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Presynaptic glycine receptors as a potential therapeutic target for hyperekplexia disease

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Abstract

While postsynaptic GlyRs as α/β heteromers attract the most research attention, little is known about the role of presynaptic GlyRs, likely α homomers, in diseases. Here, we demonstrate that DH-CBD, a nonpsychoactive cannabinoid, can rescue GlyR functional deficiency and exaggerated acoustic and tactile startle responses in mice bearing the point-mutations in the α1 GlyRs responsible for a hereditary startle/hyperekplexia disease. The GlyRs expressed as α1 homomers either in HEK-293 cells or at presynaptic terminals of the calyceal synapses in auditory brainstem are most vulnerable to hyperekplexia mutation-induced impairment. Homomeric mutants are more sensitive than heteromers to DH-CBD, suggesting presynaptic GlyRs as a primary target. Consistent with this, DH-CBD selectively rescues impaired presynaptic GlyR activity and diminished glycine release in the brainstem and spinal cord of hyperekplexic mutant mice. Thus,

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COMPETING INTEREST STATEMENT

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

W. X. and L. Z. conducted mutagenesis and animal behavioral tests. W.X. conducted patch-clamp recording in HEK 293 cells. S-R. C., Y-L. Z, H. C., D-P. L., H-L. P. conducted spinal slice recordings, L-M. He, W. X. and L-G. Wu conducted brain stem calyceal recording, K-J. C. and K. C. R. synthesized DH-CBD, G. E. H. constructed genetically engineered mouse lines, J.P. provided transgenic R271Q mouse line, L. Z initiated, designed and supervised the project and wrote the manuscript.

presynaptic α GlyRs emerge as a potential therapeutic target for dominant hyperekplexia disease and other diseases with GlyR deficiency.

INTRODUCTION

Glycine has been defined as a major inhibitory neurotransmitter in spinal cord for nearly half a century [1]. The glycine receptors (GlyRs) are the last therapeutic orphan among members of the Cys-loop ligand-gated ion channel superfamily including γ-aminobutyric acid type A ($GABA_A$), nicotinic acetylcholine (nACh) and 5-HT_3 receptors. Native GlyRs may consist of either homomers or heteromers. While all α subunits (α1, α2, α3 or α4) are capable of forming functional homomeric channels, the β subunit can form functional channels only upon co-assembly with the α subunits [2]. Postsynaptic GlyRs have been well defined as heteromeric α1β subunits because the β subunit binds to gephyrin, a postsynaptic scaffolding protein essential for the clustering and targeting of GlyRs in postsynaptic membrane [3, 4]. These receptors are thought to be the primary target for several neurological diseases such as pain, anxiety, drug addiction and hyperekplexia disease [5].

Presynaptic GlyRs are first described in calyceal synapses in the medial nucleus of the trapezoid body (MNTB) in rat brainstem [6]. These presynaptic GlyRs are subsequently found to express in spinal cords and ventral tegmental area (VTA) [7, 8]. Different from postsynaptic heteromeric GlyRs, presynaptic GlyRs are likely formed by homomeric α subunits [7, 8]. A recent study has characterized the anatomical segregation of presynaptic and postsynaptic GlyRs in detail in calyx of held neurons from auditory brainstem [9]. This study has provided strong evidence to show that the GlyRs at presynaptic terminals of calycreal synapses are composed of homomeric α1 subunits. Although presynaptic GlyRs are proposed to modulate neurotransmitter release under physiological condition, little is known about the roles of presynaptic GlyRs in pathological processes.

Missense point-mutations in the human α1 GlyR subunit gene disrupt GlyR function and result in familial hyperekplexia-startle disease[10, 11]. Although rare, this disease is characterized by excessive startle reaction to unexpected auditory and tactile stimuli followed by muscle stiffness[12]. Among dozens of point-mutations associated with hyperekplexia disease, the most frequently occurring mutation causing human dominant hyperekplexia disease is the R271Q/L mutation in the α1 GlyR subunit [13]. Mice carrying the R271Q mutation exhibit severe hypersensitivity to tactile and acoustic stimuli, closely resembling human startle disease[14]. In addition to the R271Q mice, other lines of genetically engineered mice carrying Q266I, S267Q and M287L mutations in the α1 subunit also display hyperekplexic behaviors[15, 16]. Despite overwhelming evidence for functional deficiency of GlyRs in startle disease, current therapeutic agents do not target GlyRs [12]. In addition, the role of presynaptic GlyRs in startle disease has been largely ignored because our knowledge about presynaptic GlyRs is very limited.

Allosteric positive modulators of GlyRs have been proposed to present a therapeutic potential in the treatment of diseases with GlyR deficiency[17]. This appears to be the case as recent studies have shown that a chemically modified cannabinoid, dehydroxyl-cannabidiol (DH-CBD), can suppress acute and chronic pain by specifically targeting α3 GlyRs[18, 19].

We designed the present study to address the following questions. Can DH-CBD treat exaggerated startle response by restoring deficiency in GlyR function? What is the role of presynaptic α1 GlyRs in hyperekplexia disease?

RESULTS

GlyR deficiency and exaggerated startle responses

The α1R271Q mutation substantially impaired α1 GlyRs when expressed in HEK-293 cells. Gly at 1 mM produced the maximal amplitude of I_{Gly} in HEK-293 cells expressing wild type (WT) α1 GlyRs, whereas Gly even at 10 mM evoked relatively small currents in cells expressing the R271Q mutant receptors (α1R271Q GlyRs) (Fig. 1a). The R271Q mutation reduced the maximal amplitude of I_{Gly} (Gly I_{max}) by 71% and significantly increased the Gly EC_{50} value (Fig. 1b). In line with a previous study[14], there was a pronounced reduction in both frequency and amplitude of glycinergic spontaneous IPSCs (Gly sIPSCs) in spinal slices from adult mice carrying the α1R271Q missense mutation (Fig. 1c,d). The R271Q mutant mice only survived as heterozygotes and displayed exaggerated startle response to both acoustic (Fig. 1e) and air-puff stimuli (Fig. 1f). However, these hyperekplexia mice could be trained to perform at a level similar to their wildtype (WT) littermates in the rotarod test when they were handled gently and carefully (Supplementary Fig. 1).

DH-CBD restores GlyR deficiency-induced behaviors

DH-CBD is a synthetic cannabinoid slightly modified from cannabidiol (CBD), the major nonpsychoactive component of marijuana (Fig. 2a). DH-CBD has been shown to be more efficacious than CBD in potentiating I_{Gly} in HEK-293 cells expressing GlyRs and in suppressing pain hypersensitivity in mice through a GlyR-dependent mechanism [18]. DH-CBD was continuously applied for at least 5 min with intermittent applications of Gly in our entire electrophysiological experiments. DH-CBD at 10 μM substantially recovered Gly I_{max} in HEK-293 cells expressing the α1R271Q mutant GlyRs (Fig. 2a). DH-CBD at 10 μM significantly reduced the Gly EC_{50} value and increased the maximal I_{Gly} for the α1R271Q GlyRs (Fig. 2b). The rescue of α1R271Q mutant GlyR deficiency was dependent on DH-CBD concentrations over a range from 100 nM to 30 μM (Supplementary Fig. 2).

