The Startle Disease mutation, α1S270T, predicts shortening of glycine-ergic synaptic currents

Running Title: Startle mutation α1 S270T in human glycine receptors

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Key points summary
Loss-of-function mutations in proteins found at glycinerigic synapses, most commonly in the α1 subunit of the glycine receptor (GlyR), cause the startle disease/hyperekplexia channelopathy in man. It was recently proposed that the receptors responsible are presynaptic homomeric GlyRs, rather than postsynaptic heteromeric GlyRs (which mediate glycinerigic synaptic transmission), because heteromeric GlyRs are less affected by many startle mutations than homomers.

- We examined the α1 startle mutation S270T, at the extracellular end of the M2 transmembrane helix.
- Recombinant heteromeric GlyRs were less impaired than homomers by this mutation when we measured their response to equilibrium applications of glycine.
- However, currents elicited by synaptic-like millisecond applications of glycine to outside-out patches were much shorter (7- to 10-fold) in all mutant receptors, both homomeric and heteromeric. Thus the synaptic function of heteromeric receptors is likely to be impaired by the mutation.
ABSTRACT

Human startle disease is caused by mutations in glycine receptor (GlyR) subunits or in other proteins associated with glycinergic synapses. Many startle mutations are known, but it is hard to correlate the degree of impairment at molecular level with the severity of symptoms in patients. It was recently proposed that the disease is caused by disruption in the function of presynaptic homomeric GlyRs (rather than postsynaptic heteromeric GlyRs), because homomeric GlyRs are more sensitive to loss-of-function mutations than heteromers.

Our patch-clamp recordings from heterologously-expressed GlyR characterised in detail the functional consequences of the α1S270T startle mutation, which is located at the extracellular end of the pore lining M2 transmembrane segment (18'). This mutation profoundly decreased the maximum single-channel open probability of homomeric GlyR (to 0.16; cf. 0.99 for wild-type) but reduced only marginally that of heteromeric GlyR (0.96; cf. 0.99 for wild-type). However, both heteromeric and homomeric mutant GlyR became less sensitive to the neurotransmitter glycine.

Responses evoked by brief, quasi-synaptic pulses of glycine onto outside-out patches were impaired in mutant receptors, as deactivation was approximately 10- and 7-fold faster for homomeric and heteromeric GlyRs, respectively.

Our data suggest that the α1S270T mutation is likely to affect the opening step in GlyR activation. The faster decay of synaptic currents mediated by mutant heteromeric GlyRs is expected to reduce charge transfer at the synapse, despite the high equilibrium open probability of these mutant channels.
Zhiyi Wu received his BSc in Neuroscience from University College London, where he participated in the research project that led to this paper. His interest in understanding how receptors work as molecules and in relating function and structure has led him to undertake a DPhil in the Computational Biochemistry lab of Prof Phil Biggin at Oxford University. Zhiyi is currently working on proton-mediated amino acid and protein trafficking.
INTRODUCTION

Glycine receptors mediate fast inhibitory synaptic transmission in the brainstem and spinal cord, where they are essential for motor control (Lynch, 2004). In man, inherited disruption of glycineric mechanisms produces hyperekplexia or startle disease, a neurological disorder that presents soon after birth with generalised stiffness and exaggerated startle responses to non-noxious stimuli. The generalised hypertonia fades with age, but the exaggerated startle reflexes, followed by short episodes of hypertonia, persist through life (Bakker et al., 2006; Bode & Lynch, 2014). Hyperekplexia can result from loss-of-function mutations in most of the key proteins present at glycineric synapses. These include not only the α1 and β subunits of the glycine receptor (GlyR), which are the constituents of the adult synaptic channel, but also the neuronal glycine transporter (GlyT2) and GlyR anchoring proteins, such as gephyrin and collybistin (Davies et al., 2010). Mutations in the GlyR α1 subunit are the most common cause of hyperekplexia, and their widespread location in the protein has highlighted the role of different channel domains in signal transduction (Lynch et al., 1997a; Lewis et al., 1998; Lape et al., 2012). Our work on the human startle disease mutant α1K276E (Lape et al., 2012) and the spasmodic mouse mutant α1A52S (Plested et al., 2007) showed that these mutations shorten the time single GlyR channels are active, and therefore the decay of currents produced by fast, quasi-synaptic glycine applications to outside-out patches (Wyllie et al., 1998). Comparable speeding up of the synaptic current decay has been described for glycineric currents in spinal slices from mice with the spasmodic mutation (Graham et al., 2006), and in artificial glycineric synapses in culture, where the postsynaptic GlyRs are heterologously expressed to have a defined composition (Zhang et al., 2015).

Despite this agreement between molecular and cellular results, the severity of symptoms in patients or in transgenic mice is but poorly correlated with the degree of loss-of-function at receptor level and with the reduction in charge transfer expected at glycineric synapses. The picture is made more complex by the demonstration that hyperekplexia can be caused also by some (relatively mild) gain-of-function mutations in the α1 subunit (Zhang et al., 2016).

Recently, it has been hypothesized that the form of GlyR responsible for the symptoms of startle disease is not the adult synaptic receptor, the α1β GlyR heteromer, but the homomeric α1 GlyR (Xiong et al., 2014), which is also functional in heterologous expression and occurs presynaptically in the CNS, at locations that include the calyx of Held (Turecek & Trussell, 2001). The proposed role of the homomeric receptor is based on the finding that, for a subset of startle mutations, loss of function is much less pronounced in heteromeric (postsynaptic)
GlyRs than in (presynaptic) homomers. In other words, the startle phenotype can often be substantially “rescued” by co-expression of wild-type β subunits (Xiong *et al.*, 2014). This presynaptic hypothesis of course cannot explain why startle pathology occurs with mutations of other glycineric synapse proteins that are exclusively postsynaptic, such as the β GlyR subunit and scaffolding proteins.

It is important to note that, of the many startle mutations identified, only a handful have been characterized in detail in synaptic-like conditions, such as glycineric synapses in culture (Zhang *et al.*, 2015), or with fast Gly applications (Lape *et al.*, 2012). There is a clear need of more information, especially on mutations that can be rescued by β subunit expression (a feature that has not been systematically investigated since the original report). Here we present the first detailed functional characterization of α1S270T, a hyperekplexia mutation that causes a dominant form of startle disease (Lapunzina *et al.*, 2003). We chose this mutation because it is one of those whose function has been reported to be rescued by β subunit expression (Xiong *et al.*, 2014).

