The synthetic cannabinoid dehydroxylcannabidiol restores the function of a major $GABA_A$ receptor isoform in a cell model of hyperekplexia

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The functions of the glycine receptor (GlyR) and $GABA_A$ receptor ($GABA_AR$) are both impaired in hyperekplexia, a neurological disorder usually caused by GlyR mutations. Although emerging evidence indicates that cannabinoids can directly restore normal GlyR function, whether they affect $GABA_AR$ in hyperekplexia remains unknown. Here we show that dehydroxylcannabidiol (DH-CBD), a synthetic nonpsychoactive cannabinoid, restores the GABA- and glycine-activated currents (I_{GABA} and I_{Gly}, respectively) in HEK293 cells coexpressing a major $GABA_AR$ isoform ($\alpha_1\beta_2\gamma_2$) and GlyRα_1 carrying a human hyperekplexia-associated mutation (GlyRα_1^{R271Q}). Using coimmunoprecipitation and FRET assays, we found that DH-CBD disrupts the protein interaction between $GABA_AR$ and GlyRα_1^{R271Q}. Furthermore, a point mutation of GlyRα_1, changing Ser-296 to Ala-296, which is critical for cannabinoid binding on GlyR, significantly blocked DH-CBD-induced restoration of I_{GABA} and I_{Gly} currents. This S296A substitution also considerably attenuated DH-CBD-induced disruption of the interaction between GlyRα_1^{R271Q} and $GABA_AR$. These findings suggest that, because it restores the functions of both GlyRα_1 and $GABA_AR$, DH-CBD may represent a potentially valuable candidate drug to manage hyperekplexia.

Hyperekplexia, also called startle disease, is a rare hereditary neurological disorder characterized by exaggerated startle reflex and muscular stiffness to an unexpected stimulus, such as a tactile or sound stimulus (1). Mutations in genes coding for α and β subunits of inhibitory glycine receptor (GlyR)[2] (*GLRA1* and *GLRB*) and glycine transporter GlyT2 (*SLC6A5*) are the primary cause of hyperekplexia disease (2–8). GlyR, as a ligand-gated chloride channel, mainly mediates inhibitory neurotransmission in the spinal cord and brain stem (9, 10). So far, four α subunits (α_{1-4}) and one β subunit of GlyR have been identified (11–13). The α subunits are mainly located at the spinal cord, brain stem, cortex, hippocampus, amygdala, and striatum and mediate physiological functions such as motor function, pain, and breathing (11, 14–17). Of all human hyperekplexic mutations of GlyR (2, 18–20), R271Q is one of the most common types of mutations (21–23). This mutation significantly impairs GlyRα_1 function, reflected by reduced amplitude of glycine-activated currents (I_{Gly}) and increased EC_{50} values of GlyRα_1 (24, 25).

$GABA_AR$, another widely distributed inhibitory ligand-gated ion channel in the central nervous system (26, 27), has widely been proved to be colocalized with GlyR in spinal cord and brain stem neurons (28–33). For instance, GlyR is colocalized with α_1 and γ_2 subunits of $GABA_AR$ in the hypoglossal nucleus of mice (32). Double-immunofluorescence staining also showed that $GABA_A$ receptor–positive cells exhibit prominent glycine receptor immunoreactivity in spinal cord neurons (33). Emerging evidence indicates that $GABA_AR$ is functionally impaired in hyperekplexia disease (34, 35). For instance, electrically evoked GABAergic inhibitory postsynaptic currents are decreased in the spinal cord of GlyRα_1^{R271Q} mutant mice (34). Consistent with this, we recently verified decreased activity of $GABA_AR$ in hyperekplexic transgenic mice carrying the R271Q or S267Q mutation (36). Such functional impairment can ultimately be attributed to the direct protein interaction between $GABA_AR$ and mutant GlyRα_1. Benzodiazepines, first-line medications for treating hyperekplexia disease in the clinic (37–39), restored the function of $GABA_AR$ but not GlyR (40, 41). However, medicines that target both receptors have not yet been developed.

As positive GlyR allosteric modulators, cannabinoids can directly potentiate GlyR function in a cannabinoid receptor 1 or 2 (CB1/2R)–independent manner (42–47). Dehydroxylcannabidiol

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This article contains Figs. S1–S3.

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[2] The abbreviations used are: GlyR, glycine receptor; $GABA_AR$, GAB_AA receptor; DH-CBD, dehydroxylcannabidiol; CBD, cannabidiol; CFB, cyan fluorescent protein; IP, immunoprecipitation.