Next, we examined if DH-CBD can treat exaggerated startle reflex in R271Q mutant mice. The most typical sign of α1R271Q mutant mice that resemble human hyperekplexia is exaggerated startle reflexes to sudden noise (Supplementary video 1). The startle responses to different sound stimuli (80–120 dB) were measured in the α1R271Q mutant mice and WT littermates. WT mice were nearly insensitive to all levels of the sound stimulus in a range from 80 dB to 120 dB (Fig. 2c). In contrast, α1R271Q mutant mice exhibited exaggerated startle reflexes to the sound stimulus at all intensity levels (Fig. 2c). For instance, the average values of startle response to acoustic sound stimulus (120 dB) were 135 ± 42 mV in the WT mice and 1,656 ± 222 mV in the α1R271Q mutant mice. Intraperitoneal injection of DH-CBD inhibited the exaggerated startle response of α1R271Q mutant mice in a dose-dependent manner (Fig. 2c and Supplementary Video 2). Similarly, α1R271Q mutant mice displayed exaggerated responses to tactile air-puff stimuli, suggesting that there is a deficiency in sensory-motor reflex at the spinal level in these mice

(Fig. 2d). This tactile-induced exaggerated reflex was significantly suppressed by intraperitoneal injection of DH-CBD at 50 mg/kg (Fig. 2d). Similarly, these mice were supersensitive to touching and handling as they displayed a hind feet clenching behavior and exaggerated tremor when picked up by the tail (Fig. 2e). Intraperitoneal injection of DH-CBD at 30 mg/kg markedly alleviated this symptom. While the WT mice righted themselves in less than 1 s, α1R271Q mutant mice needed more than 20 s to get back on their feet (Fig. 2f,g, Supplementary Video 3). Intraperitoneal injection of DH-CBD reversed the delay of righting reflex in α1R271Q mutant mice in a concentration dependent manner (Fig. 2f,g, Supplementary Video 4).

DH-CBD restoration: a site/genotype specific effect

We next constructed 8 additional point-mutations in the α1 GlyR. Each of them was found to cause dominant familial startle disease in rodents or humans [11, 14, 16]. All of these point-mutations increased the Gly EC_{50} values (Fig. 3a). DH-CBD significantly reduced the Gly EC_{50} values in 7 of 9 hyperekplexic mutant receptors when expressed in HEK-293 cells (Fig. 3a). The impact of hyperekplexic mutations on Gly I_{max} appeared to be less consistent and varied substantially (Fig. 3b). For instance, the S270T and M287L mutations resulted in an increase of the Gly EC_{50} values, whereas these mutations did not significantly alter the Gly I_{max} when expressed in HEK-293 cells. DH-CBD either partially or totally rescued the reduced Gly I_{max} of the R218Q, R271Q and K276E mutants but not significantly affected that of the other hyperekplexic mutant receptors. Thus, DH-CBD appeared to rescue the Gly EC_{50} in an extent more consistent than its rescue of the Gly I_{max} of hyperekplexic mutant receptors. In addition, two mutant α1 GlyRs, α1Q266I and α1S267Q, were completely insensitive to DH-CBD, suggesting that the rescue of GlyR dysfunction by DH-CBD is site-specific.

We next asked if the efficacy of DH-CBD rescue of GlyR function is correlated with its restoration of exaggerated startle behaviors. We therefore collected 4 individual mouse lines that carry DH-CBD sensitive (α1R271Q and α1M287L) and DH-CBD insensitive (α1Q266I and α1S267Q) point-mutations. Consistent with previous observations [14–16], all 4 heterozygous mutant mouse lines showed significantly increased startle response to sound stimuli of 120 db (Fig. 3c). Intraperitoneal injection of DH-CBD (50 mg/kg, i.p.) inhibited the startle response in both α1R271Q and α1M287L mutant mice (Fig. 3c). In contrast, α1S267Q and α1Q266I mutant mice were insensitive to DH-CBD inhibition of startle response. Strychnine at 100 nM produced a rightward, parallel shift in the Gly concentration response curve of WT α1 GlyRs expressed in HEK-293 cells, respectively (Supplementary Fig. 3a). In line with a previous study [20], strychnine (1.0 mg/kg, i.p.) increased startle response in WT C57BL/6J mice (Supplementary Fig. 3b). DH-CBD did not significantly alter strychnine-induced *in vitro* or *in vivo* effects, respectively. There was a strong correlation between the efficacy of DH-CBD restoration of GlyR dysfunction *in vitro* and the efficacy of DH-CBD restoration of exaggerated startle behaviors *in vivo* (Fig. 3d, γ^2=0.93, p=0.0005, linear regression).

β subunit partially reduces mutant α1GlyR deficiency

Addition of the β subunits remarkably rescued the α1R271Q mutation-induced deficiency in HEK-293 cells coexpressing the β subunit with the α1R271Q subunit (Fig. 4a). This rescue was dependent on the ratio of the β and α1R271Q subunits (Fig. 4b). With increasing the ratio of the β subunit relative to the αR271Q mutant subunit transfected into cells, the Gly EC_{50} value was decreased and the Gly I_{max} was increased. Coexpression of the β subunit with α1R271Q mutant subunit in a ratio of 3:1 completely rescued the impaired maximal efficacy of Gly. On the other hand, the β subunit did not significantly alter the expression levels of GlyR proteins at the cell surface (Supplementary Fig. 4), suggesting that the rescue induced by the β subunit is not due to alteration of receptor trafficking and receptor protein translation. Similar to its rescue of the deficiency in α1R271Q mutant receptors, the β subunit also rescued the deficiency in GlyR function caused by the other hyperekplexic mutations when co-expressed with each of 8 hyperekplexic α1 mutant receptors (Fig. 4c). Compared to homomeric α1 mutants, heteromeric α1/β mutant GlyRs were either less or insensitive to DH-CBD (Fig. 4d). Addition of the β subunit also rescued the reduced Gly I_{max} of the most but not all mutant receptors (Fig. 4e). On the other hand, DH-CBD did not significantly alter the Gly I_{max} for these heteromeric mutant α1/β GlyRs (Fig. 4f).

DH-CBD rescues spinal diminished glycine release

The above data suggest that GlyR homomers but not heteromers are the receptors most sensitive to cannabinoids. This idea favors a hypothesis that presynaptic GlyRs but not postsynaptic GlyRs are the primary therapeutic target for cannabinoid treatment of exaggerated startle disease. To test this hypothesis we first examined the effects of DH-CBD on glycinergic transmission by recording the Gly sIPSCs in spinal cord slices of adult heterozygous α1R271Q mutant and WT mice. While the frequency and amplitude of the Gly sIPSC were profoundly reduced in the mutant mice, bath application of DH-CBD at 20 μM only increased the diminished frequency but not the amplitude of Gly sIPSC (Fig. 5a). This suggests that DH-CBD rescues glycinergic deficiency by targeting presynaptic GlyRs.

