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Novel missense mutations in the glycine receptor β subunit gene (GLRB) in startle disease

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A B S T R A C T
Startle disease is a rare, potentially fatal neuromotor disorder characterized by exaggerated startle reflexes and hypertonia in response to sudden unexpected auditory, visual or tactile stimuli. Mutations in the GlyR α1 subunit gene (GLRA1) are the major cause of this disorder, since remarkably few individuals with mutations in the GlyR β subunit gene (GLRB) have been found to date. Systematic DNA sequencing of GLRB in individuals with hyperekplexia revealed new missense mutations in GLRB, resulting in M177R, L285R and W310C substitutions. The recessive mutation M177R results in the insertion of a positively-charged residue into a hydrophobic pocket in the extracellular domain, resulting in an increased EC50 and decreased maximal responses of α1β GlyRs. The de novo mutation L285R results in the insertion of a positively-charged side chain into the pore-lining 9’ position. Mutations at this site are known to destabilize the channel closed state and produce spontaneously active channels. Consistent with this, we identified a leak conductance associated with spontaneous GlyR activity in cells expressing α1βL285R GlyRs. Peak currents were also reduced for α1βW310C GlyRs although glycine sensitivity was normal. W310C was predicted to interfere with hydrophobic side-chain stacking between M1, M2 and M3. We found that W310C had no effect on glycine sensitivity, but reduced maximal currents in α1β GlyRs in both homozygous (α1βW310C) and heterozygous (α1ββW310C) stoichiometries. Since mild startle symptoms were reported in W310C carriers, this may represent an example of incomplete dominance in startle disease, providing a potential genetic explanation for the ‘minor’ form of hyperekplexia.

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Introduction
Inhibitory glycine receptors (GlyRs) are ligand-gated chloride channels enriched in the spinal cord, brainstem and retina, consisting of heteropentameric combinations of ligand-binding GlyR α subunits (α1−α4) together with the GlyR β subunit (Lynch, 2009). Each GlyR subunit is composed of an N-terminal extracellular domain and four α-helical membrane-spanning domains (M1–M4). M3 and M4 are linked by a long intracellular domain containing binding sites for a variety of intracellular factors. Although GlyR α and β subunits both play an active role in agonist binding (Dutertre et al., 2012; Grudzinska et al., 2005), the GlyR β subunit was, until recently, widely assumed to play solely a structural role in heteromeric GlyRs. In part, this was due to the key role of the GlyR β subunit in mediating high-affinity interactions with the synaptic clustering molecule gephyrin (Meyer et al., 1995), which in turn controls the dynamic localization of GlyRs at synaptic sites (Feng et al., 1998). More recently, the GlyR β subunit was also reported to interact with the proteins Vacuolar Protein Sorting 35 (Vps35) and Neurobeachin (Nbea), indicating a new role in GlyR trafficking (del Pino et al., 2011). However, the atypical M2 domain of the GlyR β subunit also confers resistance to the effects of picrotoxinin (Pribilla et al., 1994) and the insecticide
lindane (Islam and Lynch, 2012) as well as influencing the main-state single-channel conductances of heteromeric α1β GlyRs (Bormann et al., 1993).

Initial cross-linking studies of affinity-purified native GlyRs suggested that heteromeric GlyRs exist in a 3α:2β subunit combination (Langusch et al., 1988). However, three innovative studies have recently revealed that the GlyR β subunit represents the major component of heteromeric GlyRs, which are more likely to exist in a 2α:3β stoichiometry. Grudzinska et al., 2005 compared the effects of mutations affecting predicted glycine binding residues in GlyR α1 and β subunits. Co-expression of mutant GlyR α1 with wild-type β subunits resulted in a full rescue of the EC50 value for glycine, whilst co-expression of wild-type GlyR α1 with mutant β subunits resulted in a decrease in EC50. A subsequent study (Dutertre et al., 2012) revealed that the GlyR β subunit contributes more to agonist binding site formation than had been previously realized. Experiments with GlyR α1β subunit concatamers also revealed that functional heteromeric GlyRs can be produced when these were co-expressed with GlyR β subunit monomers but not when expressed alone, or with GlyR α1 subunit monomers (Grudzinska et al., 2005). This result was consistent with either a 2α:3β or a 1α:4β stoichiometry. However, quantification of [35S]methionine incorporation into recombinant αβ versus α1 subunit GlyRs suggested a 2α:3β stoichiometry and the subunit order β-α-β-α-β. This stoichiometry and subunit arrangement has recently been confirmed by imaging single antibody-bound GlyR α1β heteromers using atomic force microscopy (Yang et al., 2012).