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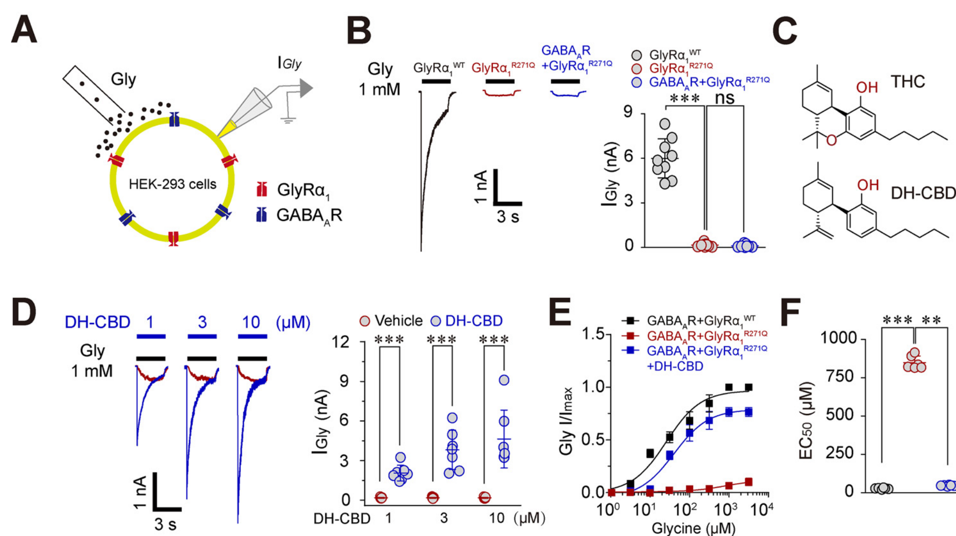

Figure 1. Effects of DH-CBD on mutant GlyRα_1 in HEK293 cells. *A*, schematic of patch clamp recording on HEK293 cells. *B*, representative trace records and average values of I_{Gly} activated by 1 mM glycine in HEK293 cells expressing $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and/or R271Q mutant α_1 GlyR ($n = 9$). *C*, chemical structure of Δ^9-tetrahydrocannabinol (*THC*) and DH-CBD. *D*, representative trace records and average values of I_{Gly} activated by 1 mM glycine in HEK293 cells coexpressing $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and R271Q mutant α_1 GlyR with or without 1 μM, 3 μM, and 10 μM DH-CBD preincubation ($n = 7$). *E*, dose–response curves of I_{Gly} in HEK293 cells coexpressing $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and R271Q mutant α_1 GlyR with or without 10 μM DH-CBD preincubation. The data were normalized to I_{max} of the WT GlyR group ($n = 6$). *F*, EC_{50} values of I_{Gly} induced by increasing glycine concentrations in HEK293 cells coexpressing $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and R271Q mutant α_1 GlyR with or without 10 μM DH-CBD preincubation ($n = 6$). Data are represented as mean ± S.D. **, $p < 0.01$; ***, $p < 0.001$; based on unpaired *t* tests; *ns*, not significant ($p > 0.05$).

(DH-CBD), a synthetic nonpsychoactive cannabinoid, significantly alleviates the abnormal startle reflex and muscle stiffness in hyperekplexic transgenic mutant mice (25). Such effects were blocked by a point mutation (Ser→Ala) of GlyRα_1 Ser-296, which is essential for DH-CBD binding. Considering the restorative effects of cannabinoids on GlyRα_1, we wondered whether it can restore the function of $GABA_AR$ in hyperekplexia disease. In this study, we coexpressed $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and R271Q mutant GlyRα_1 in a HEK293 cell model and combined various approaches, such as electrophysiological recording, FRET assays, and coimmunoprecipitation, to explore possible effects of DH-CBD on the function of GlyRα_1 and $GABA_AR$ in hyperekplexia disease.

Results

DH-CBD restores the function of hyperekplexic mutant GlyRα_1

We first examined the effect of the R271Q mutation on GlyRα_1 function using single-cell patch clamp recording (Fig. 1*A*). The R271Q mutation significantly reduced the I_{Gly} in HEK293 cells expressing GlyRα_1^{R271Q} in the presence and absence of $GABA_AR$ (Fig. 1*B*). DH-CBD is a synthetic nonpsychoactive cannabinoid modified from Δ^9-tetrahydrocannabinol (48), a major component of cannabis (Fig. 1*C*). We then evaluated the effect of DH-CBD on I_{Gly} in HEK293 cells coexpressing GlyRα_1^{R271Q} and $GABA_AR$. Consistent with a previous report (25), DH-CBD at 1, 3, and 10 μM dose-dependently caused a 10- to 30-fold increase in I_{Gly} (Fig. 1*D*). Furthermore, DH-CBD also considerably restored the dose–response curve (Fig. 1*E*) and decreased the EC_{50} values of GlyRα_1^{R271Q} (Fig. 1*F*).