Unlike the postsynaptic GlyRs that typically hyperpolarize mature neurons upon activation, stimulation of presynaptic GlyRs leads to depolarization of presynaptic terminals and an increase in glycine release due to chloride efflux [6, 7]. To further address whether DH-CBD acts on presynaptic glycinergic activity, we examined glycinergic miniature IPSCs (Gly mIPSCs) in spinal dorsal horn neurons of α1R271Q mice. To record Gly mIPSCs, tetrodotoxin (TTX, 1 μM) was added to the external solution in addition to glutamate and $GABA_A$ receptor antagonists. Similar to our observation in the Gly sIPSCs, DH-CBD profoundly increased the frequency, but not the amplitude, of Gly mIPSCs in spinal slices from the R271Q mutant mice (Fig. 5b). The cumulative probability analysis of Gly mIPSCs revealed that DH-CBD shifted the distribution pattern of the inter-event interval, but not amplitude, of mIPSCs to the left (Fig. 5c). We next tested the effect of DH-CBD on paired-pulse ratio (PPR) of evoked glycinergic IPSCs in the α1R271Q spinal slices (Fig. 5d). DH-CBD significantly increased PPR, suggesting a mechanism involving presynaptic modulation of GlyRs. The restoration of the deficiency in glycinergic transmission by DH-CBD appeared to be point-mutation specific since DH-CBD did not alter the diminished frequency and amplitude of Gly sIPSC in spinal slices from α1Q266I mutant mice

(Supplementary Fig. 5). This observation is in line with our previous observation that DH-CBD-potentiation of hyperekplexic mutant GlyRs was a site-specific effect.

The above unexpected finding reveals a presynaptic mechanism for DH-CBD-modulation of GlyRs. To further test this idea, we applied a low concentration of picrotoxin (PTX) in an attempt to differentiate the presynaptic from postsynaptic effect of DH-CBD on GlyRs. Low concentrations of PTX have been shown to preferentially inhibit homomeric α GlyRs without significantly altering heteromeric αβ GlyRs expressed in HEK-293 cells [21, 22]. Similarly, PTX at low doses is also found to selectively affect presynaptic GlyRs, which are likely homomers, in the spinal cord and brainstem [7, 9, 23]. We first examined the sensitivity of homomeric and heteromeric α1R271Q mutant GlyRs to PTX-induced inhibition when expressed in HEK-293 cells. The homomeric α1R271Q GlyRs were substantially more sensitive than heteromeric α1R271Q/β receptors to PTX-induced inhibition (Fig. 5e). For example, PTX at 30 μM significantly reduced I_{Gly} by 55% in cells expressing α1R271Q mutant homomers, whereas PTX at this dose had no effect on I_{Gly} in cells expressing α1R271Q/β1 mutant heteromers. This finding is in line with the idea that PTX at low concentrations (i.e. 30 μM) preferentially inhibits homomeric or presynaptic GlyR activity. We next tested whether PTX at 30 μM can block the effect of DH-CBD on Gly sIPSCs because DH-CBD similarly potentiated the frequency of both Gly sIPSCs and mIPSCs in the spinal slices from the α1R271Q mice. In these slices, PTX at 30 μM completely eliminated the DH-CBD-induced potentiating effect on the Gly sIPSC frequency (Fig. 5f). In contrast, PTX did not significantly alter the Gly sIPSC amplitude in the α1R271Q spinal slices (Supplementary Fig. 6).

DH-CBD selectively rescues presynaptic GlyR deficiency

The Calyx of Held is one of the very few sites in mammalian CNS where direct recording of presynaptic channel conductance can be achieved[24]. The Calyx of Held and its postsynaptic target, medial nucleus of the trapezoid body (MNTB), function as a critical relay in brainstem auditory circuitry. Moreover, these calyceal terminals seem to be an ideal preparation for us to study homomeric α1 subunits because of lacking the β, α2 and α3 GlyR subunits [9]. We next recorded using whole-cell patch-clamp from both ^{I}Gly presynaptic and postsynaptic terminals in Calyx of Held neurons from postnatal (P) 12-P18 mice (Fig. 6a). Application of 1 mM Gly evoked strychnine-sensitive and nearly maximal currents in postsynaptic MNTB principle neurons as early as after P12 in WT mice (Fig. 6b). On the other hand, there was no detectable current when exposed to 3 mM of Gly in calyceal terminals from P12 mice. I_{Gly} became detectable in only 30% of calyceal terminals from P14 mice and the amplitude of I_{Gly} reached the maximal after P16. This expression pattern is coincident with the timing when the α1 subunits emerge and replace embryonic dominant α2 subunits in brainstem and spinal cord [2]. It should be mentioned that a previous study reported a very similar observation that presynaptic GlyR activity in rat calyx depends on developmental stages [25].

Among 4 mutant mouse lines, only the M287L mouse line can yield homozygous offspring. These mutant homozygous mice displayed seizure-like behaviors in response to acoustic and tactile stimuli after P14 (Supplementary Fig. 7a). This abnormal behavior in the mutant mice

(P16–20) was fully reversed shortly after intraperitoneal injection of DH-CBD (50 mg/kg) (Supplementary Fig. 7b). In calyceal neurons isolated from homozygous M287L mice of P16–18, the M287L mutation substantially impaired the function of presynaptic GlyRs (Fig. 6c). Both Gly I_{max} and the apparent Gly affinity of presynaptic GlyRs from the M287L mutant mice were significantly reduced as compared with presynaptic GlyRs from their WT littermates. In contrast, the M287L point-mutation did not significantly alter the functionality of postsynaptic GlyRs in principle neurons (Fig. 6d). DH-CBD at 10 μM significantly enhanced the average amplitude of I_{Gly} recorded in presynaptic M287L terminals from 65 ± 15 pA to 181 ± 43 pA (Fig. 6e). In contrast, DH-CBD did not significantly alter postsynaptic I_{Gly} recorded in MNTB neurons from M287L mutant mice (Fig. 6f). This is consistent with our above observation that the homomeric M287L mutant receptor but not its heteromeric counterpart is cannabinoid sensitive receptors.

Thus, 9 hyperekplexic mutant α1 GlyRs can be classified as cannabinoid sensitive and insensitive receptors based on their response to cannabinoid potentiation of I_{Gly} and rescue of startle behavior (Supplementary Fig. 8a). Addition of the β subunits alone can substantially rescue the disrupting effect induced by all startle dominant mutations in the α1 subunits (Supplementary Fig. 8b). Cannabinoids appear to restore the glycinergic dysfunction induced by hyperekplexic point-mutations in homomeric α1 GlyRs, most likely located in the presynaptic sites (Supplementary Fig. 8c).

DISCUSSION

Despite of abundant evidence for presynaptic GlyRs in CNS, it is far from clear to what extent they play a role in physiological and pathological processes. This study highlights the potential significance of presynaptic GlyRs in both pathophysiological mechanism and therapeutic target of dominant hyperekplexia disease. First, hyperekplexic point-mutations in the α1 subunits disrupted the function of homomers more significantly than that of heteromers when expressed in HEK-293 cells and in segregated pre- and postsynaptic sites of calyceal/MNTB synapses. Second, hyperekplexic mutant homomers were more sensitive than mutant heteromers to DH-CBD-induced rescue. Third, DH-CBD only potentiated presynaptic homomeric α1 GlyRs without significantly altering postsynaptic GlyR activity in the Calyx of Held of auditory brainstem from hyperekplexic mutant mice. Consistent with this observation, DH-CBD preferentially rescued the diminished frequencies without significantly affecting the amplitudes of Gly sIPSCs and mIPSCs in spinal cord slice. Such rescue by DH-CBD was completely abolished by PTX at a concentration preferentially blocking homomeric GlyRs, suggesting DH-CBD rescue is mediated by presynaptic GlyRs. Finally, an increase in PPR induced by DH-CBD indicates an enhanced probability of neurotransmitter release in the spinal cord slice of adult hyperekplexic mutant mice.