In our experiments, we found that α1S270T reduced macroscopic GlyR sensitivity to glycine to a similar extent in hommeric and heteromeric GlyRs. At single channel level, maximum open probability was reduced by almost 5-fold only in homomers. In heteromers, a high open probability was reached despite the mutation, but it required very high glycine concentrations, 30-fold greater than those likely to be reached at the synapse. Loss of gating function manifested itself also in the faster decline of GlyR currents activated by millisecond, quasi-synaptic application of high glycine concentrations. This faster decay was similar in homomeric and heteromeric GlyRs.
METHODS

Cell culture and transfection  Human embryonic kidney 293A cells (HEK293A; ATCC cat# PTA-4488, RRID:CVCL0045) were used for transient expression of wild type or mutant homeric α1 or heteromeric α1β GlyRs. The S270T hyperekplexia missense mutation was introduced into the human α1 GlyR subunit using the QuickChange site-directed mutagenesis kit (Agilent Technologies, cat# 200514). The entire open reading frame of the plasmids was sequenced by Source Bioscience UK to confirm successful mutation incorporation. HEK293A cells were cultured in 25 cm² vented culture flasks containing 5 ml of Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco cat# 41966-029) supplemented with foetal bovine serum (Gibco cat# 10500-064), non-essential amino acids (Gibco cat# 11140-050) and penicillin/streptomycin (Gibco cat# 15140-122). Cells were maintained at 37 ºC, 95% air/5% CO₂ in a humidified incubator and passaged every 2-3 days in order to maintain ~70% confluence.

HEK293A cells were transiently transfected using the calcium phosphate-DNA precipitation method. The DNA mixture consisted of pcDNA3.1 plasmids with inserts coding for the enhanced Green Fluorescence Protein (eGFP; GenBank accession number U55763.1), and the human glycine receptor subunits α1 (wild-type or bearing the S270T mutation) with or without the β subunit (GenBank accession numbers P23415.2 or P48167.1 for wild-type α1 and β, respectively). Plasmid without an open reading frame (“empty”) was added to the transfection mixture to optimize the expression level (Groot-Kormelink et al., 2002). In each case we used a total of 6 µg of cDNA per dish. For wild type hommeric receptors, the final mixture of cDNA contained 2% α1, 18% eEGFP and 80% empty pcDNA3.1 plasmid. For mutant homomorphic receptors, the proportion of empty plasmid was reduced to 62% and the proportion of α1S270T subunit plasmid increased to 20%. In order to express heteromeric receptors (wild-type or mutant), the cDNA mixture contained 2% α1, 80% β and 18% eGFP plasmids. Cells were washed 5-16 hrs later and electrophysiological experiments were performed 1-2 days after transfection.

Patch-clamp recordings and analysis  Macroscopic and single-channel currents were recorded from transfected HEK293A cells in the whole-cell, outside-out and cell-attached patch-clamp configurations at 20ºC. Transfected cells were visualised using an inverted fluorescence microscope (Olympus IX71, Olympus). Patch electrodes were pulled from thick-walled borosilicate glass capillary tubes (GC150F-7.5; Harvard Apparatus) on a Flaming/Brown puller (Model-P97, Sutter Instruments). Pipettes for single-channel recordings were coated

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near the tip with Sylgard 184 (Dow Corning). Electrodes were fire-polished at a microforge to give a final resistance of 3-5 MΩ for whole-cell recordings and 5-15 MΩ for cell-attached or outside-out recordings (when filled with the appropriate solution).

Cells were bathed in an extracellular solution consisting of (in mM): Na D-glucuronate (20), NaCl (112.7), KCl (2), CaCl₂ (2), MgCl₂ (1.2), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 10), glucose (40) (all Sigma, UK) and tetrathylenonium chloride (TEACl; 10) (Alfa Aesar cat# A15215) adjusted to pH 7.4 with NaOH (osmolarity ~320 mOsm/l). To record macroscopic currents the pipette was filled with a 30 mM chloride intracellular solution, containing (in mM): K gluconate (101.1), ethylene glycol tetraacetic acid (EGTA; 11), CaCl₂ (1), MgCl₂ (1), HEPES (10), MgATP (2), sucrose (40), KCl (6) (all Sigma) and TEACl (20), adjusted to pH 7.4 with NaOH (osmolarity ~320 mOsm/l). The pipettes for cell-attached recordings were filled with extracellular solution containing the required concentration of agonist. All solutions were prepared in bi-distilled water or, for cell-attached single channel recordings, in high-performance liquid chromatography grade (HPLC) water (VWR Chemicals), and filtered through a 0.2 µm cellulose nitrate membrane filter (Whatman cat# 7182-004) before use. Glycine or sarcosine-containing solutions were prepared by diluting a 1 M glycine (Sigma cat# 50049) or 1 M sarcosine (Sigma cat# S7672) stock in extracellular solution.

Sarcosine was tested at 100 mM for glycine contamination by an HPLC assay: samples were resuspended in 10 µl 50% EtOH, reacted for 30 min with 90% EtOH:triethyamine:phenylisothiocyanate (7:2:1), evaporated to dryness at room temperature and redissolved in 100 µl 5% acetonitrile in 0.1 M ammonium acetate. A 150x4.6 mm Hypersil C18 column was used. Molecules of interest were detected at 254 nm. Sarcosine was found to be contaminated by about 10 µM glycine per 100 mM. Sarcosine (approx. 7 g) was therefore purified by re-crystallizing it three times from 95% ethanol obtaining 2.37g of purified compound, whose ¹H-NMR and elemental analysis were consistent with those expected for sarcosine. The glycine contamination of a 100 mM solution of purified sarcosine was below the detection limit of the HPLC assay (ca 1 µM). Only purified sarcosine was used in the single channel experiments.

Whole-cell recording Whole-cell currents were recorded from isolated transfected cells held at a holding potential of −60 mV (corrected for the junction potential of 11 mV) with an Axopatch 200B amplifier (Molecular Devices) and filtered at 5 kHz by the amplifier’s low pass 4-pole Bessel filter. Recordings were digitized with a Digidata 1440A (Molecular...
Devices) at a sampling frequency of 20 kHz and acquired to PC using Clampex 10.2 software (Molecular Devices; RRID:SCR_011323) for offline analysis. Access resistance was below 10 MΩ and was compensated by at least 70%.

Agonist solutions were applied to the cells for approximately 1-2 s by a U-tube application system (Krishtal & Pidoplichko, 1980), with a 10-90% exchange time <1 ms (as tested by application of 50% diluted extracellular solution to the open tip pipette). A saturating test dose of glycine was applied at the beginning of each recording and repeated until a stable peak response was observed. This saturating agonist concentration was applied also every third or fourth application to check response stability throughout the recording. Different agonist concentrations were applied in random order to obtain concentration-response curves. The peak current amplitudes for each glycine application were measured using Clampfit 10.2 software (Molecular Devices; RRID:SCR_011323). Response stability was analysed by plotting the current amplitudes of the saturating test doses against time. Only cells where the test response rundown was less than 30% during the experiment were accepted for further analysis. No correction for rundown was applied. The concentration-response data for each cell were then fitted with the Hill equation using the CVFIT programme (https://github.com/DCPROGS/CVFIT/releases/tag/v1.0.0-alpha) to estimate $EC_{50}$ (agonist concentration required to elicit 50% maximal response), $n_H$ (Hill-slope) and $I_{max}$ (maximum peak current). The responses for each cell were then normalised relative to their respective fitted $I_{max}$ and pooled for display of the concentration-response curves in the figures.