DH-CBD restores the function of $GABA_AR$ in the presence of GlyRα_1^{R271Q}

Compared with WT GlyRα_1, coexpression of GlyRα_1^{R271Q} significantly decreased the GABA-activated current (I_{GABA}) in HEK293 cells (Fig. 2*A*). DH-CBD at 3 μM and 10 μM, but not 1 μM, remarkably restored I_{GABA} (Fig. 2*B*). Additionally, GlyRα_1^{R271Q} obviously shifted the dose–response curve of I_{GABA} to the right and increased the EC_{50} values of $GABA_AR$ (Fig. 2, *C* and *D*). Such effects were eliminated by preincubation of DH-CBD (Fig. 2, *C* and *D*). DH-CBD-induced restoring effect on $GABA_AR$ seem to depend on GlyRα_1 because DH-CBD at 10 μM could not affect I_{GABA} in HEK293 cells expressing $GABA_AR$ alone (Fig. 2*E*). Previous reports showed that cannabidiol (CBD), another nonpsychoactive cannabinoid, could also potentiate GlyR function (46, 47). We then incubated HEK293 cells with CBD. CBD at 10 μM significantly restored GlyRα_1^{R271Q}-decreased I_{GABA} in HEK293 cells (Fig. S1).

DH-CBD interrupts the protein interaction between $GABA_AR$ and GlyRα_1^{R271Q}

We have reported previously that the decreased activity of $GABA_AR$ in hyperekplexia was due to the protein interaction between $GABA_AR$ and mutant GlyRα_1 (36). Such effects may specifically depend on the type of GlyRα_1 mutations because GlyRα_1 carrying a nonhyperekplexic K385A mutation showed very weak binding with $GABA_AR$ and had no effect on I_{GABA} (Fig. S2), which was similar to WT GlyRα_1. Next we performed a coimmunoprecipitation assay to measure the effects of DH-CBD on the protein interaction between both receptors using HEK293 cells coexpressing GlyRα_1^{R271Q} and $GABA_AR$. Preincubation of DH-CBD significantly reduced the amount of GlyRα_1^{R271Q} protein coimmunoprecipitated with $GABA_AR$ (Fig. 3*A*).

Next we verified this effect of DH-CBD in HEK293 cells by FRET, a powerful technique for studying protein interactions in living cells with advanced spatial and temporal resolution (49). To perform the FRET assay, cyan fluorescent

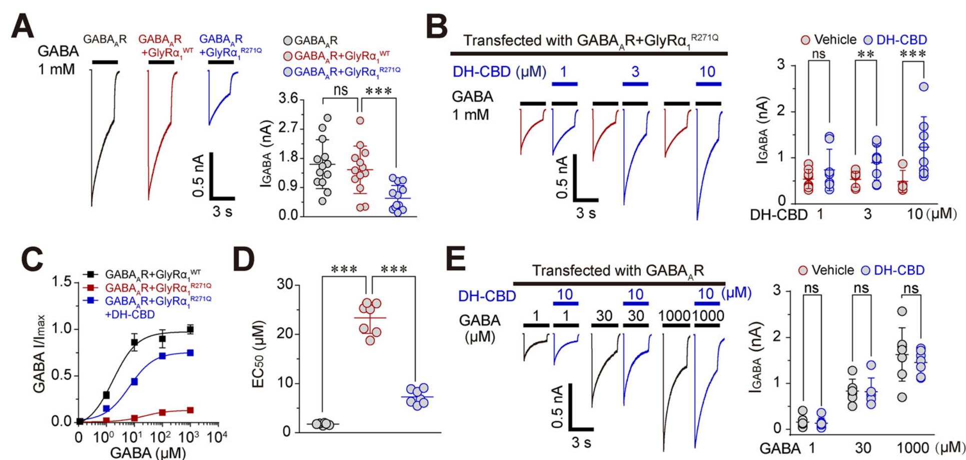

Figure 2. Effects of DH-CBD on GABA$_A$R in HEK293 cells coexpressing GlyRα$_1^{R271Q}$ and GABA$_A$R. *A*, representative trace records and average values of I_{GABA} activated by 1 mM GABA in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and WT or R271Q mutant α_1 GlyR (n = 13). *B*, representative trace records and average values of I_{GABA} activated by 1 mM GABA in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and R271Q mutant α_1 GlyR with or without 1 μM, 3 μM, and 10 μM DH-CBD preincubation (n = 8). *C* and *D*, dose–response curves (*C*) and EC$_{50}$ values (*D*) of I_{GABA} in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) (n = 6) and R271Q mutant α_1 GlyR with or without 10 μM DH-CBD preincubation (n = 7). The data were normalized to I_{max} of the GABA$_A$R + GlyRα$_1^{WT}$ group. *E*, representative trace records and average values of I_{GABA} induced by 1 μM, 30 μM, and 1000 μM GABA in HEK293 cells expressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) alone (n = 7) with or without 10 μM DH-CBD preincubation (n = 6). Data are represented as mean ± S.D. **, $p < 0.01$; ***, $p < 0.001$; based on unpaired *t* tests; *ns*, not significant ($p > 0.05$).