One could also argue that extrasynaptic GlyRs may be a target of hyperekplexia disease and DH-CBD. However, there is no good evidence for homomeric extrasynaptic α1 subunits. Most extrasynaptic GlyRs characterized previously were homomeric α2 subunits in the brain during early developmental stage[2]. On the other hand, extrasynaptic GlyRs in brainstem are found to be α1β heteromers[9]. These receptors are usually localized either in postsynaptic or in presynaptic soma but not at presynaptic terminals[4, 9]. Our previous studies

have shown that the α3 GlyRs are the target of cannabinoids in the treatment of acute and chronic pain [18, 19]. This raises a question whether or not the α3 subunit is also involved in DH-CBD rescue of hyperekplexic mutation-induced *in vivo* effect. It is unlikely to be the case. The pain sensitivity to thermal stimuli is unchanged in R271Q mutant mice [26], suggesting that there is no α3GlyR deficiency-dependent behavioral change in these mutant mice. Although DH-CBD can potentiate both wild type α1 and α3 GlyRs, DH-CBD does not affect the baseline of either locomotor activity and startle response in normal mice [18, 19]. In this regard, the potentiation of the α3GlyRs by DH-CBD is unlikely contributable to its rescue of exaggerated startle response in α1R271Q mutant mice.

Consistent with recent studies [27, 28], co-expression of the β subunits partially but significantly restored the functional deficiency in some hyperekplexic mutant GlyRs expressed HEK-293 cells. Our data also suggest that homomeric like/presynaptic α1 GlyRs in native neurons sustain more damage than heteromeric/postsynaptic GlyRs in channel function from hyperekplexia mutations. In general, these mutant homomers were more sensitive to DH-CBD rescue. One hypothesis for this β subunit-dependent rescue is that a cluster of amino acid residues in the transmembrane domains of the β subunit may shield or compromise the gating impairment from hyperekplexic point-mutations in the α1 subunit [27]. There is strong evidence to indicate a reduction of GlyR function in both chronic inflammatory and neuropathic pain in animals [29, 30]. Yet, it is unclear about the role of presynaptic GlyRs in the regulation of pain transmission. Future studies should be carried out to determine whether presynaptic GlyRs are a potential target in chronic pain. We emphasize the presynaptic GlyRs in this study because the effect of DH-CBD on glycinergic input is presynaptic. In addition to the deficiency in presynaptic GlyR function (diminished glycine release), the postsynaptic GlyR deficiency (reduced amplitude of Gly IPSCs) was observed as well in spinal cord slices from the R271Q mice. Thus, both impaired pre- and postsynaptic α1GlyRs should account for diminished glycinergic synaptic transmission caused by the R271Q mutation.

Hyperekplexia is usually introduced as a neuromotor disorder. However, there is a significant underestimation of the impact of the sensory dysfunction in this disease due to a loss of glycinergic innervation/transmission in either sensory neurons or from sensory neurons to motoneurons in spinal cord. This could be especially true for hyperekplexia patients bearing dominant mutations in the α1 subunits. These patients usually display hyperreflexia and exaggerated startle response to noise, tactile (such as air), handling (feeding, touching the head or nose) and even visual stimuli shortly after birth [31–33]. It appears that an increase in acoustic startle responses in hyperekplexia mutant mice does not always extend to more general motor behaviors such as balance and coordination in rotarod test reported in previous and our current studies [15]. This strongly suggests that disinhibition by diminished glycinergic input is a major cause of hyperekplexia. Consistent with this notion, hyperekplexia mutations largely impaired the function of α1GlyRs expressed at the presynaptic terminals of auditory brainstem and spinal dorsal horn neurons. On the other hand, DH-CBD restored glycinergic transmission deficiency in both brainstem and spinal cord by targeting, at least in part, presynaptic α1GlyRs.

There are a few hyperekplexic mutations detected in postsynaptic proteins such as gephyrin and the GlyR β subunit [11, 34]. However, about 35% of hyperekplexia patients do not carry mutations in genes encoding postsynaptic proteins such as gephyrin and the GlyR β subunits [13]. In addition, most hyperekplexic mutations occurring in the α1 subunits are linked to dominant familial startle disease, whereas mutations detected in the β subunit and glycine transporter genes are principally inherited in a recessive startle disease [11, 35, 36]. In addition to the R271Q, 5 additional cannabinoid sensitive mutants tested in this study are inherited in human dominant hyperekplexia disease [11]. The majority of them such as V260M, P250T, S270T and R271Q were very sensitive to DH-CBD rescue when expressed as homomers, but less or insensitive to DH-CBD when coexpressed with the β subunits as heteromers. In this regard, the notion that presynaptic GlyRs as an important therapeutic target should be applicable to dominant familial hyperekplexia. However, this hypothesis should be tested further once either a genetic or pharmacological approach selectively for homomeric α1 subunit becomes available in the future.

Therapeutic mechanisms and applications of nonpsychoactive cannabinoids have been a renewed topic of recent research [17, 37]. The results presented here have revealed a new potential for nonpsychoactive cannabinoids in the treatment of a severe hereditary neurological disease. Unlike $GABA_A$ acting agents that are plagued by various side effects, [38], DH-CBD does not produce significant psychoactive or sedative effects even at high concentrations [19]. In addition, clonazepam, a commonly used agent in the treatment of hyperekpleixa, is not always effectively in the control of some symptoms in this disease [39]. More than 30 hyperekplexia missense, nonsense and frame-shift mutations in the α1 GlyRs have been found to link to hyperkeplexia disease [11]. Our data suggest that DH-CBD specifically targets cannabinoid-sensitive GlyRs, which are likely homomers located at presynaptic sites. It is worth noting that the M287L mutation produced more functional deficiency of GlyRs when expressed in calyeal neurons than in HEK-293 cells. This is consistent with a recent study showing decreased glycine-mediated currents in isolated neurons of brain stem from the M287L mutant mice [40]. One possibility to explain this discrepancy is that a neuron-specific post-translational modification may alter the sensitivity of GlyRs to hyperekpelxic mutations in *vivo*. This idea is supported by a previous study showing different functional properties of the R271Q mutant α1GlyR receptors when expressed in dorsal horn neurons and HEK-293 cells [41].

Taken together, we have provided new evidence showing that presynaptic GlyRs are an emerging target for the mechanism and therapeutics of hyperekplexia disease. These presynaptic-like GlyRs are proposed to play a role in ethanol action and reward mechanisms in the brain [8, 42–44]. Thus, these GlyRs residing at presynaptic terminals should represent a previously underestimated target potentially important for various diseases involving GlyR deficiency or GlyR signaling pathways.

METHODS

Animals

Unless otherwise indicated, male hyperekplexic GlyR mutant mice and their wild-type littermates weighing between 20 and 28g (7–12 weeks old) were used in all experiments.

