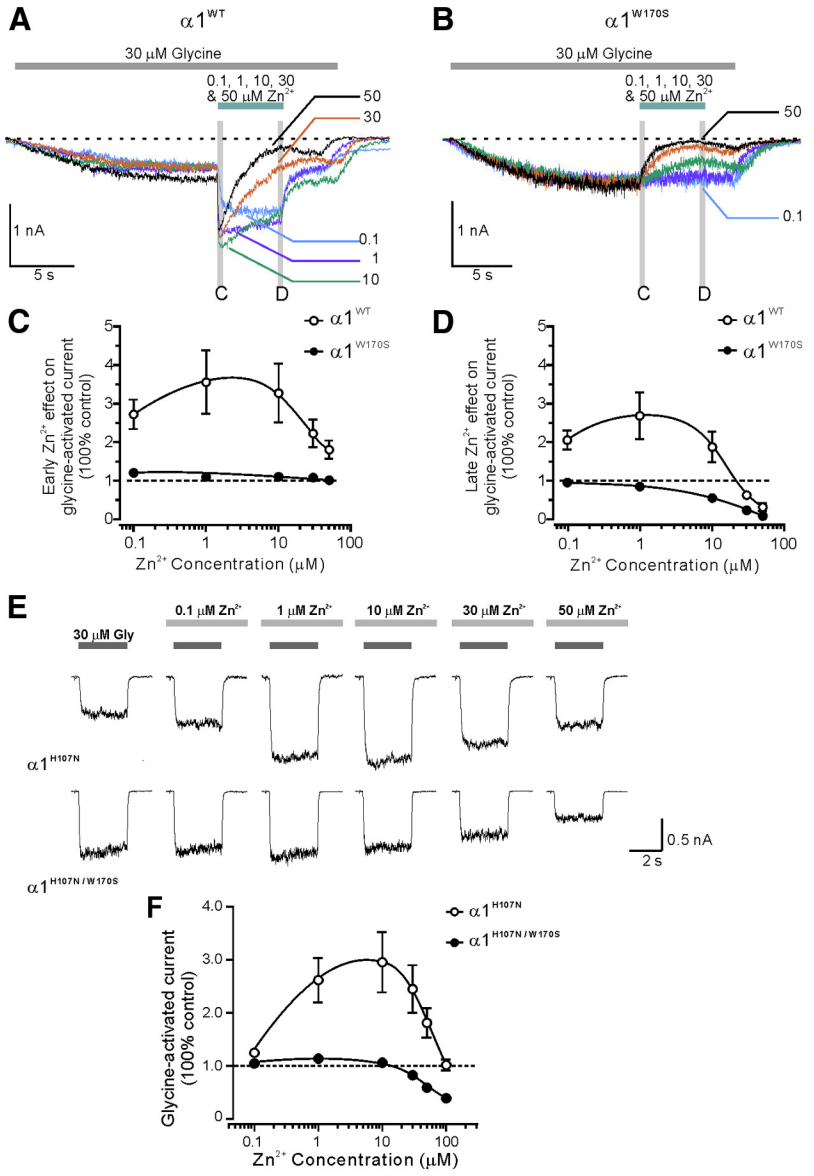

Figure 3. Separation of Zn^{2+}-mediated potentiation and inhibition indicated that the W170S mutation mainly impaired Zn^{2+}-mediated potentiation of GlyRs. ***A***, Examples of $\alpha1^{WT}$-mediated currents showing that, after GlyR activation by glycine (EC_{10}), subsequent Zn^{2+} application (5 s) at higher concentrations (30 and 50 μM) evoked an initial potentiation followed by inhibition. ***B***, In $\alpha1^{W170S}\beta$ GlyRs, the same application of Zn^{2+} only evoked inhibitory effects. Zn^{2+}-mediated early effect was measured as the maximal potentiation of glycine-activated currents during the first 300 ms of Zn^{2+} perfusion (***C***). Zn^{2+}-mediated late effect was measured as the maximal inhibition of glycine-activated currents during the last 300 ms before Zn^{2+} was washed out (***D***). ***C***, ***D***, Averaged dose–responses for Zn^{2+}-mediated early and late effects on glycine-activated currents in homomeric $\alpha1^{WT}$ ($n = 6$) and $\alpha1^{W170S}$ receptors ($n = 4$). ***E***, Top, Using the low Zn^{2+} potentiation background H107N, Zn^{2+} application induced only potentiation but no significant inhibition of glycine-activated currents in the homomeric $\alpha1^{H170N}$ receptors. Bottom, Zn^{2+}-mediated potentiation was ablated in the $\alpha1^{H170N/W170S}$ double-mutant receptors. ***F***, Averaged dose–responses for Zn^{2+}-mediated modulations in $\alpha1^{H170N}$ ($n = 5$) and $\alpha1^{H170N/W170S}$ receptors ($n = 16$).