protein (CFP, the donor) and yellow fluorescent protein (YFP, the acceptor) were conjugated to the N-termini of the GlyRα$_1^{R271Q}$ and GABA$_A$Rα$_1$ subunits separately (Fig. 3*B*). CFP-tagged GlyRα$_1^{R271Q}$ and YFP-tagged GABA$_A$R were functionally expressed in HEK293 cells (Fig. 3, *C* and *D*) and could be detected by a dual-channel spinning disk confocal microscope (Fig. S3). DH-CBD did not change the YFP/CFP emission ratio in HEK293 cells coexpressing CFP-tagged GlyRα$_1^{WT}$ and YFP-tagged GABA$_A$R (Fig. 3*F*). However, after 30–60 min of incubation, DH-CBD significantly increased the YFP/CFP emission ratio in HEK293 cells coexpressing CFP-tagged GlyRα$_1^{R271Q}$ and YFP-tagged GABA$_A$R (Fig. 3*E*), suggesting a changed protein interaction pattern between GlyRα$_1^{R271Q}$ and GABA$_A$R induced by DH-CBD treatment.

The S296A mutation diminishes DH-CBD–induced restoration of GlyRα$_1$ and GABA$_A$R function in hyperekplexia

Ser-296 in the third transmembrane domain of the GlyRα$_1$ subunit has been suggested to be essential for DH-CBD–GlyRα$_1$ interaction (46, 48, 50). The S296A mutation in GlyRα$_1$ blocked the rescuing effects of DH-CBD on both I_{Gly} and I_{GABA} in HEK293 cells coexpressing GABA$_A$R and GlyRα$_1$ carrying the R271Q and S296A mutations (Fig. 4, *A* and *B*). The S296A mutation also significantly inhibited the restoring effects of DH-CBD on the protein interaction between GlyRα$_1^{R271Q}$ and GABA$_A$R (Fig. 4*C*). Benzodiazepines are routine drugs for treating hyperekplexia in the clinic, targeting GABA$_A$R (37, 38, 51–53). However, unlike DH-CBD, diazepam could not affect the protein interaction between GABA$_A$R and GlyRα$_1^{R271Q}$ (Fig. 4*D*).

Discussion

Benzodiazepines are the most commonly used drugs for the treatment of hyperekplexia disease in the clinic (37, 38, 51–53). Our recent study also reported that diazepam can restore the function of pre- and extrasynaptic GABA$_A$R in hyperekplexia disease (36). Benzodiazepines are effective and relatively safe to treat this disease, especially at low doses (37–39). However, benzodiazepines may cause sedative effects even at low doses (54), which is far from ideal for long-term treatment and children. In addition, there is a chance of tolerance after months of benzodiazepine use, even at low doses (55). Therefore, there is a need to develop new therapeutic avenues targeting both GlyRα$_1$ and GABA$_A$R with minimal side effects for the treatment of hyperekplexia disease. The data presented in this study provide evidence that DH-CBD may be a more appropriate candidate medicine for treating hyperekplexia disease under certain conditions, such as the GlyRα$_1$ R271Q mutation. DH-CBD restores the function of hyperekplexic mutant GlyRα$_1$ and GABA$_A$R by interrupting the protein interaction between these two receptors, whereas benzodiazepines only restore GABA$_A$R function (53).

Emerging evidence shows that Ser-296 of the GlyR α$_1$ subunit is a critical site for cannabinoid action (46, 48, 50). In this study, the S296A site mutation blocked DH-CBD–induced disruption of the protein interaction between GlyRα$_1^{R271Q}$ and GABA$_A$R. Such an effect likely leads to restoration of GABA$_A$R function in HEK293 cells. Considering the fact that DH-CBD had no effect on GABA$_A$R alone expressed in HEK293 cells, we suppose that DH-CBD–induced functional restoration of GABA$_A$R must be achieved by first acting on GlyRα$_1$. However, the detailed mechanism of how DH-CBD diminishes the hijacking effect of mutant GlyRα$_1$ on GABA$_A$R needs further investigation. Several techniques may help to illustrate the potential mechanism, such as molecular dynamics simulation and protein crystal structure analysis, and may provide the detailed protein structure of the GlyRα$_1$–GABA$_A$R complex and the potential binding sites of DH-CBD on the GlyRα$_1$–GABA$_A$R protein complex.