Defects in the adult GlyR isoform (αβ) also have an important role in disease. Mutations in GLRA1, encoding the GlyR α1 subunit, are the major genetic cause of startle disease/hyperekplexia in humans (Chung et al., 2010; Shiang et al., 1993, 1995) and cause similar disorders in mice (Buckwalter et al., 1994; Holland et al., 2006; Ryan et al., 1994; Traka et al., 2006) and Poll Hereford cattle (Pierce et al., 2001). In humans, hyperekplexia affects newborn children and is characterized by exaggerated startle reflexes and hypertonia in response to sudden, unexpected auditory, tactile or visual stimuli. By contrast, GLRB mutations are less frequent, but recessive mutations have been discovered in three families with hyperekplexia (AI-Owain et al., 2012; Lee et al., 2012; Rees et al., 2002), in the mouse mutant spastic (Kingsmore et al., 1994; Müllhardt et al., 1994) and the zebrafish bandoneon mutant (Hirata et al., 2005). However, one conundrum is why so few GLRB mutations are found in startle disease relative to GLRA1, or SLC6A5, encoding the presynaptic glycine transporter GlyT2, which are the primary and secondary genetic causes of startle disease respectively (Carta et al., 2012; Giménez et al., 2012; Rees et al., 2006). Here we report the characterization of three new missense mutations in GLRB, resulting in M177R, L285R and W310C substitutions in the extracellular domain, and the M2 and M3 membrane-spanning domains, respectively. We present detailed molecular modeling and functional analysis of these new GlyR β subunit mutations, revealing novel pathogenic mechanisms and modes of inheritance.

Materials and methods

Patients and cases

49 individuals with a clinical diagnosis of hyperekplexia (non-habituating startle response, positive nose tap test, history of neonatal/infantile hypertonicity) but lacking mutations in GLRA1 and SLC6A5 were ascertained by referral from pediatricians from international centers. DNA analysis of GLRB was performed at the Laboratory for Diagnostic Genome Analysis (LDGA) of the Center of Human and Clinical Genetics of the Leiden University Medical Center. The LDGA is NEN-EN-ISO 15189:2007 accredited by the Dutch Accreditation Council. Informed consent for participation in the study and publication of clinical and genetic data was obtained by the referring clinicians (MV at Sahlgrenska University Hospital and FMC at Imperial College London).

Genetic analysis

DNA was extracted from whole blood taken from affected individuals and relatives using a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA). Primers recognizing the ten GLRB coding exons and flanking splice sites were designed using the Lightsscanner Primer Design Software package, version 1 (Idaho Technology Inc, Salt Lake City, Utah). To avoid allelic dropout, all primers were placed in intronic regions that were devoid of single-nucleotide polymorphisms (SNPs) as revealed by National Center for Biotechnology Information databases. Primer designs are provided in Supplementary table 1. Amplified PCR fragments were sequenced on an ABI PRISM 3730 using the BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) and analyzed using SeqScap software for comparative sequence analysis (Applied Biosystems). Single nucleotide variants (SNVs) were assessed against those found in NCBI dbSNP Build 136 and release 20100804 from the 1000 genomes project.