These animals bred specifically for this study were backcrossed to C57BL/6J mice for at least 6 generations. They were housed 2 per cage on a 12:12 h light/dark cycle and not used in different behavioural tests. All behavioural tests were double-blind and performed during the light cycle (9:00 am–5:00 pm). These mice were adapted to the experimental environment at least 2 hours prior to the tests. All animal studies were carried out under the protocols approved by Animal Care and Use Committees of the University of Pittsburgh, the University of Texas MD Anderson Cancer Center and the National Institute on Alcohol Abuse and Alcoholism. The α1Q266I, α1M287L, α1S267Q and α1R271Q mutant mice were generated as previously described [14, 16, 40, 45]. All genetically engineered mice studied were heterozygous for the mutant α1 subunit. Genotyping of the α1Q266I mutant mice was done using the following primers: Forward primer: 5′ GCC TGC TCA TCG TCA TCC TG 3′; Reverse primer: 5′ CCA ATC TGA TCT GTG CAA TCC T3′. Genotyping of the $\alpha1^{S267Q}$ mutant mice was done using the following primers: Forward primer: 5′ GCT TTA ACT TCT GCC CTA TGG 3′; Reverse primer: 5′ GTT GTT GTT AAC TTG TTT ATT G 3′. Genotyping of the α1M287L mutant mice was done using the following primers: Forward primer: 5′ GAA TCT TCC AGG CAA CAT TTC AG 3′; Reverse primer: 5′ AGT ATC CCA CCA AGC CAG TCT TT 3′. Genotyping of the α1R271Q mutant mice was done using the following primers: Forward primer: 5′ CTC ATC TTT GAG TGG CAG GA 3′; Reverse primer: 5′ GCA TCC ATG TTG ATC CAG AA-3′. Wild type mice (α1WT) and mutant (α1R271Q, α1M287L, α1Q266I and α1S267Q) heterozygous mice used for behavior experiments were produced from heterozygous and corresponding wild type breeding pairs.

Site-directed mutagenesis

Point-mutations of the human α1 GlyR were introduced using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The authenticity of the DNA sequence through the mutation sites was confirmed by double stranded DNA sequencing using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc).

Electrophysiological recording

HEK-293 cell transfection and recording—HEK-293 cells were cultured as described previously [46]. The plasmid cDNAs coding for the wild type and mutant GlyR subunits were transfected using the SuperFect Transfection Kit (Qiagen, Hidden, CA). Electrophysiological recordings were carried out 2 days after transfection. HEK-293 cells were treated with 0.25% (w/v) Trypsin and 0.53 mM EDTA 2 hrs prior to recording. The HEK-293 cells were lifted and continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH; ~340 mosmol with sucrose). Patch pipettes (3–5 MΩ) were filled with the intracellular solution that contained 120 mM CsCl, 4 mM $MgCl_2$, 10 mM ethyleneglycol bis-(-aminoethylether)-**N,N,N′N′**-tetraacetic acid (EGTA), 10 mM HEPES, 0.5 mM Na-GTP and 2 mM Mg-ATP (pH 7.2 with CsOH, ~280 mosmol). Membrane currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier (Axon) at 20–22°C. Cells were held at −60 mV unless otherwise indicated. Data were acquired using pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Data were filtered at 1 KHz and digitized at 2 KHz. Bath solutions were applied through 3 barrel square glass tubing (Warner

Instrument, Hamden, CT) with a tip diameter of ~700 μm. Drugs were applied using a Warner fast-step stepper-motor driven system. The solution exchange time constants were ~4 ms for an open pipette tip and 4–12 ms for whole-cell recording.

Spinal cord slice preparation and recording—Lumbar spinal cord slices at the L5–L6 level were prepared from adult mice as we described previously [47, 48]. The lumbar spinal cord was removed through laminectomy during isoflurane-induced anesthesia and sliced (400 μm) using a vibratome. The slices were continuously superfused with artificial cerebrospinal fluid containing (in mM) 117.0 NaCl, 3.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 11.0 glucose, and 25.0 $NaHCO_3$ (bubbled with 95% O_2/5% CO_2). Neurons in the lamina II of the spinal cord were visualized using a fixed-stage microscope (BX50WI; Olympus, Tokyo, Japan) with differential interference contrast/infrared illumination. We obtained all whole-cell patch-clamp recordings at 34 °C using glass pipettes filled with a solution containing (in mM) 110 Cs_2SO_4, 5 TEA, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, and 10 lidocaine N-ethyl bromide; adjusted to pH 7.2–7.4 with 1 M CsOH (290–300 mOsm). Spinal slices were recorded at the holding potential of 0 mV. DH-CBD was applied by puff application directly to the recorded neuron using a positive pressure system (4 psi, 15 ms; Toohey Company, Fairfield, NJ). Spontaneous glycinergic IPSCs were recorded in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μM bicuculline, and 50 μM AP-5, which were bath applied during the recording period. Glycinergic miniature IPSCs (mIPSC) were recorded in the presence of TTX (1 μM). The input resistance was continuously monitored, and the recording was abandoned if it changed more than 15%. All spinal slice recordings were performed under a double-blind condition. Data acquisition and analysis of postsynaptic currents were done as described previously [47, 48].

Calyceal slice preparation and recording—Parasagittal brainstem slices (100 μm thick) containing the medial nucleus of the trapezoid body were prepared from 12 to 18 day old mice of either sex using a vibratome (7000 SMZ, Campden Instrument). For the dissection and storage of slices, we used a solution containing (in mM): 95 NaCl, 25 NaHCO3, 25 glucose, 50 sucrose, 2.5 KCl, 1.25 NaH2PO4, 0.1 CaCl2, and 3 MgCl2, 0.4 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate (95% O2/5% CO2). The slices were incubated for 30 min at 37°C and then held at room temperature (22–24°C). Whole-cell current recordings were made from calyx of Held terminals and principal neurons of the medial nucleus of the trapezoid body (MNTB). Sampling intervals and filter settings were 20 μs and 2.9 kHz, respectively. Cells were visualized by differential interference contrast microscopy through a 40x water-immersion objective using an upright Olympus microscope. All experiments were made with the EPC-10 amplifier. Holding potential was 0 mV. Bath solution (~22–24°C) containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 25 dextrose, 1.25 NaH2PO4, 0.4 ascorbic acid, 3 myo-inositol, 2 sodium pyruvate, 25 NaHCO3, pH 7.4 when bubbled with 95% O2–5% CO2. The presynaptic and postsynaptic pipette solution contained (in mM): 125 Cs-gluconate, 20 CsCl, 4 MgATP, 10 Na2-phosphocreatine, 0.3 GTP, 10 HEPES, 0.05 BAPTA, pH 7.2, adjusted with CsOH. The BAPTA concentration (50 μM) mimicked the endogenous calcium buffer capacity at calyces [49].

Startle response measurement

Acoustic startle response test—Startle responses were measured using SR-LAB test stations and software (San Diego Instruments, San Diego, CA). The method of startle response measurement was modified from a previous study [16]. Briefly, test stations were both standardized and calibrated. Individual mice were then placed in the Plexiglas holding cylinder for a 5-min acclimation period. A background noise level of 70 dB was maintained over the duration of the test session. The recording period of acoustic startle response consisted of six blocks. Each block consisted of 6 trials: one control trial with no stimulus (baseline) and the other five trials with single 40 ms sound of 80, 90, 100, 110, 120 dB presented in a random order with a 10–20 s inter-trial interval. The entire session comprised 36 trials and took ~15 min. The startle amplitude was measured every 1 ms over a 65 ms period beginning at the onset of the startle stimulus trial. The startle response was identified as the maximum startle amplitude (Vm) to a given stimulus minus the Vm to no stimulus (baseline).

Tactile startle response test—The San Diego Instruments, Inc (San Diego, CA) "SR-LAB" system was used to deliver the tactile air puff stimulus and to measure the flinch response of the mouse. The mouse was placed in a Plexiglas cylinder, located within a ventilated sound-attenuating chamber to reduce noise contamination, and left undisturbed for a 5 min acclimation period. Air puff stimuli of 1.5 psi or 5 psi controlled by a regulator were delivered to the back of mice for 100 ms duration. Vibrations occurring in the Plexiglas cylinder caused by the whole-body startle response were recorded over 200 ms after presentation of the stimulus and transduced into analog signals by a piezoelectric unit attached to the platform. The entire session comprised 6 trials with a 1–2 min inter-trial interval.