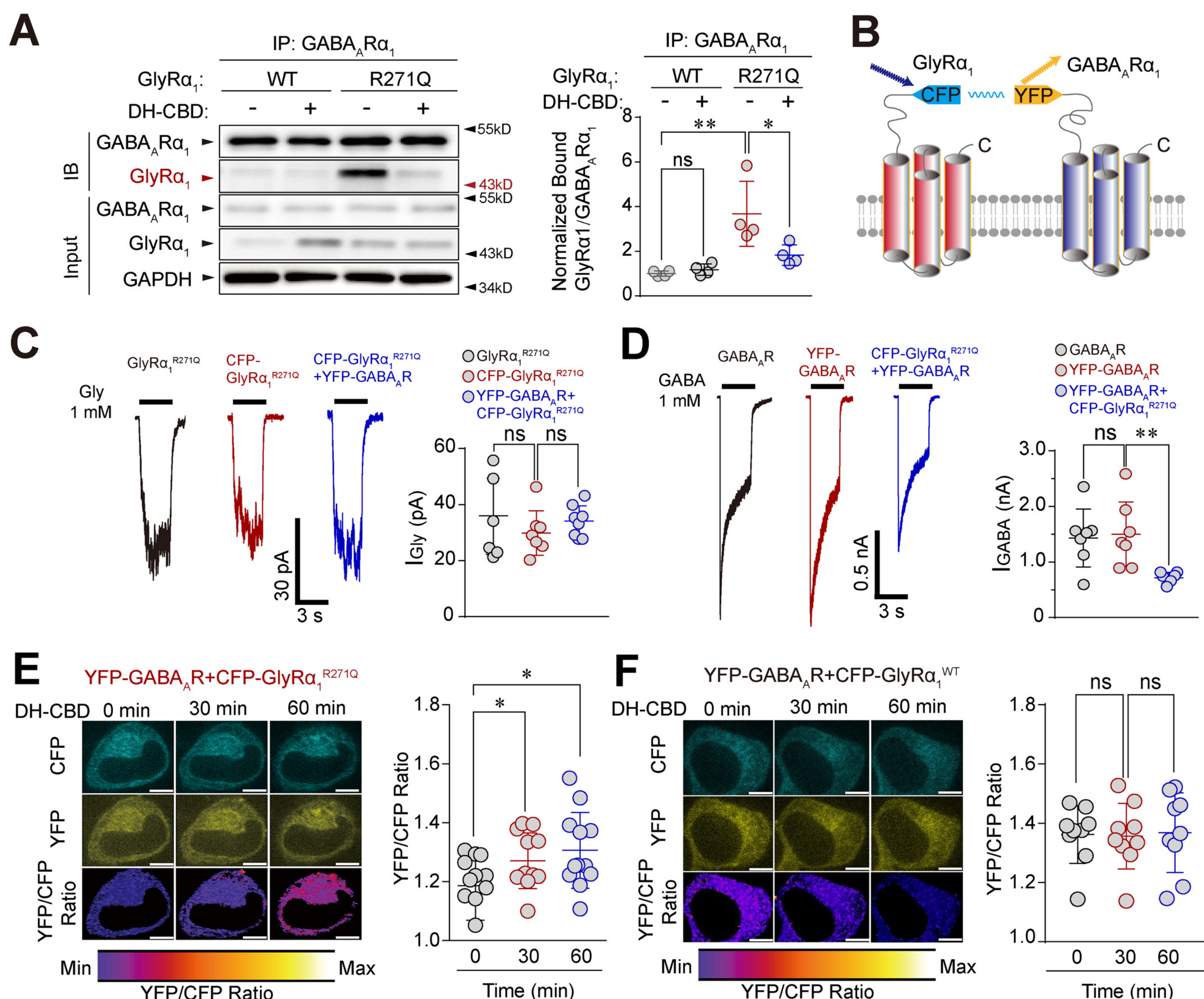

Figure 3. Effects of DH-CBD on protein interaction between $GABA_AR$ and $GlyR\alpha_1^{R271Q}$. *A*, $GlyR\alpha_1$ protein was purified using $GABA_AR\ \alpha_1$ antibodies in HEK293 cells coexpressing $GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and WT/R271Q mutant α_1 GlyR with or without 10 μM DH-CBD preincubation. Input represents the same protein immunoblots (*IB*) extracted from cell lysates prior to co-IP. Shown is quantification of WT and R271Q mutant GlyR α_1 binding to $GABA_AR\ \alpha_1$ subunits with or without 10 μM DH-CBD preincubation ($n = 4$). The data were normalized to the WT group without DH-CBD preincubation. *B*, schematic of plasmid structures and FRET. *C*, representative trace records and average values of I_{Gly} activated by 1 mM glycine in HEK293 cells expressing $GlyR\alpha_1^{R271Q}$ ($n = 7$) or CFP-$GlyR\alpha_1^{R271Q}$ alone ($n = 7$) or YFP-$GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and $GlyR\alpha_1^{R271Q}$ ($n = 8$). *D*, representative trace records and average values of I_{GABA} activated by 1 mM GABA in HEK-293 cells expressing $GABA_AR$ ($n = 7$) or YFP-$GABA_AR$ ($\alpha_1\beta_2\gamma_2$) alone ($n = 7$) or YFP-$GABA_AR$ ($\alpha_1\beta_2\gamma_2$) and CFP-$GlyR\alpha_1^{R271Q}$ ($n = 6$). *E*, representative images and quantification of HEK293 cells coexpressing CFP-tagged $GlyR\alpha_1^{R271Q}$ and YFP-tagged $GABA_AR$ ($n = 12$). These images were collected separately via CFP and YFP channels 0, 30, and 60 min after 10 μM DH-CBD preincubation. *Scale bars* = 5 μm. *F*, representative images and quantification of HEK293 cells coexpressing CFP-tagged $GlyR\alpha_1^{WT}$ and YFP-tagged $GABA_AR$ ($n = 9$). These images were collected separately via CFP and YFP channels 0, 30, and 60 min after 10 μM DH-CBD preincubation. *Scale bar* = 5 μm. Data are represented as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; based on unpaired *t* tests; *ns*, not significant ($p > 0.05$).