Homology modeling of the human GlyR

Fold recognition of human GlyR α1 and β subunits was performed with HHPred (Söding et al., 2005), identifying the structure of the Caenorhabditis elegans glutamate-gated chloride channel (GluCl; PDB: 3RHW) (Hibbs and Gouaux, 2011) as the best template (p-value: 0). A profile–profile alignment between the human GlyR α1 subunit and the GluCl α subunit was generated using MUSCLE (Edgar, 2004) and T-COFFEE (Notredame et al., 2000) resulting in 44.4% sequence identity. A separate alignment was generated for the human GlyR β subunit and GluCl resulting in 38.5% sequence identity. A short part of the sequence at the N-terminus of the β subunit (residues 1–30), and the extended loop region between M3 and M4 of both the α1 and β subunits (residues 312–396 and 337–453, respectively) could not be accurately modeled due to lack of sequence identity and were therefore removed. The two alignments were inspected manually and combined to include all five subunits together to re-create the entire pentameric structure in the stoichiometry 2α1:3β with the subunit arrangement [-α-β-α-β-α-β]. Using MODELLER-9v10 (Eswar et al., 2003), 50 models were built based on the combined alignment, restraining disulfide bonds between the cysteine residues involved in the cys-loops within each subunit (C138–C152 and C198–C209 in the α1 subunit and C161–C175 and C221–C233 in the β subunit). Each model was assessed by MODELLER with the Discrete Optimized Protein Energy (DOPE) statistical potential score (Shen and Sali, 2006) and the optimal model was selected based on the lowest score (normalized DOPE Z score: −0.222). A further assessment of model quality was performed using QMEAN (Benkert et al., 2011) The QMEAN Z score for the human GlyR α1β model was −3.43, indicating a reasonable model (Benkert et al., 2011). Selected non-synonymous substitutions were modeled into the GlyR homology model with the swapaa command in Chimera (Pettersen et al., 2004) using the Dunbrack backbone-dependent rotamer library (Dunbrack, 2002) and taking into account the lowest clash score, highest number of H-bonds and highest rotamer probability.

Site-directed mutagenesis and expression constructs

Full-length human GlyR α1 and β subunits were cloned into the vector pRK5 as previously described (Chung et al., 2010). Mutations were introduced into pRK5–hGlyRs cDNA using the QuikChange site-directed mutagenesis kit (Agilent). All expression constructs were confirmed by Sanger sequencing of the entire coding region.
Wild-type or mutant GlyR α1β heteromers were transiently expressed in human embryonic kidney (HEK293) cells transfected using magnetofection (CombiMag; O2 Biosciences, Lipofectamine 2000; Invitrogen). GlyR α1 and β subunit expression constructs were transfected at a DNA ratio of 1:10 to promote the formation of heteromeric α1β GlyRs. 32 h after transfection, cell surface-expression of GlyR β subunits was measured using a cell membrane-impermeable reagent Sulfo-NHS-LC-Biotin (Pierce Biotechnology) as previously described (Chung et al., 2010). Proteins in the whole-cell lysates or cell-surface proteins were analyzed by Western blotting with an antibody against the GlyR β subunit (1:90; AbCam ab123886). An anti-β-actin antibody (1:5000; AbCam) was used as a control to confirm that intracellular proteins were not labeled with biotin. The intensity of the immunoreactivity signal was quantified using ImageJ (http://rsweb.nih.gov/ij/).

**Fluorescence-based imaging**

Cells were imaged using an automated fluorescence-based screening system using EYFP/CFP, fluorescence quench as an indicator of anion influx. In this technique, iodide flowing into the cell binds to and quenches EYFP/CFP fluorescence, thus providing an indication of the relative activity levels of membrane anion channels (Kruger et al., 2005). Briefly, HEK AD293 cells were transfected with the plasmid DNAs for wild-type and mutant GlyRs and with the DNA for EYFP/CFP, as described in the results and plated into a 384-well plate. Unless otherwise indicated, all GlyR plasmid DNAs were transfected in equimolar ratios for both the fluorescence and patch-clamp electrophysiological assays. Within the following 24–32 h, the culture medium in the wells was replaced with extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4 using NaOH). After 30 min, fluorescence images of each well were obtained twice, before and after the application of NaI solution (140 mM NaI, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose, pH 7.4 using NaOH) containing varying concentrations of glycine. Values were pooled from 3 to 4 experiments with three wells each containing ≥200 cells. To determine the glycine dose–response curve, an empirical three or four parameter Hill equation was fitted by a non-linear least squares algorithm using SigmaPlot 11.0 software. Throughout this study, % quench is defined as the (initial fluorescence – final fluorescence) × 100/initial fluorescence. Thus, a treatment that completely abolished all fluorescence would yield a 100% quench.