**Fast agonist application** Macroscopic currents evoked in outside-out patches by fast agonist application pulses were recorded at a pipette holding potential of $-100$ mV ($-111$ mV when corrected for a junction potential of 11 mV). The internal solution was the same as the one for whole cell recordings and contained 30 mM chloride. Internal chloride concentration has a profound effect on the kinetics of GlyRs (Pitt et al., 2008; Moroni et al., 2011). The value of 30 mM was chosen as a compromise between matching the physiological chloride concentration of a few mM and maintaining a large current signal. Glycine, dissolved in extracellular solution, was applied to outside-out patches with a theta tube (Hilgenberg GmbH cat# 1407201), cut to a final diameter of $\approx 150$ μm at the tip. The tube was driven by a piezo stepper PZ-150M (Burleigh Instruments Inc). The exchange time was measured by the application of diluted bath solution (eg 30:70 bath solution: water) before the experiment (to optimize the electrode position) and after the rupture of the patch. The rise and decay times for these open-tip currents were measured using Clampfit 10.2 as the times from 20 to 80%
of the peak response. Patches in which the open tip response had a 20–80% exchange time slower than 200 μs were rejected from further analysis.

In order to study the kinetics of macroscopic currents, 10–20 responses were recorded in response to pulses of glycine applied at intervals of at least 10 s and averaged. Only experiments in which the rundown between the first and last three responses was < 30% were included in the analysis. The time course of the macroscopic currents was characterised by fitting the risetime between 20 and 100% and the decay time from 80 to 20% of the peak response with one or more exponentials.

**Single-channel recording**  Low-noise single-channel currents were recorded in the cell-attached configuration at a pipette holding potential of +100 mV with an Axopatch 200B amplifier and filtered at 10 kHz using the amplifier’s low pass 4-pole Bessel filter. The data was digitized with a Digidata 1322A (Molecular Devices) at a sampling frequency of 100 kHz and acquired to PC using Clampex 10.2 for offline analysis.

**Single channel current amplitude and cluster P_{open} measurements**  In order to compare single-channel current amplitudes and cluster open probability, single-channel recordings were filtered offline using the Clampfit 10.2 low-pass Gaussian filter with a final cut-off of 5 kHz and resampled at 50 kHz. At high agonist concentration, channel openings occurred in clusters delimited by long closed intervals, likely to be desensitised. These clusters are likely to originate from the activity of a single ion channel molecule and were used for P_{open} measurements. Clusters longer than 100 ms with more than 10 openings were selected for analysis. The gap between clusters was at least 300 ms for the homomeric α1S270T GlyR and at least 100 ms for the heteromeric α1S270Tβ GlyR and both wild-type receptors. Channel activity in the selected clusters was idealised using the half-amplitude threshold method implemented in Clampfit 10.2 and open probability was calculated as the ratio of cluster open time over total cluster length. The amplitude of single channel currents was measured in Clampfit 10.2 as the average of all detected opening amplitudes inside a cluster.

**STATISTICS**

Results are reported as the mean ± standard deviation (SD), where n represents number of cells, clusters or patches as indicated. Non-parametric randomisation test (two-tail, non-paired; 50000 iterations) was used to determine P values for the difference being greater than
or equal to the observed difference (DC-Stats software: https://github.com/DCPROGS/DCSTATS/releases/tag/v.0.3.1-alpha). Where appropriate, the 95% confidence intervals for the differences between means are given. For the fit of the Hill equation to \( P_{\text{open}} \) cluster data, a single set of data was fitted, thus we report result as fit estimate ± approximate standard deviation estimated from the covariance matrix and two-unit likelihood intervals for each of the fitted parameters (CVFIT software: https://github.com/DCPROGS/CVFIT/releases/tag/v1.0.0-alpha).

RESULTS

Whole-cell concentration-response curves
Figure 1A shows the whole-cell current responses obtained when glycine (0.05-50 mM) was applied by U-tube to HEK293 cells expressing wild-type or mutant \( \alpha1S270T \) GlyRs. The traces show that these inward currents activated and desensitised quickly during the application, especially at the highest concentrations. The graphs in Figure 1B are the concentration-response curves plotted from the peak amplitude of the agonist responses, normalised to the fitted maximum of the glycine curve for each receptor form. The glycine \( EC_{50} \) values estimated from fitting the Hill equation to the data points were very similar for wild-type homomeric and heteromeric receptors: 0.24±0.06 mM \( (n=10 \text{ cells, see Table 1}) \) and 0.23±0.03 mM \( (n=5 \text{ cells}) \), respectively. The \( \alpha1S270T \) mutation increased the glycine \( EC_{50} \) in both homomeric and heteromeric GlyRs, by 4.5- and 3.7-fold \( \text{to } 1.1±0.3 \) and 0.9±0.1 mM, \( n=8 \text{ cells; } P=0.00002 \) and 0.0006, respectively). Thus, the glycine sensitivity of the mutant heteromeric receptor was not rescued to wild-type values by the co-expression of the \( \beta \) subunit (Table 1).

Figure 1 here
Table 1 here

Cell-attached single-channel recordings at saturating agonist concentration
Impairment in gating is the most common phenotype associated with startle disease mutations. Direct measurement of the maximum single-channel open probability, \( P_{\text{open}} \), elicited by high agonist concentrations is a sensitive and reliable test of whether a mutation has affected channel gating (Colquhoun, 1998). The traces in the left-hand panels of Figure 2 are typical examples of cell-attached single-channel activity at saturating glycine concentrations. At 10 mM glycine, openings of wild type \( \alpha1 \) or \( \alpha1\beta \) GlyRs (Figure 2A and
2C, respectively) occurred in clusters, flanked by long periods of inactivity likely due to desensitisation. The traces show also that the amplitude of homomeric openings was higher than that of heteromeric GlyR (5.6 ± 0.5 vs. 2.5 ± 0.2 pA, respectively; \( P << 10^{-6} \), two-tailed randomisation test), as expected, given the higher conductance of homomeric receptors (Bormann et al., 1993).

The average maximum cluster \( P_{\text{open}} \) was high, with very little variability, for both homomeric and heteromeric receptors, as shown by the histograms in Fig 2B and D (0.99 ± 0.02 and 0.993 ± 0.006, \( n = 45 \) clusters from 4 patches and 87 clusters from 4 patches respectively; Table 2).

**Figure 2 here**  
**Table 2 here**

For mutant GlyRs, we chose 100 mM as a saturating glycine concentration, on the basis of the whole cell concentration-response curves in Figure 1B. The traces in Figure 2E show that openings of homomeric α1S270T GlyR also occurred in clusters, but the appearance of the clusters was clearly different. In the homomeric mutant, the maximum \( P_{\text{open}} \) was much lower (0.16 ± 0.1, \( n = 128 \) clusters from 10 patches; \( P << 10^{-6} \), two-tailed randomisation test) than in wild type receptors. Furthermore, the cluster appearance was much less consistent, with a wide spread of \( P_{\text{open}} \) values (range 0.005 – 0.826) as shown in the histogram in Figure 2F. This is clearly visible in the two clusters shown in the top trace of Figure 2E, where the cluster on the left has a \( P_{\text{open}} \) of 0.54 and the one on the right 0.06 (200 ms of each cluster marked by a bar are shown also at faster time scale in Fig. 2E, middle and bottom traces).