This study showed that DH-CBD had no direct effects on $GABA_AR$ in HEK293 cells. However, a previous report revealed that CBD and 2-arachidonoyl glycerol, an endogenous cannabinoid, can enhance the function of $GABA_AR$ in *Xenopus* oocytes (56). There may be two possible reasons for this contradiction. One may be the use of two distinct cell models in the two studies, amphibian oocytes and mammalian cell lines, which may carry quite different genomes, proteomes, and plasma membrane components (57, 58). Another possible reason is that, although CBD and DH-CBD are very similar in structure, the subtle structural differences between them still lead to differences in their direct action on $GABA_AR$.

To illustrate the therapeutic effects of DH-CBD on hyperekplexia disease, the R271Q site mutation was selected as for this study because, of all reported $GlyR\alpha_1$ gene mutations, R271Q is the most common mutation causing hyperekplexia disease (21–23). In addition to R271Q mutant $GlyR\alpha_1$, $GlyR\alpha_1$ carrying many other mutations, such as R218Q, P250T, V260M, S270T, and K276E, is also responsive to DH-CBD (25). These mutations, especially R271Q, have a high prevalence among all hyperekplexic patients. For example, in a clinical study, it was found that 10 of 17 hyperekplexic patients carried the $GlyR\alpha_1$ R271Q mutation (23). However, several site mutations in the GlyR β subunit and GlyT2 can also cause hyperekplexia (2–7).