**Electrophysiology**

Glycine-gated currents were measured using whole-cell patch-clamp electrophysiology at a holding potential of −40 mV. Spontaneous single-channel currents were recorded from outside-out excised patches, held at −70 mV and in the absence of agonist. HEK AD293 cells were transiently transfected with GlyR subunit expression constructs and plated on coverslips. Cells were continually superfused with extracellular solution (composition as above) and visualized with an inverted fluorescence microscope. Transfected cells were identified by co-transfection with an EGFP expression construct. Patch pipettes were pulled to fine tip resistances of 1–4 MΩ (whole-cell) and 6–12 MΩ (outside-out) when filled with a standard intracellular solution (145 mM CsCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, 10 mM EGTA, pH 7.4 with CsOH). Whole-cell currents, which were filtered at 1 kHz and digitized at 2 kHz, were recorded using an Axon MultiClamp 700B amplifier and pClamp10.2 software. Single-channel currents, which were filtered at 5 kHz and digitized at 20 kHz, were recorded using an Axon Axopatch 200B and pClamp10.2 software. Drugs diluted in extracellular solution were applied to cells or patches via gravity-forced perfusion using parallel small tubes. Solution exchange was complete within 200 ms.

**Results**

**Mutation analysis of GLRB in hyperekplexia**

Individuals with startle disease lacking mutations in GLRA1 and SLC6A5 were screened for genetic variation in GLRB coding exons and donor and acceptor splice junctions by Sanger DNA sequencing. Sequence variants were assigned as potentially pathogenic after cross-referencing with known GLRB mutations and common GLRB polymorphisms found in dbSNP and the 1000 genomes project. This analysis revealed two novel sequence variants in GLRB (Fig. 1A, B, Table 1) in two individuals. Individual 1 harbored a two base missense mutation (c.920_921ΔinsGA) resulting in a L285R substitution in the second membrane-spanning domain (M2) of the GlyR β subunit. Since this change was found in the heterozygous state (Fig. 1A, B) and neither parent carries the mutation, this mutation appears to be de novo. By contrast, individual 2 harbored a different missense mutation (c.G996T), resulting in a W310C substitution in the third membrane-spanning domain (M3) of the GlyR β subunit. At first glance, this mutation appeared to show recessive inheritance, since both parents were heterozygous carriers. However, clinical assessments and functional data suggest an alternative mechanism of inheritance for this mutation (see below). Recently, another recessive mutation c.T596G, resulting in a M177R substitution, was reported (Al-Owain et al., 2012) in nine patients in a large family of Saudi Arabian origin with hyperekplexia and esotropia, an eye misalignment disorder where one or both eyes turn inward.

**Clinical assessment**

Individual 1 is a male of white British descent born at term plus 11 days. His weight and height were on the 3rd centile at delivery. Apneas were observed within 40 min and hypertonia presented within the first hour after birth. These symptoms were severe enough to necessitate intubation, his rigidity made ventilation very difficult and startle was prominent during this time. The classical nose-tap reflex was also present. Magnetic resonance imaging (MRI) revealed subtle brain abnormalities, including a mild increase in signal on T2-weighted sequences in the white matter and obvious dentate nuclei; however the basal ganglia had an unremarkable appearance. At 3 months of age stereotyped but unusual dystonic posture movements were observed, including an arm-raising phenomenon that was followed by a period of generalized hypertonia during which voluntary movements were impossible. At 9 months, his gross motor performance was at the level of 7 months. Although this had broadly normalized by 14 months, dystonic movements were still prominent. Minor motor delay remained a feature for some time and when last seen he was still hyperekplexic and had prominent cervical hypertonia, although exaggerated startle was less prominent; he also had an alternating strabismus. Now at 9 years, he remains small for his age. He still has very mild motor delay, but stiffness is not noticeable and he plays football regularly. He is making good progress in school and socially. He is mildly myopic but no longer has strabismus. No epileptic seizures have been observed.