Righting reflex test

Each mouse was placed on its side on a flat surface, and the time it took to turn over to rest in the normal position with all four feet on the ground was recorded. The cut-off time was set as 30 s. This experiment was repeated three times and the average time recorded was identified as the righting reflex time.

Rotarod test

A computer-interfaced rotarod accelerating from 4–40 rotations per min over 300 s was used (ENV-575M, Med Associates). The shaft diameter was 3.2 cm. The α1R271Q mutant mice were handled with an extreme care in a quiet room. The α1R271Q mutant mice and their WT littermates were allowed to stand on a lowly rotating (4 rpm) rotarod for 30 s prior to acceleration. These mice were trained three trials per day with a 20 min interval for 3 consecutive days. Each trial ended when the mouse fell off the rotarod or after 300 s had elapsed. The time that each mouse maintained its balance on the rotating rod was measured as latency to fall.

Western blot of total and surface α1GlyR proteins

HEK-293 cells expressing WT and mutant GlyRs were incubated with *N*-hydroxysuccinimide-SS-biotin (NHS-SS-biotin; Thermo) at a concentration of 0.8 mg/ml in PBS for 1 hr at 4°C under a non-permeabilized condition. The cells were then washed thoroughly with ice-cold PBS. The cells were homogenized and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was incubated with 100 μl neutravidin-linked beads (Pierce) by end-over-end rotation for 2 hrs at 4°C. Labeled proteins eluted from the beads were resolved by electrophoresis and then transferred onto a PVDF membrane (Invitrogen). The surface and total proteins were detected with a polyclonal antibody (1:200) directed to the extracellular N-terminal domain of the α1GlyR (Catalog#: AV13003, Sigma) [19]. Horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG-HRP, Pierce) was applied at a concentration of 1:20,000. Blots were developed using SuperSignal® West Dura Extended Duration Substrate (Pierce). A Kodak DC290 camera was used to capture field images.

Statistical analysis

No statistical methods were used to predetermine sample sizes but our sample sizes are similar those described in previous publications[9, 18, 19, 50]. As indicated above, the data from behavioral tests and spinal slice recordings were collected with the investigators blind to either genotypes of the animals or specific treatments given to animals. There was no blinding to the conditions of the other experiments. For behavioral experiments, animals from different genotypes were randomly picked for testing. For electrophysiological experiments, the spinal and brain stem neurons or transfected HEK cells were randomly picked for patch clamp recordings.

Statistical analysis of concentration-response data was performed with the use of the nonlinear curve-fitting program (Prism 5.0). Data were fit using the Hill equation $I/I_{\max}=\mathrm{Bottom}+(\mathrm{Top}-\mathrm{Bottom})/(1+10\hat{}((\mathrm{LogEC}_{50}-\mathrm{Log}[\mathrm{Agonist}])*\mathrm{Hill\ Slope}))$ where *I* is the 50current amplitude activated by a given concentration of agonist ([Agonist]), *I* is the max maximum response of the cell, and EC is the concentration eliciting a half-maximal 50 response. Average values are expressed as mean ± standard error (SE). Data were statistically compared by the unpaired *t*-test, or analysis of variance (ANOVA) using GraphPad Prism 5.0, as indicated in specific figures. P value of < 0.05 was considered to be significant. Data distribution was assumed to be normal but this was not formally tested.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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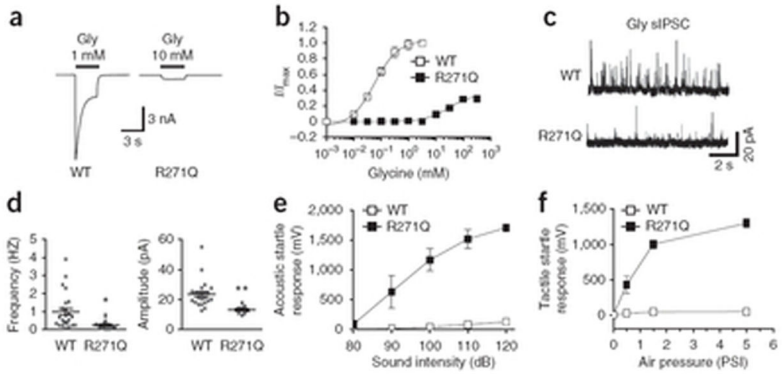

Figure 1.

The α1R271Q mutation impairs GlyR function and causes exaggerated startle behavior in mice. (**a**) Trace records of I_{Gly} in HEK-293 cells expressing WT and α1R271Q mutant receptors. (**b**) The Gly concentration-response curves for the WT (n=9) and α1R271Q (n=6) mutant receptors. The Gly EC_{50} values are 60 ± 8 μM for the WT receptors and 21,000 ± 3,215 μM for the α1R271Q mutant receptors. (p=0.0006, t(10)=9.003, unpaired t-test). (**c**) Trace records of Gly sIPSCs in spinal dorsal horn neurons isolated from the WT and α1R271Q mutant mice. (**d**) The average values and data points of Gly sIPSC frequency and amplitude. (WT, n=23 from 6 mice; R271Q, n=11 from 5 mice) (Frequency, WT vs R271Q, * p=0.022, t(32)=2.4; Amplitude, WT vs R271Q, ** p=0.0031, t(32)=3.227; unpaired t-test) (**e**) The average values of startle response induced by white noise ranging from 80 to 120 decibel (dB) in WT (n=8) and α1R271Q (n=8) mice. Maximum startle amplitude (Vm) as a function of sound intensity. (**f**) The average values of startle responses induced by air puff in WT (n=5) and α1R271Q (n=10) mice.

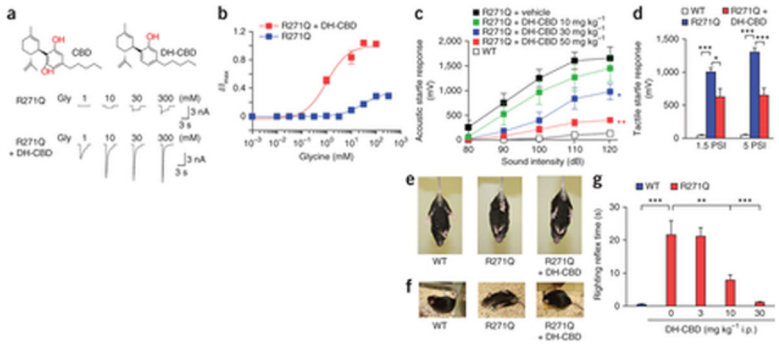

Figure 2.

DH-CBD rescues the α1R271Q mutation-induced GlyR deficiency and hyperreflexia in mice.