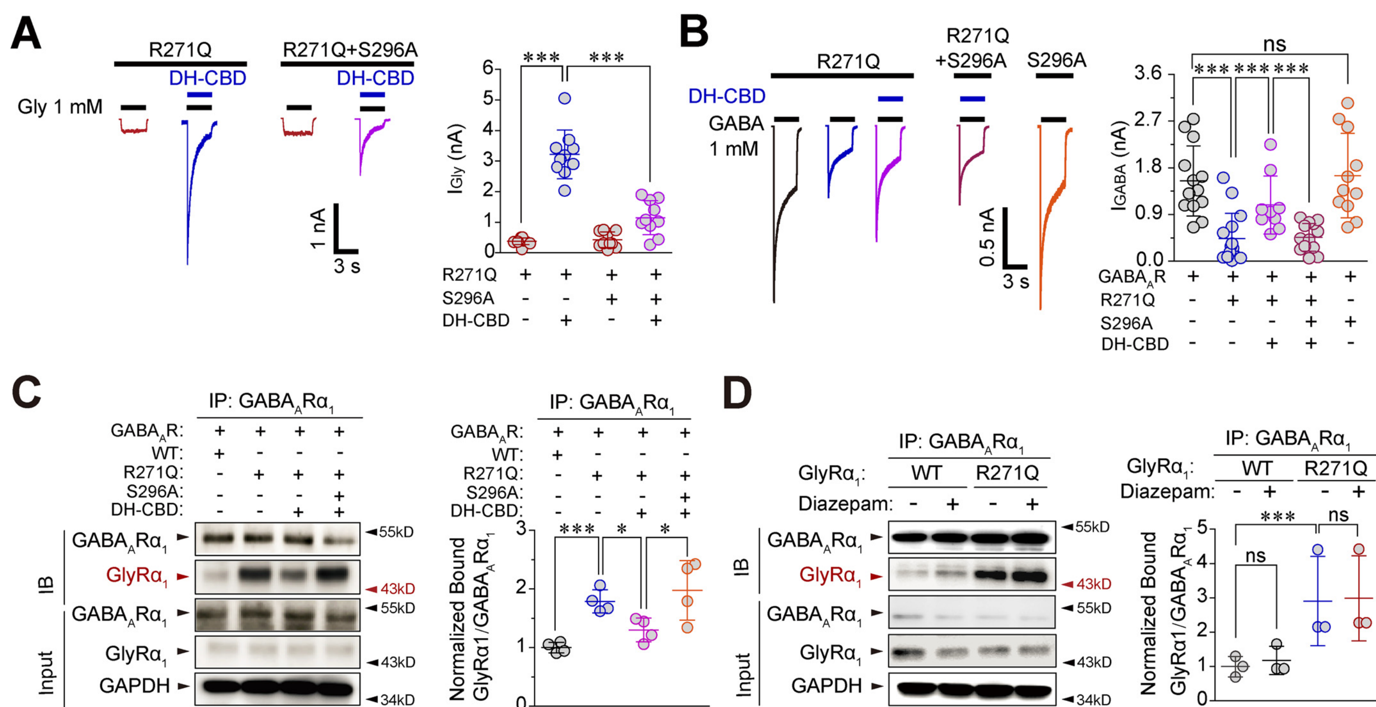

Figure 4. Effects of GlyRα_1^{S296A} site mutation on DH-CBD–induced restoration on the interaction between GlyRα_1 and GABA$_A$R. *A*, representative trace records and average values of I_{Gly} activated by 1 mM glycine in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and GlyRα_1 carrying both R271Q and S296A mutation with or without 10 μM DH-CBD preincubation ($n = 10$). *B*, representative trace records and average values of I_{GABA} activated by 1 mM GABA in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and GlyRα_1 carrying the R271Q and S296A mutations with or without 10 μM DH-CBD preincubation ($n = 13$, 15, 9, 14, and 11). *C*, GlyRα_1 protein was purified using GABA$_A$R α_1 antibodies in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and GlyRα_1 carrying the R271Q and S296A mutations with or without 10 μM DH-CBD preincubation. *Input* represents the same protein immunoblots (*IB*) extracted from cell lysates prior to co-IP. Shown is quantification of WT and S296A mutant GlyRα_1^{R271Q} subunits binding to GABA$_A$R α_1 subunits with or without 10 μM DH-CBD preincubation ($n = 4$). The data were normalized to the WT group without DH-CBD preincubation. *D*, GlyRα_1 protein was purified using GABA$_A$R α_1 antibodies in HEK293 cells coexpressing GABA$_A$R ($\alpha_1\beta_2\gamma_2$) and GlyRα_1 carrying the R271Q and S296A mutations with or without 10 μM diazepam preincubation. *Input* represents the same protein immunoblots extracted from cell lysates prior to co-IP. Shown is quantification of WT and S296A mutant GlyRα_1^{R271Q} subunits binding to GABA$_A$R α_1 subunits with or without 10 μM diazepam preincubation ($n = 3$). The data were normalized to the WT group without diazepam preincubation. Data are represented as mean ± S.D. *, $p < 0.05$; ***, $p < 0.001$; based on unpaired *t* tests; *ns*, not significant ($p > 0.05$).

Whether these hyperekplexia-causing mutations can affect the function of GABA$_A$R remains unknown and needs to be investigated further. In addition, considering the fact revealed by this study that DH-CBD–induced functional restoration of GABA$_A$R is based on GlyRα_1, DH-CBD may not influence GABA$_A$R under conditions involving mutations of the GlyR β subunit and GlyT2 mutations. Thus, the therapeutic effect of DH-CBD may be based on gene sequencing results of individual hyperekplexic patients. For instance, DH-CBD or other cannabinoids may be an effective and precise medical treatment option for patients carrying GlyRα_1 mutations, especially Arg-271 mutations. However, for patients carrying GlyR β subunit– or GlyT2-related mutations, benzodiazepines may still be a preferential choice.

Experimental procedures

Ethics approval

All procedures were approved by the Institutional Animal Use and Care Committee of the School of Life Sciences, University of Science and Technology of China.

Electrophysiological recording

HEK293 cells were cultured as described previously (36). Plasmids coding *Rattus* GABA$_A$R ($\alpha_1\beta_2\gamma_2$) in the pUNI vector (59) and human GlyRα_1 (NM_000171) in the pcDNA3.1+ vector were cotransfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen). 48 h later, patch clamp recordings were performed. Trypsin (0.25% (w/v)) was used to digest the cells 2 h before recording. Then the cells were patched and recorded with external solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH, ~320 mosmol with sucrose). The patch pipettes (3–5 megaohm) used for patching were filled with intracellular solution containing 140 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 0.5 mM Na-GTP, and 2 mM Mg-ATP (pH 7.2 with CsOH, ~280 mosmol). The equivalent vehicle and DH-CBD/CBD were added 30 min before recording. Membrane currents were collected using an Axopatch 200B amplifier (Axon). The holding potential was held at −60 mV. Data were acquired using pClamp 10.4 software (Molecular Devices, Sunnyvale, CA). Drugs were applied using a Warner Fast-Step Stepper Motor-driven system when recordings were performed (SF-77B, Warner).