We next examined the effect of the mutation on the heteromeric channel, which is the form of GlyR that is relevant to adult synaptic transmission. Typical clusters of single channel openings of mutant heteromeric GlyR at 100 mM glycine are shown in Figure 2G (top trace). The open probability of these clusters was much higher than for the mutant homomer (0.96 ± 0.03; \( n = 131 \) clusters from 4 patches; \( P << 10^{-6} \), two-tailed randomisation test), and very close to the wild-type value (see also Fig 2H), although with much higher cluster-to-cluster variability (compare 2D and 2H). Thus, the β subunit does rescue the receptor maximum \( P_{\text{open}} \) to a value similar to that of wild type, but this value was reached at a very high glycine concentration (Fig. 1B).
The partial agonist sarcosine

Because of the heterogeneity in the single channel activity of the mutant, we felt it useful to confirm that gating is impaired in the whole population of α1S270T mutant GlyRs. It is not possible to detect this effect simply by comparing the absolute size of maximum currents in whole-cell experiments, because we do not know how many channels are on the cell surface. The robust way to prove by a macroscopic experiment that gating is impaired in the whole channel population is to show that the maximum response to a partial agonist, relative to that of the full agonist glycine in the same cell, is reduced by the mutation. We therefore obtained whole-cell concentration response curves to sarcosine (N-methyl-glycine) (Zhang et al., 2009), which is a partial agonist in homomeric wild-type GlyRs (Safar et al., 2017). Figure 3A and B show that the S270T mutation reduced both the potency of sarcosine (from 14 ± 6 mM to 23 ± 3 mM; \( n = 6 \) cells; \( P = 0.0076 \), two-tailed randomisation test) and its maximum response relative to glycine (from 60 ± 20% to 8 ± 4%; \( P = 0.00014 \), two-tailed randomisation test). This marked reduction in sarcosine maximum (7.5-fold) confirms that the α1S270T mutation impairs channel gating.

In wild type homomeric α1 GlyRs, a near-saturating concentration of sarcosine (100 mM) elicits clusters of openings, with a mean \( P_{\text{open}} \) of 0.58 ± 0.2 (Safar et al., 2017).

Figure 3 around here

Figure 3C shows activity evoked by 100 mM sarcosine in α1S270T homomeric GlyRs. Mutant homomeric channels displayed only sparse openings in response to 100 mM sarcosine and no clusters could be detected (\( n = 3 \) patches). Channel openings were generally very short in duration and mostly failed to reach full amplitude. Measurement of the few fully-resolved longer openings present gave an amplitude of 3.9 ± 0.5 pA (\( n = 11 \) openings from 3 patches). Since no clusters could be identified in these recordings, we measured the \( nP_{\text{open}} \) of the entire record at 100 mM sarcosine and estimated its value to be approximately 0.01 (Table 2).

Concentration-dependence of homomeric α1S270T GlyR single-channel activity

In order to obtain a robust confirmation of the effects of the mutant on agonist potency vs. efficacy, we obtained single-channel recordings of homomeric mutant GlyRs in the presence of 0.3, 1 and 10 mM glycine (Figure 4A-C). At low glycine concentrations (up to 0.3 mM), individual channel activations were sparse and occurred as single openings, or bursts of few openings as shown in Figure 4A (top and bottom traces at slower and faster time scales, respectively). The longer shut times in these records were concentration-dependent, and
therefore likely to reflect primarily long sojourn(s) in un-liganded states (rather than desensitization). At this low concentration we could not measure single channel open probability because we could not identify stretches of openings and shuttings that reflect the activity of one channel molecule.

At glycine concentrations equal to or higher than 1 mM, the mutant receptors enter into long-lived desensitised states (Sakmann et al., 1980; Beato et al., 2004), which manifest as concentration-independent shut times between clusters of channel activations (in wild-type receptors clustering was observed from 50 μM, (Beato et al., 2004). Each cluster reflects the activity of a single GlyR molecule that emerges for a time from desensitization and this allows us to measure single channel $P_{\text{open}}$ in the cluster. We observed substantial variability in the $P_{\text{open}}$ at every concentration, as expected from the variability seen at saturating glycine concentrations (Figure 2E and F). Nevertheless, the effect of increasing glycine concentration was clear. Figure 4B depicts the first 4 seconds of a long cluster elicited by the lowest cluster concentration (1 mM glycine). This cluster was preceded by a long shut interval of 31 seconds (only ~1 second of which are shown here). At higher agonist concentration (10 mM glycine), the clusters are shorter and their variability more obvious, as shown by Figure 4C.

The three clusters in 4C are separated by shuttings longer than 100 ms and have $P_{\text{open}}$ of 0.28, 0.02 and 0.06. Two stretches from the highest and lowest $P_{\text{open}}$ clusters are depicted at a slower time scale (Figure 4C, middle and bottom traces). Higher $P_{\text{open}}$ clusters were different, because they had shorter intracluster shut intervals and longer open times. This was reflected in a strong positive correlation between cluster $P_{\text{open}}$ and cluster mean open time (e.g. $r = 0.68$ for clusters in 10 mM glycine). In addition to that, many long (1 to 10 ms) sojourns in a lower conductance level were detectable.

The histograms in Figure 4D show the wide scatter of for the cluster $P_{\text{open}}$ values at all glycine concentrations (1 to 10 mM). Plotting the mean cluster $P_{\text{open}}$ values gives a concentration-response curve with a shallow concentration dependence (Figure 4E; cf. the reference $P_{\text{open}}$ curve for the wild type (Beato et al., 2004).

**Figure 4 here**

The fit with a Hill equation (dashed line) gave a maximum $P_{\text{open}}$ of $0.16 \pm 0.01$ (2-unit likelihood interval 0.14 - 0.22), $EC_{50}$ of $0.9 \pm 0.4$ mM (2-unit likelihood interval n/a – 1.7 mM) and Hill slope of $1.5 \pm 0.8$ (2-unit likelihood interval 0.2 – n/a; Table 3). Note that $EC_{50}$ and Hill slope were poorly defined, probably because we could not identify clusters at
concentrations lower than 1 mM. With these limitations, the increase in glycine $EC_{50}$ produced by the mutation was similar in the single channel and in the whole cell-experiments (approximately 8.5-fold and 4.5-fold, respectively).

Table 3 here

Concentration dependence of heteromeric α1S270Tβ GlyR single-channel activity

We repeated the same single channel experiments for the heteromeric α1S270Tβ GlyR, obtaining cell-attached recordings in the presence of 10 µM to 100 mM glycine (Figure 5).