(**a**) Chemical structure of CBD and DH-CBD. Trace records of I_{Gly} without and with DH-CBD (10 μM) in HEK-293 cells expressing WT and the α1R271Q GlyRs. (**b**) Gly concentration-response curves with or without DH-CBD (10 μM) in HEK-293 cells expressing α1R271Q GlyRs. ($GlyEC_{50}$ values: 21 ± 3.2 mM without DH-CBD (n=6) and 1.2 ± 0.1 mM with DH-CBD (n=5), p=0.0007, t(9)=5.05, unpaired t-test). (**c**) DH-CBD restoration of startle responses induced by different levels of acoustic sound in α1R271Q mutant mice (WT n=6, R271Q n=8, R271Q+DH-CBD, n=8). * p<0.05, ** p<0.01, F(1, 13)=5.3, F(1, 13)=14.74, Two-way ANOVA compared to vehicle injected group. (**d**) DH-CBD (50 mg/kg, i.p.) restoration of startle responses induced by different levels of tactile air-puff stimuli in α1R271Q mutant mice (1.5 PSI: WT n=6, R271Q n=10, R271Q+DH-CBD n=6; 5 PSI: WT n=6, R271Q n=6, R271Q+DH-CBD n=8). * p<0.05, *** p<0.001, F(5, 31)=25.6, One-way ANOVA followed by Tukey's post hoc test. (**e**) Hind feet clenching behavior in the α1R271Q mutant mouse and the restoration of this behavior by DH-CBD (i.p. 30 mg/kg). (**f**) Photo images of DH-CBD restoration of righting reflex behavior. (**g**) Concentration dependence of DH-CBD restoration of prolonged righting reflex time in the α1R271Q mutant mice (WT n=8; R271Q+DH-CBD (0–30mg/kg), n=10). ** p<0.01, *** p<0.001, F(4, 39)= 27.1, one-way ANOVA followed by Tukey's post hoc test.

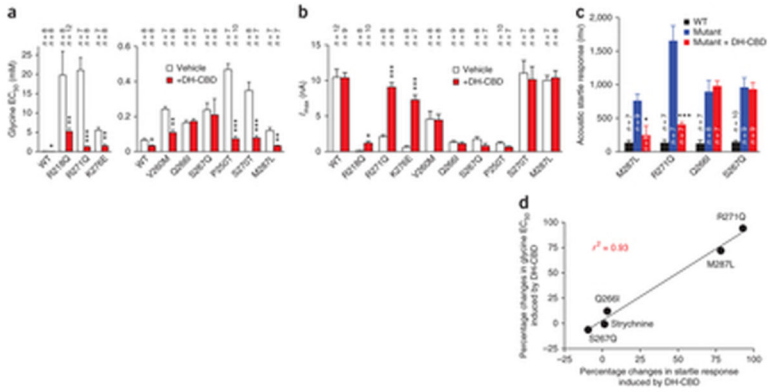

Figure 3.

Site-specific restoration of hyperekplexic GlyR dysfunction and startle response by DH-CBD.

(**a**) The average EC_{50} values of Gly concentration-response curves without and with DH-CBD (10 μM) in HEK-293 cells expressing various hyperekplexic mutant α1 subunits. * p<0.05, ** p<0.01, *** p<0.001, t(12–18)>2.2, unpaired t-test. (**b**) The average E_{max} values of Gly concentration-response curves without and with DH-CBD (10 μM) for various hyperekplexic mutant α1 subunits. * p<0.05, *** p<0.001, t(13–19)>2.2, unpaired t-test. (**c**) The average startle responses to acoustic stimuli in wild type (WT) littermates, α1M287L, α1R271Q, α1Q266I, and α1S267Q mutant mice injected with vehicle (*blue*) and injected with DH-CBD at 50 mg/kg, i.p. (*red*). * p<0.05, *** p<0.001, t(12–15)>2.2 between vehicle and DH-CBD injection in the mutant mice, unpaired t-test. (**d**) Correlation analysis of DH-induced percentage change of the Gly EC_{50} values and the DH-CBD-induced percent changes of the startle response in mice carrying corresponding mutant GlyRs (p=0.0005, linear regression).

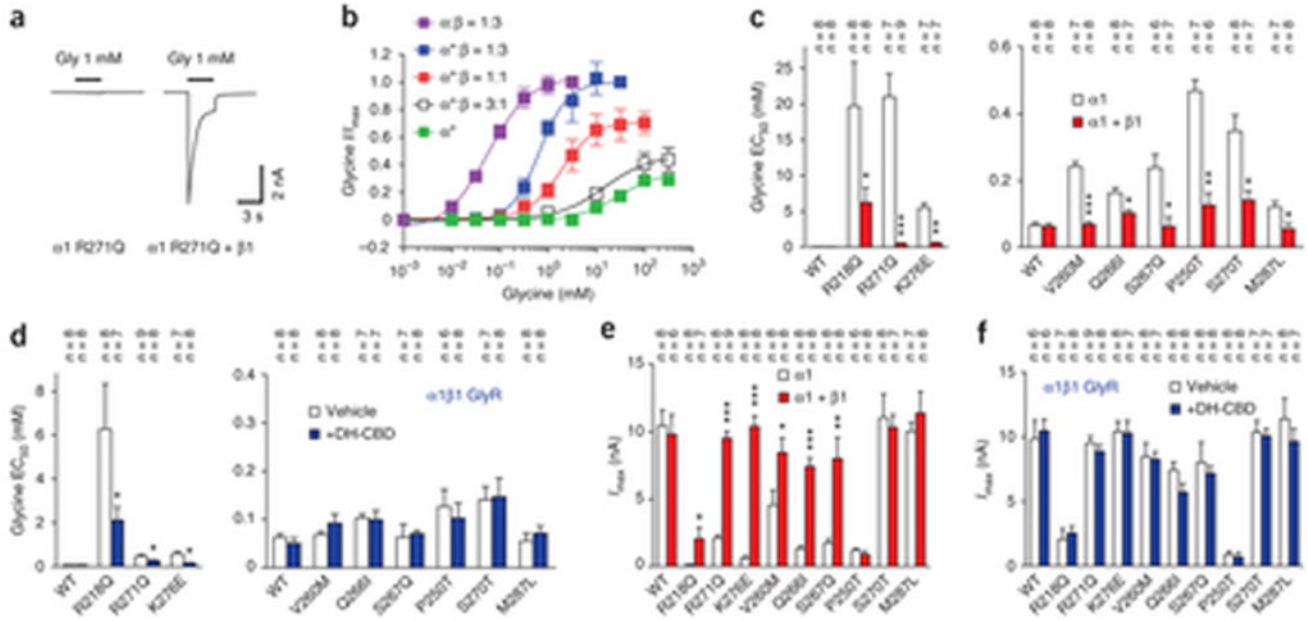

Figure 4.

Differential sensitivity of homomeric and heteromeric GlyRs to hyperekplexic mutations and DH-CBD. (**a**) Trace records of Gly (1 mM)-activated current in HEK-293 cells expressing homomeric α1R271Q or heteromeric α1R271Q/β GlyRs. (**b**) Gly concentration-response curves of the WT or α1R271Q and β1 GlyR cDNAs transfected at different ratio (2 μg/ml WT (n=6) or α1R271Q cDNA plus 6 (n=8), 2 (n=6), 0.67 (n=6) or 0 (n=5) μg/ml β1 cDNA) in HEK-293 cells. (**c**) The EC_{50} values of homomeric and heteromeric hyperekplexic mutant GlyRs (mutant α1 cDNA: β1 cDNA=1:3). (**d**) The Gly EC_{50} values of heteromeric mutant α1β (1:3) subunits without and with DH-CBD (10 μM). (**e**) The Gly I_{max} values of homomeric and heteromeric hyperekplexic mutant GlyRs expressed in HEK-293 cells. (**f**) The Gly I_{max} values of heteromeric mutant α1β subunits without and with DH-CBD (10 μM). *p< 0.05, **p< 0.01, ***p<0.001, t(11–15)>2.4, unpaired t-test (c–f).