Site-directed mutagenesis

The R271Q, S296A, and K385A site mutations of α_1 GlyR were introduced using the QuikChange Site-Directed Mutagenesis Kit (Takara, Inc.). The complementary DNA sequences were determined through dsDNA sequencing with a genetic analysis system (Sangon, Inc.).

Coimmunoprecipitation

Plasmids coding GlyRα_1 and $GABA_AR$ were cotransfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen). DH-CBD and equivalent vehicle were added separately to the medium 30 min before the cells were collected. 48 h after transfection, the cells were collected and treated with buffer containing 1 M Tris-HCl (pH 7.5), 1% protease inhibitor mixture (Roche), 1 M NaCl, and 5% sodium deoxycholate. To confirm the protein expression level, 60 μl of whole-cell lysate was collected as the input before immunoprecipitation. The input has always been regarded as a standard in coimmunoprecipitation experiments. Then the primary antibody against $GABA_AR$ α_1 protein was incubated with IgG-agarose beads overnight at 4 °C. The mixtures were collected and incubated with the remaining cell lysates overnight. After five washes with cell lysis buffer, 100 μl of loading buffer was added, followed by 5 min of boiling. The samples were then used for Western blotting. Samples were loaded on SDS-PAGE gels (12%) and then transferred to a PVDF membrane (NEN, Boston, MA) for 90 min. Then the membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) nonfat milk for 1 h. Primary antibodies against $GABA_AR$ α_1 (1:100, 06-868, Merck), GlyR α_1 (1:500, NB300-113, Novus), and GAPDH (1:5000, 60004-1-AP, Proteintech) were used for overnight incubation. After three 5-min washes, the membrane was incubated with secondary antibodies against rabbit (1:5000, ab6721, Abcam) for 2 h at room temperature. The membrane was washed three times, and the protein bands were imaged using ECL reagent (Thermo Fisher Scientific). The gray values were analyzed using ImageJ software (National Institutes of Health).

FRET and live imaging

The CFP-GlyRα_1^{WT}, CFP-GlyRα_1^{R271Q}, and YFP-$GABA_AR\alpha_1$ plasmids were created by inserting complementary DNA sequences coding CFP and YFP at the N terminus of GlyRα_1 and $GABA_AR\alpha_1$ in vector pcDNA3.1+. For live imaging, cells were plated on 18 × 18 mm glass coverslips (CITOGLAS) coated with poly-D-lysine (Sigma-Aldrich). The coverslips were mounted in custom-designed chambers using L-15 medium without phenol red (Thermo Fisher). The temperature was maintained at ~37 °C using an air stream incubator. The CFP-GlyRα_1^{WT}, CFP-GlyRα_1^{R271Q}, and YFP-$GABA_AR\alpha_1$ plasmids were then transfected equivalently using Lipofectamine 2000 according to the manufacturer's instructions and used for analysis 48 h later. The equivalent vehicle and DH-CBD were added separately to the transfected cells 2 h before live imaging. DH-CBD at 10 μM was used in this experiment. The concentration of vehicle (ethanol) was less than 0.1%. The cells were observed using a Nikon Ti-Eclipse inverted microscope equipped with a charge-coupled device camera (Andor), a spinning disk confocal microscope (Yokogawa), and a laser merge module equipped with 445-, 488-, and 594-nm lasers (ILE, Andor). Fluorescence images were collected using iQ3 software (Andor). CFP and YFP were excited at 445 and 488 nm, respectively. CFP and YFP emissions were acquired simultaneously with a beam splitter (OPTOSOLIT II). The YFP/CFP emission ratio in each image was calculated after background subtraction by MATLAB and averaged over multiple cells. Experiments were repeated multiple times with similar results. The fluorescence intensity was analyzed using ImageJ software.

Drugs

All chemicals, including diazepam, glycine, and GABA, were from Sigma-Aldrich. The external solution was prepared the day before the experiment. Before electrophysiological recordings, the agonists, modulators, and antagonists were diluted with external solution. DH-CBD and CBD were synthesized according to a procedure described previously (50). DH-CBD was dissolved with ethanol and diluted by external solution before recording.

Statistical analysis

Transfected HEK293 cells were picked randomly for electrophysiological experiments. Concentration–response data analysis was performed using the nonlinear curve fitting program. Data were fit using the following Hill equation: I/I_{max} = bottom + (top − bottom)/(1 + 10 (logEC$_{50}$ − log[agonist]) × Hill slope). I_{max} is the maximum current. Data were statistically analyzed by unpaired t tests using GraphPad Prism 6.0 (GraphPad Software). Data are presented as mean ± S.D. $p < 0.05$ was considered significant.

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