Individual 2 is a female of Turkish origin with consanguineous parents. She exhibited neonatal hypertonia and irregular breathing necessitating continuous positive airway pressure (CPAP) treatment for several days following birth. In addition, she had episodes of neonatal bradycardia and an excessive startle reflex, although consciousness was unaltered during startle episodes. She had a short period of generalized stiffness following the startle response during which voluntary movements were impossible. MRI imaging performed at 2 months of age showed bilateral periventricular cystic changes, in
keeping with periventricular leucomalacia (necrosis of white matter). She was not dysmorphic and is not currently of small stature, at only −1 SD below the mean. At 4 months of age she had screaming periods without a clear loss of consciousness. Concurrently, she had spasms in the jaw and unusual movements of her arms. At that time her EEG was normal without focal signs. At 16 months of age she had an abnormal EEG with clear high-amplitude spike and wave activity and occasional hypsarrythmia (high amplitude and irregular waves and spikes in a background of chaotic and disorganized activity). She was treated with nitrazepam and both the seizures and EEG changes disappeared. She currently takes clonazepam and has no further symptoms. She does not have gaze palsy, eye movement disorders, nor any apparent learning difficulties. According to the family her parents and maternal uncle had ‘light symptoms’ in early life. However, further details of their early childhood have been lost over time and bound up within cultural restrictions surrounding

Table 1

| Position | Exon | Protein precursor/mature | Type          | SIFT | Polyphen-2 | Overall prediction |
|----------|------|--------------------------|---------------|------|------------|--------------------|
| t.596G   | 6    | M199R/M177R               | Missense      | Damaging (0) | Possibly damaging (0.802) | Pathogenic         |
| G752A    | 8    | G251D/G229D               | Missense      | Damaging (0) | Probably damaging (1.0) | Pathogenic         |
| IVS5+5G>A| –    | –                        | Splice site   | –    | –          | Pathogenic         |
| 921ΔInsGA| 9    | L307R/L285R               | Missense      | Damaging (0) | Probably damaging (0.997) | Pathogenic         |
| 921ΔInsGA| 9    | L307R/L285R               | Missense      | Damaging (0) | Probably damaging (0.999) | Pathogenic         |
| 921ΔInsGA| 9    | L307R/L285R               | Missense      | Damaging (0) | Probably damaging (0.990) | Pathogenic         |

Fig. 1. Human GlyR β subunit mutations identified in individuals with startle disease/hyperekplexia. Amino acid sequences of the human GlyR α1 and β subunits indicating the positions of putative membrane-spanning domains (grey shaded boxes) defined by alignments with GluCl (Hibbs and Gouaux, 2011) and amino-acid residues affected by hyperekplexia mutations. Mutation key: dominant mutation: red; recessive: blue; de novo: orange. Green lettering denotes key ligand-binding residues in the GlyR α1 and β subunits. Purple lettering denotes the gephyrin-binding motif between M3 and M4 of the GlyR β subunit.

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disclosure. Her older sister (untested) has clear symptoms and her younger brother born in 2010 (tested) is also affected. The parents (both heterozygous for W310C) do not currently show any hyperekplexia symptoms.