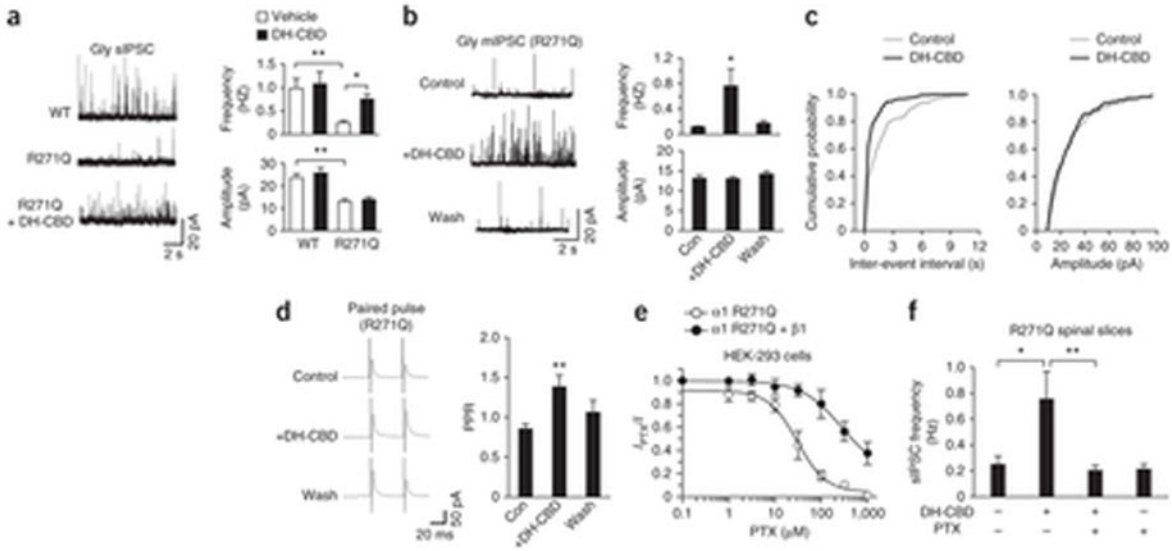

Figure 5.

DH-CBD rescue of diminished glycine release in spinal slices from the α1R271Q mutant mice. (**a**) Trace records of Gly sIPSC in spinal slices from WT and α1R271Q mutant mice before and after DH-CBD (20 μM). The bar graphs representing the average frequency and amplitude of Gly sIPSC (WT, vehicle n=24 cells of 6 mice, DH-CBD n=12 cells of 4 mice; R271Q, vehicle n=14 cells of 5 mice, DH-CBD n=17 cells of 5 mice). * $p<0.05$, ** $p<0.01$, $F(3, 60)=7.2$, one-way ANOVA followed by Tukey's post hoc test. (**b**) Trace records and average frequency and amplitude of the Gly mIPSCs before and after DH-CBD (20 μM). A significant difference in the Gly mIPSC frequency before and after DH-CBD (n=10 cells of 3 mice, * $p=0.03$, $t(18)=2.37$, unpaired t-test). (**c**) Cumulative plot analysis of the distribution of the inter-event interval and amplitude of Gly mIPSCs without and with DH-CBD. (**d**) Trace records and ratio of paired pulse responses recorded in spinal neurons before and after DH-CBD (20 μM) (n=7 cells of 3 mice, ** $p=0.0072$, $t(12)=3.23$, unpaired t-test). (**e**) PTX inhibition of I_{Gly} in HEK-293 cells expressing human α1R271Q GlyRs without (n=6) and with (n=6) β1 subunits. (**f**) The effect of PTX (30 μM) on Gly sIPSC frequency in the absence (n=12 cells of 4 mice) and presence (n=12 cells of 4 mice) of DH-CBD in the spinal slices of α1R271Q mutant mice. *$p<0.05$, **$p<0.01$, $F(3, 41)=7.5$, one-way ANOVA followed by Tukey's post hoc test.

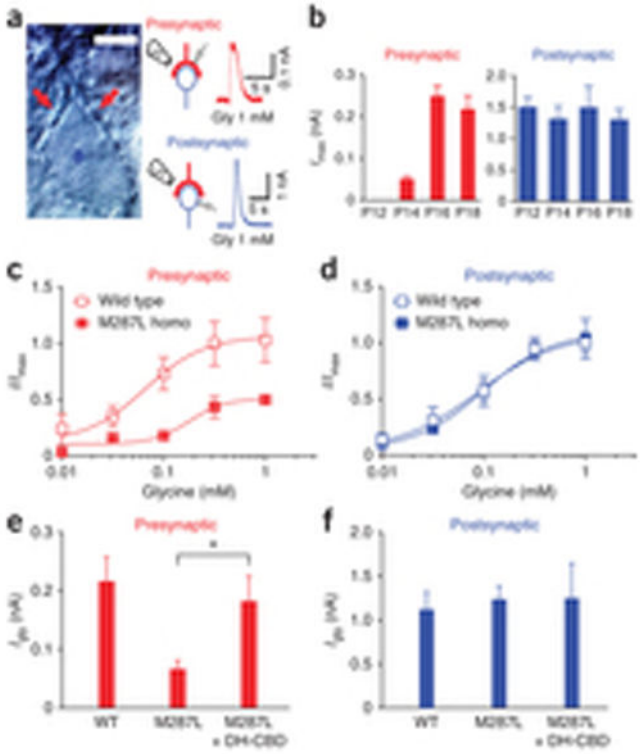

Figure 6.

Differential sensitivity of presynaptic and postsynaptic GlyRs to hyperekplexic mutation and DH-CBD rescue. (**a**) Photo imaging of a calyx associated with a postsynaptic neuron. The scale bar (*solid white*) represents 10 μM. The schemes illustrate the recording configurations of calyceal terminal and MNTB principle neuron. I_{Gly} recorded from either presynaptic terminal (*red trace*) or postsynaptic membrane of MNTB principle neuron (*blue trace*). (**b**) The average maximal amplitudes of I_{Gly} recorded from presynaptic calyceal terminals (*red*, P12, n=4 cells of 3 mice; P14, n=7 cells of 3 mice; P16, n=4 cells of 3 mice; P18, n=3 cells of 2 mice) and postsynaptic MNTB neurons (*blue*, P12, n=6 cells of 3 mice; P14, n=4 cells of 2 mice; P16, n=5 cells of 4 mice; P18, n=5 cells of 2 mice) during development from P12 to P18. (**c**) Gly concentration-response curves recorded from calyceal terminals of wild type littermates (*open squares*, n=7 cells of 3 mice) and M287L homozygous mutant mice (*solid squares*, n=6 cells of 4 mice). (**d**) Gly concentration-response curves recorded from MNTB principle neurons of wild type littermates (*open squares*, n=7 cells of 3 mice) and M287L mutant mice (*solid squares*, n=7 cells of 3 mice). (**e**) The average amplitudes of I_{Gly} (Gly, 300 μM) from calyceal presynaptic terminals of WT (n=7 cells of 3 mice) and mutant mice in absence (n=9 cells of 5 mice) and presence (n=9 cells of 5 mice) of DH-CBD (* p=0.023, t(16)=3.62, unpaired t-test). (**f**) The average amplitudes of I_{Gly} (Gly, 100 μM) from MNTB principle neurons of WT (n=10 cells of 3 mice) and M287L mutant mice in the absence (n=6 cells of 3 mice) and presence (n=8 cells of 5 mice) of DH-CBD (p=0.98, t(12)=0.031, unpaired t-test).