Figure 5 here

At the lowest glycine concentrations of 10 µM (data not shown) and 30 µM (Fig 5A), the α1S270Tβ GlyR activations appeared as single openings or bursts of openings (see the second, expanded trace) and clusters could not be identified (note that, in wild-type heteromeric GlyR, clear clustering was observed at 30 µM (Burzomato et al., 2004). The expanded stretch of record shows also that the majority of openings were very short and rarely reached full amplitude.

At higher glycine concentrations (≥ 100 µM) channel activity appeared as clusters of openings separated by long shut intervals lasting hundreds of milliseconds or seconds (Figure 5B-C). Cluster-to-cluster variability in open probability was seen at most concentrations, but was more prominent at the lower ones (see Figure 5E). The top trace in Figure 5B (top trace) shows two clusters recorded at 100 µM glycine. Stretches for each of these clusters are shown in Figure 5B (middle and bottom traces) at a faster time scale. The first cluster was formed by shorter openings and longer shuttings than those in the second cluster. The $P_{\text{open}}$ of the first and the second cluster was 0.195 and 0.493, respectively, and the average $P_{\text{open}}$ at 100 µM glycine was 0.18 ± 0.09 for 81 clusters from 5 patches.

Even at 10 mM glycine, a concentration near the maximum of the macroscopic dose response curve, clusters with different behaviour were obvious (see Figure 5C). These clusters belonged to two distinct types. The first two clusters in the trace in 5C (top) had low $P_{\text{open}}$ (0.591 and 0.684), whereas the last two had a $P_{\text{open}}$ of 0.996 and 0.989. It was not clear whether the different clusters came from different population of channels or represented different gating modes of the same molecule. The latter seems more likely, as both patterns were observed in same patch and in a few cases both patterns appeared in the same cluster (as shown by the traces at 100 mM in Figure 5D).
Figure 5E shows the distribution of $P_{\text{open}}$ values for all heteromeric $\alpha_{1270}$ GlyR clusters. At lower concentrations, the spread of $P_{\text{open}}$ values was particularly high, ranging from 0.125 to 0.993 at 3 mM glycine. As the agonist concentration increased, the proportion of low $P_{\text{open}}$ clusters decreased, disappearing at 100 mM. At 10 mM glycine, the majority of clusters (62 out of 73) had $P_{\text{open}}$ higher than 0.8 (average 0.95 ± 0.05), and only 11 clusters had $P_{\text{open}}$ lower than 0.8 (average 0.58 ± 0.1). The overall pattern is clearly different from that observed in homomorphic GlyRs. Despite the large number of clusters analysed (between 21 and 131 for each concentration), there were no clearly defined peaks, and it was not possible to fit the distribution to identify Gaussian components.

Using all the clusters collected gave rise to the concentration- $P_{\text{open}}$ curve shown in Figure 5F, where the data points for the mutant (filled circles) fitted by a Hill equation (dashed line) are shown together with a reference concentration-response curve for the wild-type heteromeric receptor (solid line). The mutant curve reached a fitted maximum $P_{\text{open}}$ similar to that of wild-type GlyRs (0.91 ± 0.02; 2-unit likelihood interval 0.85 - 0.94), but had a shallow Hill slope (1.1 ± 0.1; 2-unit likelihood interval 0.97 – 1.9) and a higher EC50 (320 ± 30 µM; 2-unit likelihood interval 230 – 360 µM).

**Glycine concentration jumps**

During synaptic transmission, postsynaptic receptors are exposed to high concentrations of glycine (2-3.5 mM) for less than 1 ms (Beato, 2008). In order to simulate such conditions, we recorded GlyR macroscopic currents elicited by the fast application of short glycine pulses by a theta tube to outside-out patches. The aim of these recordings was to assess how the $\alpha_{1270}$ mutation changes the time course of current deactivation in quasi-synaptic conditions.

*Figure 6 here*

*Table 4 here*

The traces in the top panels of Figure 6 are examples of GlyR macroscopic currents recorded from outside-out patches exposed to 2 ms pulses of 3 mM glycine. Both homomeric and heteromeric wild-type GlyRs (grey traces in Fig. 2A and B, respectively) activated quickly, with time constants (fitted from 20% to peak) of 0.13 ± 0.04 ($n = 6$) and 0.2 ± 0.1 ms ($n = 5$; $P = 0.057$, two-tailed randomisation test; Table 2, data plotted in Figure 6C). Given that the 20-80% solution exchange time was on average 0.12 ± 0.04 ms ($n = 25$ patches), these risetimes are the expression of the solution exchange, rather than the receptor response. The
deactivation phase after the end of the pulse for homomeric and heteromeric GlyRs had time constants of $12 \pm 5$ and $10 \pm 6$ ms, respectively (fitted from 80 to 20% of peak with a single exponential; $n=7$ and 5 patches; $P = 0.51$, two-tailed randomisation test; Fig. 6D). Figure 6 shows that introducing the $\alpha1S270T$ mutation speeded up the deactivation of both homomeric and heteromeric GlyR in response to 3 mM glycine (black traces in Figure 6A and 6B, plot in Fig 6D) to $1.2 \pm 0.3$ and $1.5 \pm 0.6$ ms, respectively ($n=6$ and 7; $P = 0.0018$ and $P \ll 10^{-6}$, two-tailed randomisation test, compared to wild type homomeric and heteromeric GlyR). The time course of the onset phase was not affected by the mutation. These results indicate that synaptic currents mediated by homomeric or heteromeric GlyR bearing the $\alpha1S270T$ mutation would be much shorter.

DISCUSSION

We studied the effects on human recombinant homomeric and heteromeric GlyRs of the naturally occurring mutation $\alpha1S270T$, which is known to cause dominantly inherited startle disease/hyperekplexia in man (Lapunzina et al., 2003). Its functional effects have been reported to be much less severe in heteromeric GlyRs (Xiong et al., 2014). We show here that the function of mutant GlyR was rescued by $\beta$ subunit co-expression only in equilibrium measurements of glycine efficacy. Experiments more relevant to the conditions during synaptic transmission, such as outside-out current responses to fast glycine concentration jumps, show that the function of GlyR was equally impaired for heteromeric and homomeric receptors, and mutants deactivated much more quickly than wild-type channels.

Can structural data shed light on how the $\alpha1S270T$ mutation impairs GlyR function?