inhibition without significantly affecting Zn^{2+}-mediated potentiation (Miller et al., 2005a, b). Therefore, we generated the double-mutation $\alpha1^{H107N/W170S}$ on the background of $\alpha1^{H107N}$ ($\alpha1^{H107N}$, $EC_{50} = 41.6 \pm 3.1$ μM, $n = 2$; $\alpha1^{H107N/W170S}$, $EC_{50} = 136.9 \pm 6.5$ μM, $n = 7$). In $\alpha1^{H107N}$ GlyRs, Zn^{2+} produced significant potentiation of glycine-induced responses without showing obvious inhibition at concentrations up to 100 μM (Fig. 3*E*,*F*). In contrast, Zn^{2+}-mediated potentiation was abolished in $\alpha1^{H107N/W170S}$ receptors. At Zn^{2+} concentrations of 50–100 μM, the W170S mutation even partially recovered the sensitivity of $\alpha1^{H107N}$ to Zn^{2+}-mediated inhibition (Fig. 3*F*). Together, these data revealed that W170 residue is likely a required site for Zn^{2+}-mediated potentiation in $\alpha1$ GlyRs.

Neuronal expressed $\alpha1^{W170S}$ GlyRs were insensitive to Zn^{2+}-mediated potentiation

The above results have shown that $\alpha1^{W170S}$-containing GlyRs lacked sensitivity to Zn^{2+}-mediated potentiation in a recombinant expression system. However, the differential protein expression profiles between HEK293T cells and neurons might result in altered receptor functional characteristics in these two systems. To confirm that $\alpha1^{W170S}$-containing GlyRs are also insensitive to Zn^{2+}-mediated potentiation when expressed in nerve cells, we overexpressed $\alpha1^{WT}$ or $\alpha1^{W170S}$ subunits in cultured cortical neurons as an endogenous expression system. Young cortical neurons mainly express $\alpha2$ subunits, which exhibit substantially lower sensitivity to Zn^{2+} potentiation than that of $\alpha1$ (Lynch, 2004; Miller et al., 2005b). In addition, $\alpha1$- and $\alpha2$-containing GlyRs have very distinct activation kinetics that can facilitate their biophysical isolation and thus also allow unambiguous confirmation of successful $\alpha1^{WT}$ or $\alpha1^{W170S}$ overexpression in neurons (Mangin et al., 2003; Mohammadi et al., 2003). To establish the accuracy of this strategy, we first examined the activation kinetics of $\alpha1^{WT}$ and $\alpha1^{W170S}$ and compared them with the kinetics of $\alpha2$-containing GlyRs expressed in HEK293T cells. $\alpha1^{WT}$ and $\alpha1^{W170S}$ GlyRs exhibit similar 10–50% rise time ($\alpha1^{WT}$, 86.7 ± 6.9 ms, $n = 8$; $\alpha1^{W170S}$; 83.6 ± 7.1 ms, $n = 8$; $p > 0.05$) after application of 3 s pulses of 30 μM glycine, whereas $\alpha2$ receptors expressed in HEK293T cells exhibited substantially slower activation kinetics (10–50% rise time: 243.9 ± 17.3 ms, $n = 8$, $p < 0.01$ compared with $\alpha1^{WT}$ or $\alpha1^{W170S}$; Fig. 4*A*–*C*). In nontransfected cortical neurons, the same glycine application induced currents with activation kinetics similar to that of $\alpha2$ receptors expressed in HEK293T cells (206.7 ± 21.6 ms, $n = 7$, $p > 0.05$ compared with $\alpha2$ in HEK293T). Neurons overexpressing $\alpha1^{WT}$ or $\alpha1^{W170S}$ receptors showed GlyR activation kinetics with rise times ranging from 50 to 290 ms, indicating variable GlyR expression profiles. Therefore, only neurons that exhibited glycine currents with rise times <100 ms were included in our analysis and were considered to predominantly express $\alpha1^{WT}$ or $\alpha1^{W170S}$ GlyRs.