Like other pentameric ligand-gated channels, GlyRs are formed by five subunits arranged around a central pore lined by the M2 $\alpha$-helices, surrounded by the other transmembrane helices (M1, M3 and M4). The extracellular end of M2 (where S270 is) and the M2-M3 loop are important in channel gating and many dominant hyperekplexia mutations are found in these areas (Bode & Lynch, 2014; Lynch et al., 1997b; Lewis et al., 1998). $S270/18'$ (green sphere in Figure 7C) is in the penultimate turn of M2, is conserved in GlyR $\alpha$ subunits (but not in $\beta$; Figure 7A) and not likely to be exposed to the pore (by analogy with GABA channels; Xu & Akabas, 1993). The adjacent R271 residue, the first startle mutation to be discovered (R to L/Q)(Shiang et al., 1993), moves during activation to stabilize the open state by interacting with Q226 (another conserved startle residue) in the M1 of the adjacent...
clockwise subunit (Du et al., 2015; Pless et al., 2007; Bode & Lynch, 2013). The same cryoEM structures suggest that the interactions of S270 are different, and that its side chain (green sticks in Figure 7C) points intrasubunit, towards M3 when the channel is resting or desensitised (yellow and light grey in the figure), and towards M1 (M227, blue sticks) of the same subunit when the channel is open (light blue). The side chain of M227 is resolved only in the desensitised structure (bottom of Fig. 7C), but modelling it in the open structure (top of Fig. 7C) suggests that its sulphur atom is close enough to the hydroxyl of S270 to form a hydrogen bond (2.2 Å, dashed line in Fig 7C top). If this hydrogen bond adds to the stability of the open state, anything that disrupts it would impair channel gating. The S270T startle mutation is a conservative change, but the additional methyl on the β carbon may affect the rotation kinetics of the side chain or impact the interactions of the adjacent R271 residue. These explanations are plausible, but do not suggest a straightforward mutational test of their validity. We must consider them with great caution, especially since there is still uncertainty as to the real conformation of the GlyR open channel (see the molecular dynamics proposals of different open states, Cerdan et al., 2018; Dämgen & Biggin, 2020).

The effects of the S270T mutation in homomeric and heteromeric GlyR

We chose to study S270T because it has not been fully characterized before and because this mutation is of special interest for the homomeric/heteromeric pathology of startle disease, given the report that its effects are rescued by co-expression of the GlyR β subunit (Xiong et al., 2014).

There are few data in the literature on the functional rescue of α subunit startle disease mutations by co-expression of the β subunit and they are inconsistent. Rea et al. (2002) found no β rescue for mutation R392H expressed in oocytes, but the effects of this mutation were attributed to failure of the receptor to reach the membrane. A very small degree of β rescue was reported for R392H expressed in heteromeric GlyR in HEK293 cells (Chung et al., 2010).

Our own work in GlyR expressed in HEK293 showed evidence of a partial functional rescue in heteromeric GlyR for both the mouse spasmodic mutation (α1A52S) and the human startle α1K276E mutation. The loss of function caused by these mutations in homomers was so extreme that they could not be characterized at single channel level, whereas some functional channels were recorded for mutant heteromeric channels (Plested et al., 2007; Lape et al., 2012). Interestingly, data from our first characterization of the K276E mutation in oocytes are
in conflict with this rescue, showing an identical loss of glycine sensitivity for homomeric and heteromeric channels (Lewis et al., 1998).

Our present work on S270T shows that there was no β rescue at whole cell level and the mutation produced essentially the same shift in the dose-response curves of homomeric and heteromeric GlyRs. We took extreme caution in repeating experiments so that all four receptors (wild type and mutant, homomer and heteromer) could be examined in the same batches of transfections. We also made sure that a fast exchange for the agonist applications was consistently obtained in whole cell U-tube applications (0.5-1 ms). This is important, because the decay of whole cell current responses was quite fast in the mutants and in wild type heteromers (Fig. 1). This decay is likely to be due to desensitization and will distort the measurement of peak currents, making it prone to substantial artefactual variation if the speed of the application system is not consistently optimized. Given that macroscopic dose-response curves are distorted by desensitization, it was particularly important to measure agonist sensitivity also at single channel level, where we can measure open probability in clusters of openings, and the long shut times caused by desensitization are excised. In the single channel experiments on homomeric GlyRs we found a decrease in the sensitivity to glycine of about 9-fold. This change was accompanied by a dramatic loss of channel gating, which took the maximum $P_{\text{open}}$ elicited by glycine from almost 1 in wild-type to 0.2 in the mutant (Table 2). This profound loss of gating transforms glycine into a partial agonist, a result that agrees with the marked reduction in the activity produced by the partial agonist sarcosine. In mutant homomer GlyRs, sarcosine failed to produce clusters of openings, eliciting a maximum $nP_{\text{open}}$ value of 0.01 (Table 2). This single-channel value is in good agreement with the whole-cell results, where the sarcosine maximum response was 8% of that to glycine. Given that the glycine maximum $P_{\text{open}}$ in the homomeric mutant was 0.16, we would expect a sarcosine $P_{\text{open}}$ of 0.012, in broad agreement with the experimental $nP_{\text{open}}$ estimate.

In the heteromer we saw a rescue of channel function, as the maximum equilibrium $P_{\text{open}}$ was restored to 0.96, a value similar to that observed in the wild type. However, this maximum open probability was reached at much higher glycine concentrations, as glycine sensitivity was decreased by about 5-fold in the mutant.
Impact at the synapse of the loss of function produced by the S270T mutation

We next addressed the question of how these changes affected glycine responses produced by applications that simulate the fast, short exposure experienced by receptors at the synapse. The best estimate available (for mammalian motoneurones in spinal cord slices) indicates that the glycine transient reaches a concentration of 2.2-3.5 mM, for a fraction of a millisecond, and decays with a time constant of 0.6-0.9 ms at room temperature (Beato, 2008). We have previously shown that fast applications of mM glycine to wild-type receptors in outside-out patches evoke currents with a decay kinetics similar to that of glycinergic synaptic currents in spinal cord slices, provided the correct intracellular chloride concentration is used in the recordings (Pitt et al., 2008). Our data here show that currents elicited with 2 ms pulses of 3 mM glycine decayed much more quickly for mutant GlyRs. The time constant of decay was speeded up by approximately 6-fold for both homomers and heteromers, from the wild type values of 12 and 10 ms to 1.2 and 1.5 ms, respectively.

The time constant of macroscopic current decay after the agonist concentration is “jumped” to zero is known to contain the same time constants as the burst distributions in single channel records at steady state (Wyllie et al., 1998). Despite the difficulty in analysing the mutant single channel records, we verified the effects of the mutation on the burst time distributions on homomeric GlyRs, which were the mutant channels that were least heterogeneous and lent themselves better to single channel analysis (Appendix Fig. 1). Here we found a shorter burst length in agreement with that observed in macroscopic concentration jumps onto outside-out patches, further confirmation that synapses where mutant receptors are expressed would suffer a substantial decrease in charge transfer.

Some questions remain. First of all, the mutation produces a dominant form of startle disease and the patients’ GlyRs will contain a mixture of mutant and wild type α subunits. Thus it is to be expected that the effects of the mutation may be attenuated in the channels in vivo, to an extent that we cannot predict from our experiments. However, charge transfer will be affected also by the amplitude of the synaptic current. A decrease in peak amplitude is plausible, given the decreased sensitivity to glycine in the heteromer. Neither the whole-cell nor the jump experiments contain the information we need, i.e. the peak open probability reached in response to the millisecond rise in glycine concentration at the synapse, because we do not know the number of channels in each recording. A robust calculation of this non-equilibrium peak open probability requires an adequate kinetic model of the mutant channel. We could not achieve this even for homomeric mutant channels (data not shown), probably
because we could analyse only high glycine concentrations (where we could check that we had the dominant mode of activity in the clusters) and because the presence of more than one open state at high glycine concentrations made it necessary to fit a complex activation mechanism.

Finally, given that we could not carry out global mechanism fits to the mutant single channel data, can we nevertheless identify which of the steps in receptor activation is likely to be most affected by the mutation? We systematically explored how changes in the values of each rate constant in the wild type GlyR activation mechanisms affected glycine EC50, equilibrium maximum $P_{open}$ and deactivation time constants (see Appendix). Impairing the first gating step (the transition to the pre-open ‘flip’ intermediate state, Burzomato et al., 2004) failed to account for the changes in deactivation we observed and could be excluded. On the other hand, impairing channel opening by decreasing the opening rate constant predicted effects that approximated well those observed for mutant homomeric GlyRs. However, the simple change we modelled (only one rate constant, similar changes at all levels of ligation) accounted only in part for the mutation effects seen in heteromeric GlyRs. It is likely that combining changes in opening and closing rate constants, or changing only the fully liganded rate constants would improve predictions, but this implies exploring a large parameter space with limited constraints by the data.

Our experiments show that the $\alpha1S270$ mutation causes substantial loss of function in both homomeric and heteromeric GlyRs. This conclusion is strengthened by the good agreement between data obtained with different patch-clamp recording modes and with different types of agonist applications, especially those that mimic synaptic conditions.
ADDITIONAL INFORMATION:

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Authors’ Contributions
LGS, RL and TG conceived and designed the experiments; ZW, RL, L J-S, BJ O’C and TG performed and analysed experiments; ZW, RL and LGS wrote the manuscript. All Authors approved the final version of the manuscript and are accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing Interests
The Authors declare that they have no conflict of interests.

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Data Availability Statement
The data that rt the findings of this study are available from the corresponding author upon reasonable request.
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### Table 1. Effects of the α1S270T startle disease mutation on the whole-cell concentration-response curves of recombinant homomeric and heteromeric GlyR.

| Agonist, receptor | $I_{max}$, nA | $EC_{50}$, mM | nH | $I_{agonist}/I_{Gly}$ | n |
|-------------------|--------------|---------------|----|------------------------|---|
| Glycine, WT α1    | 7 ± 5        | 0.24 ± 0.06   | 1.6 ± 0.2 | 1                      | 10 |
| Glycine, WT α1β   | 5 ± 5        | 0.23 ± 0.03   | 1.7 ± 0.2 | 1                      | 5  |
| Glycine, α1S270T  | 13 ± 2       | 1.1 ± 0.3     | 1.1 ± 0.2 | 1                      | 8  |
| Glycine, α1S270Tβ | 7 ± 6        | 0.9 ± 0.1     | 1.29 ± 0.08 | 1                      | 8  |
| Sarcosine, WT α1  | 5 ± 2        | 14 ± 6        | 1.5 ± 0.1 | 0.6 ± 0.2              | 7  |
| Sarcosine, α1S270T| 0.8 ± 0.4    | 23 ± 3        | 1.4 ± 0.3 | 0.08 ± 0.04            | 6  |

Hill equation fits to agonist concentration-response data from whole-cell recordings; holding potential −60 mV, internal chloride 30 mM.
Table 2. Effects of the α1S270T startle disease mutation on the properties of single-channel clusters evoked by saturating concentrations of agonist in cell-attached recordings.

| Agonist          | $P_{\text{open}}$ (range) | Amplitude, pA | Duration, s | $n$ (clusters/patches) |
|------------------|---------------------------|---------------|-------------|------------------------|
| Glycine 10 mM,   | 0.99 ± 0.02 (0.90-0.999) | 5.6 ± 0.5     | 0.6 ± 0.5   | 45 / 4                 |
| WT α1           |                           |               |             |                        |
| Glycine 10 mM,   | 0.993 ± 0.006 (0.96-1.0) | 2.5 ± 0.2     | 2 ± 2       | 87 / 5                 |
| WT α1β          |                           |               |             |                        |
| Glycine 100 mM,  | 0.16 ± 0.1 (0.005-0.83)  | 4.4 ± 0.8     | 1 ± 1       | 128 / 10               |
| α1S270T         |                           |               |             |                        |
| Glycine 100 mM,  | 0.96 ± 0.03 (0.85-1.0)   | 2.9 ± 0.6     | 0.6 ± 0.6   | 131 / 4                |
| α1S270Tβ        |                           |               |             |                        |
| Sarcosine 100 mM,| 0.58 ± 0.2 (0.29-0.94)   | 4.3 ± 0.5     | 0.6 ± 0.4   | 21 / 1                 |
| WT α1           |                           |               |             |                        |
| Sarcosine 100 mM,| < 0.01*                   | 3.9 ± 0.5     | -           | 0 / 3                  |
| α1S270T         |                           |               |             |                        |

* No clusters were observed in α1S270T GlyR single channel records in the presence of 100 mM sarcosine, thus, $nP_{\text{open}}$ was measured and is presented in the table.
Table 3. Effects of the α1S270T startle disease mutation on the concentration dependence of single channel $P_{\text{open}}$ of glycine receptors.

|       | maximal $P_{\text{open}}$ | $EC_{50}$ µM | $nH$ | $n$ (clusters at each concentration) |
|-------|---------------------------|--------------|------|--------------------------------------|
| α1 GlyR WT | 0.98                      | 106          | 1.8  | -                                    |
| α1β GlyR WT | 0.97                      | 60           | 3.4  | -                                    |
| α1S270T GlyR | 0.16 ± 0.01              | 900 ± 400    | 1.5 ± 0.8 | range: 33 – 128                    |
| α1S270Tβ GlyR | 0.91 ± 0.02              | 320 ± 30     | 1.1 ± 0.1 | range: 41 – 131                     |

Data for wild type receptors are for rat α1 and α1β GlyR, from Beato et al., 2004 and from Burzomato et al., 2004, respectively. Data for mutant receptors are shown as mean ± approximate standard deviation calculated from the covariance matrix.
Table 4. Effects of the α1S270T startle disease mutation on the activation and deactivation time course of glycine currents

|          | Peak current, pA | Activation τ, ms | Deactivation τ, ms | n  |
|----------|------------------|------------------|--------------------|----|
| WT α1    | 5000 ± 4000      | 0.13 ± 0.04      | 12 ± 5             | 6  |
| WT α1β   | 110 ± 80         | 0.2 ± 0.1        | 10 ± 6             | 5  |
| α1S270T  | 60 ± 30          | 0.4 ± 0.1        | 1.2 ± 0.3          | 6  |
| α1S270Tβ | 200 ± 200        | 0.18 ± 0.03      | 1.5 ± 0.6          | 7  |

Experimental values measured from currents elicited by the fast application of 2 ms pulses of 3 mM glycine to outside-out patches. V holding −111 mV; 30 mM internal chloride; open tip exchange time better than 200 µs.
Figure 1. The $\alpha 1$ S270T startle disease mutation reduces the glycine sensitivity of both homomeric and heteromeric GlyR. A, Representative whole-cell current responses evoked by U-tube glycine application to HEK293 cells expressing wild-type or $\alpha 1$S270T mutant homomeric or heteromeric glycine receptors. Bars above the traces show the timing of glycine applications. Currents were recorded with pipettes filled with low-$\text{Cl}^-$ solution (30 mM).