Neurons overexpressed with $\alpha1^{WT}$ showed remarkable increase in glycine-mediated currents by 0.1–10 μM Zn^{2+} (Fig. 4*D*,*E*; mean 10–50% rise time: 66.4 ± 8.9 ms, $n = 4$). In contrast, Zn^{2+} did not enhance glycine-mediated currents in neurons overexpressed with $\alpha1^{W170S}$ (Fig. 4*D*,*E*; mean 10–50% rise time: 62.9 ± 11.6 ms, $n = 5$). These results confirm our findings, using

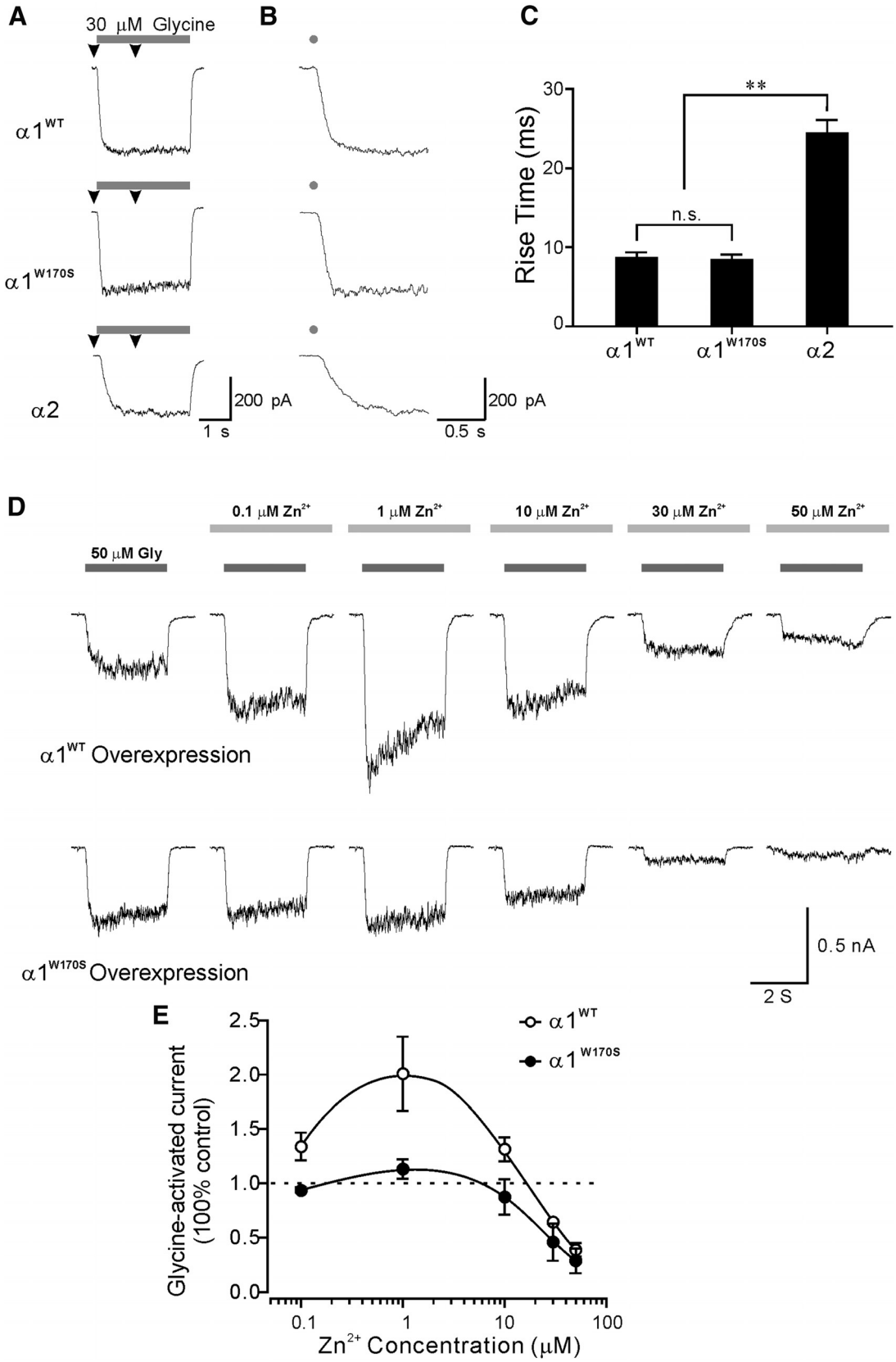

Figure 4. Neuronal expression of $\alpha1^{W170S}$ ablated Zn^{2+}-mediated potentiation of glycine-activated currents. ***A***, ***B***, Representative traces showing different activation kinetics of recombinant $\alpha1$ and $\alpha2$ receptors expressed in HEK293T cells. ***A***, Arrowheads indicate regions of the traces that are expanded in ***B***. ***B***, Gray dots indicate application of glycine. ***C***, Averaged 10–50 rise time of $\alpha1^{WT}$ ($n = 8$), $\alpha1^{W170S}$ ($n = 8$), and $\alpha2$ receptors ($n = 8$) expressed in HEK293T cells. ***D***, Top, Whole-cell recordings from a cortical neuron overexpressing $\alpha1^{WT}$ that exhibited sensitivities to Zn^{2+}-mediated biphasic modulation. Bottom, Whole-cell recordings from a cortical neuron overexpressing $\alpha1^{W170S}$ and not exhibiting sensitivity to Zn^{2+} potentiation. ***E***, Averaged dose–responses for Zn^{2+}-mediated modulation of glycine (50 μM)-activated currents in $\alpha1^{WT}$ ($n = 4$) and $\alpha1^{W170S}$ ($n = 5$) overexpressed neurons.

a recombinant expression system, that the W170S mutation also abolished sensitivity of α1 GlyRs to Zn^{2+} potentiation in neurons, and this may be a major causal factor of the symptoms of hyperekplexia in humans carrying this mutant allele.

Discussion

In the present study, we found that W170 was a novel Zn^{2+} potentiation site at the N-terminal domain of the GlyR α1 subunit. This site was identified from patients with an autosomal recessive form of hyperekplexia exhibiting homozygote W170S missense mutation (Al-Futaisi et al., 2012). We found the W170S mutation to cause complete ablation of sensitivity of α1 GlyRs to Zn^{2+} potentiation. In contrast to previously identified missense *glra1* mutations, it did not affect other basic electrophysiological properties of α1 GlyRs. After recombinant expression, the W170S mutation removed Zn^{2+} potentiation and increased the sensitivity to Zn^{2+} inhibition of current responses activated by glycine, β-alanine, or taurine. This substantial reduction of Zn^{2+}-mediated potentiation was further revealed by temporally separating the positive and negative modulatory phases and by examining the W170S mutation using the H107N mutation background, which exhibits low sensitivity to Zn^{2+} inhibition. Furthermore, abolishment of Zn^{2+} potentiation was also observed in $\alpha1^{W170S}$ GlyRs expressed in neuronal cultures, indicating that the alteration of Zn^{2+} modulation can also be observed in a native neuronal environment and might be a major causal factor of hyperekplexia in humans carrying W170S alleles.

Typically, missense mutations of *glra1* identified from hyperekplexia might reduce agonist binding sensitivity, affect channel conductance, or disrupt receptor surface expression (Saul et al., 1999; Harvey et al., 2008; Chung et al., 2010). We found that homomeric $\alpha1^{W170S}$ receptors had a twofold decrease in sensitivity to glycine compared with the WT receptors. However, in $\alpha1^{W170S}\beta$ GlyRs, the W170S mutation exhibited no reduction in sensitivity to all tested agonists, no changes in the maximal current responses, and no change in I-V relationships, suggesting that there were no defects in the intrinsic channel properties of the GlyR and that abnormalities in these properties were unlikely to be the major cause of pathogenesis in hyperekplexia patients carrying the $\alpha1^{170S}$ mutation. Instead, our results demonstrated that the major alteration of $\alpha1^{W170}$ was the ablated sensitivity to Zn^{2+}-mediated potentiation and the consequently increased sensitivity to Zn^{2+} inhibition. To our knowledge, the present study is the first demonstration of disrupted allosteric modulation of GlyRs by Zn^{2+} as an important factor in human hyperekplexia symptoms.

Endogenous free Zn^{2+} is concentrated in synaptic vesicles at certain synapses and is speculated to regulate synaptic transmission (Sensi et al., 2011). The physiological role of Zn^{2+} in synaptic transmission has been demonstrated by studies using Zn^{2+} chelators or exogenous application of Zn^{2+} to affect the duration and amplitude of glycinergic IPSCs (Suwa et al., 2001; Eto et al., 2007). Selectively ablating sensitivity of α1 to Zn^{2+} potentiation led to hyperekplexia-like phenotypes in transgenic mice carrying $\alpha1^{D80A}$ (Hirzel et al., 2006). In line with these studies, our findings suggest that synaptic Zn^{2+} plays a crucial role in glycinergic synaptic transmission and efficacy in human CNS.

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