B, The glycine concentration-response relation for wild-type (circles) or $\alpha 1$S270T mutant (triangles) glycine receptors ($n = 5$ to 10 cells). Lines are fits of the Hill equation to the data (see Table 1). Curves and data points are normalised to the fitted maxima for each curve.
Figure 2. The α1S270T startle disease mutation reduces the maximum single-channel open probability in homomeric glycine receptors, but not in heteromers.

Cell-attached single-channel currents recorded at saturating glycine concentration.

A, C, E and G, Continuous 5 s records (top traces in each panel) and 0.2 s sweeps (lower traces in each panel are expanded stretches indicated by bars in 5s traces) from wild-type α1 (A), wild-type α1β (C), mutant α1S18'T (E) and mutant α1S270Tβ (G) receptors at saturating glycine concentrations. The traces show clearly the decrease in maximum $P_{open}$ in homomeric GlyR (E).

B, D, F and H, Histograms of cluster $P_{open}$ for each receptor. Note the wide spread of the $P_{open}$ values of homomeric α1S270T receptor clusters (F).
Figure 3. The $\alpha_{1S270T}$ startle disease mutation reduces the potency and the efficacy of the partial agonist sarcosine in homomeric GlyR

A, Representative whole-cell current responses evoked by U-tube sarcosine application to HEK293 cell expressing wild-type or $\alpha_{1S270T}$ mutant homomeric GlyR. Bars above the traces show the timing of agonist applications. The first trace shows the response to 10 mM glycine used to normalise the concentration-response curve in this cell. Currents were recorded with pipettes filled with low-Cl$^-$ solution (30 mM).

B, The sarcosine concentration-response relation for homomeric wild-type or $\alpha_{1S270T}$ mutant glycine receptors. Symbols represent average data for $n = 6-7$ cells. The lines are the fits of the Hill equation to the data (see Table 1). Curves and data points are normalised to the maximum glycine-activated current in each cell. Note the marked reduction in the maximum response to sarcosine (relative to glycine) in $\alpha_{1S270T}$ homomeric receptors.

C. Traces show typical single channel openings produced by 100 mM sarcosine in mutant homomeric GlyR, recorded in the cell-attached mode. Note that sarcosine failed to evoke distinguishable clusters of openings, and channel openings were sparse and brief.
Figure 4. Concentration dependence of the open probability of homomeric $\alpha 1$S270T glycine receptor clusters.

A, B and C, Examples of continuous 5 s records (top traces in each panel) at 0.3 (A), 1 (B) and 10 mM (C) glycine. The bars over the top traces indicate the regions expanded in time and depicted in the lower traces in each panel.

D, Histograms of open probabilities of clusters recorded at 1, 3, 10 and 100 mM glycine. $P_{\text{open}}$ values were widely spread at all concentration. $P_{\text{open}}$ values were obtained for each patch from the ratio between the total open time and the total duration of the clusters, from recordings idealized by half-amplitude threshold crossing.

E, Cluster open probability plot vs. glycine concentration. The solid circles are the mean values (error bars for the SD of the mean are smaller than the symbols) for the patches included in the analysis (33-128 clusters from 2-4 patches at each concentration). The dashed line is a fit of the Hill equation to the homomeric $\alpha 1$S270T receptor data and the solid line is the concentration $P_{\text{open}}$ curve for wild-type homomeric receptor from (Beato et al., 2004).
Figure 5. Concentration dependence of the open probability of heteromeric $\alpha_{1S270T}\beta$ glycine receptor clusters.

A, B, C and D, Examples of continuous 5 s records (top traces in each panel) at 0.03 (A), 0.1 (B) and 10 mM (C and D) glycine. The bars over the top traces indicated the regions expanded in time and depicted as lower traces in each panel.

D, Histograms of open probabilities of clusters recorded at 0.1, 0.3, 1, 3, 10, 30 and 100 mM glycine. $P_{\text{open}}$ values were widely spread at all concentrations.

E, Cluster open probability plot vs glycine concentration. The solid circles are the mean values of all the analysed clusters ($n=21-131$). The solid line is the concentration open probability curve for heteromeric $\alpha_{1}\beta$ channels from (Burzomato et al., 2004).
Figure 6. Glycine receptors carrying the α1S270T startle disease mutation deactivate faster in response to synaptic-like applications of 3 mM glycine

A, B Representative current responses evoked by the fast application of 3 mM glycine (2 ms pulses) via piezo driven θ-tube to outside-out patches. Responses by wild-type (grey trace) and α1S270T (black trace) receptors are shown for homomeric (A) and heteromeric (B) GlyR and currents are normalised to their own peak.

The internal solution contained 30 mM chloride. The activation of wild-type GlyR is fast, and its speed is limited by the solution exchange time. At the end of the glycine pulse, channel deactivation was much faster for both types of mutant receptors than for wild-type ones.

C and D, The activation (C) and deactivation (D) time constants for the outside-out currents evoked by fast application of glycine. The values for each patch are shown as open circles and the mean for each receptor is indicated as a bar.
Figure 7. Location of the S270 (18') startle disease residue in the M2 transmembrane helix of the glycine receptor.

A. Sequence alignment of the M1 and M2 transmembrane helices and the M2-M3 linker region for the three functional human α GlyR subunits, the mouse α4 GlyR subunit, zebrafish α1 subunit and the β human GlyR subunit showing the position of S270 in M2 and that of M227 in M1. The extent of the M1 and M2 α-helices is indicated above the alignment. Red background indicates the residue is conserved in all sequences and red font signifies residues that have similar properties. B, Glycine receptor architecture: resting closed (yellow, Protein Data Bank code 3JAD), open (light-blue, 3JAE) and desensitised closed structures (light-grey, 3JAF, all from (Du et al., 2015)). C, The single subunit view shows (right) the position of S270 (green sphere) at the end of the M2 helix close to the extracellular side, and that of M227 (blue sphere) at the start of M1, also close to the extracellular side. This area is shown in detail in the three structures in the middle, where M1 and M2 are shown in stick format, S270 in green and M227 in blue. The M227 side chain in the open state was modelled using the mutagenesis functionality in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC), where the rotamer closest to the one adopted by M227 in the desensitised state was chosen. The dashed line in the open state shows the hydrogen bond interaction between S270 and M227 (M227:S to S270:O distance = 2.2Å).