Molecular modeling

M177R, L285R and W310C substitutions were predicted (Table 1) to be damaging or probably/possibly damaging using SIFT (Kumar et al., 2009) and Polyphen-2 (Adzhubei et al., 2010), respectively. These bioinformatics tools predict the possible impact of an amino acid substitution on the structure using the conservation of amino acid residues in sequence alignments derived from related sequences and machine learning approaches. To gain further insights into the potential pathogenic mechanisms of these mutations, we constructed a homology model of the human αβ GlyR based on the crystal structure of the GluCl α subunit from C. elegans (Hibbs and Gouaux, 2011). The GluCl structure makes a significantly better template for homology modeling of the human GlyR compared to other ligand-gated ion channel structures such as Gloeobacter violaceus GLIC (Bocquet et al., 2009), Erwinia chrysanthemi nAChR (Hill and Dutzler, 2009) or the Torpedo marmorata nAChR (Unwin, 2005) due to functional similarity (i.e., GluCl is an anion channel) and higher overall sequence identity. Profile–profile alignment between the human GlyR subunits and the GluCl α subunit generated using MUSCLE (Edgar, 2004) and T-COFFEE (Notredame et al., 2000) resulted in 44.4% (α1) and 38.9% (β1) identity. By contrast, GlyR subunits share lower sequence identity with GLIC (α1: 21.8% and β1: 24.6%) and different nAChR subunits (α1: 19.7 to 20% and β1: 23.9 to 24.6%). Our GlyR model (Fig. 2A) predicts that L285 in the GlyR β subunit is a pore-lining residue, projecting a small uncharged, hydrophobic side chain into the ion-channel lumen (Fig. 2B). The L285R mutation introduces a significantly larger, positively-charged side chain that projects into the pore lumen (Fig. 2C). Because this mutation is de novo and the individual is heterozygous for L285R, in theory heteromeric GlyRs could be formed in vivo containing 3 × wild-type, 1 ×, 2 × or 3 × mutant GlyR β subunits (Fig. 2C). Modeling of pore-lining residues at a β subunit/β subunit interface (Fig. 3A) reveals that L285 is at the 9′ position in M2, equivalent to L254 in GluCl. This mutation is predicted to disrupt the pentameric radial symmetry of the 9′ leucine ‘hydrophobic girdle’, that is essential for stabilizing the pore in the closed channel state. Disruption of this girdle is well known to induce spontaneous channel activity and/or enhance agonist sensitivity in other Cys-loop receptors (Chang and Weiss, 1998, 1999). In common with GluCl, none of the major pore-lining residues are charged, with the exception of D271 at the -5′ position at the cytoplasmic end of M2 (Fig. 3A). This is consistent with different models of anion channel function which suggest that the positive electrostatic potential at the base of the pore arises from either: i) buried basic residues at the intracellular entrance of the pore (Cymes and Grosman, 2011), such as R276 in the M2 0′ position in the GlyR β subunit (R252 in the GlyR α1 subunit) or ii) oriented peptide dipoles in the M2 α helices (Hibbs and Gouaux, 2011). Given that the electrostatic potential at the 0′ position strongly influences single-channel conductance (Chang and Weiss, 1998, 1999), it is possible that the introduction of a large, positively-charged residue at the 9′ position could have a similar effect.

By contrast, the side-chain of W310 in M3 is part of an intramembrane network of aromatic residues (phenylalanine, tyrosine, histidine and tryptophan) contributed by M1, M3 and M4 (Fig. 4A, B). Substitution W310C introduces a significantly smaller and shorter side chain into this position (Fig. 4C), with a reactive sulfhydryl group. Although we considered that C310 might form a disulfide bond with C314 in M3, one turn down the helix in our model, the distance between the two sulfur atoms (4.6 Å) precludes this interaction (Careaga and Falke, 1992). In addition, disulfide bonds rarely form within the membrane or a reducing intracellular environment. Rather, our modeling predicts that the W310C substitution disrupts aromatic stacking (Fig. 4B) – a key requirement for correct GlyR assembly and cell-surface trafficking (Haeger et al., 2010).

Fig. 2. Molecular modeling of the human αβ GlyR and mutation L285R. (A) Side view of the molecular model of the human GlyR αβ pentamer showing α1 subunits in gold and β1 subunits in blue. The positions of the inner and outer membrane surfaces are indicated by grey spheres. (B) Orthographic view of the GlyR αβ pentamer, showing the relative position of the L285 side chain in the ion-channel pore. (C) Panels depicting the multiple GlyR isoforms that could be formed on co-expression of wild-type and mutant GlyR β1 subunits harboring the L285R substitution. A red plus sign denotes the positive charge carried by the side-chain of R285.
M177 is a hydrophobic amino acid located in the GlyR extracellular domain on a β-sheet (β7). The side-chain of this residue forms part of a hydrophobic pocket (Fig. 5A) that is conserved with in the GluCl structure (Hibbs and Gouaux, 2011), which has an isoleucine at the equivalent position. The M177R substitution results in the introduction of a positively-charged, hydrophilic side chain into this pocket (Fig. 5B), which is predicted to have a significant effect on the folding of the extracellular domain. Although M177/R177 are not predicted to directly influence glycine binding (Fig. 5C, D), disruption of the local structure around M177 is predicted to cause an indirect effect on agonist binding or signal transduction. For example, M177 is located just five residues away from F182, which is involved in a cation-π interaction (Pless et al., 2008) with the amino group of glycine.

Cell-surface biotinylation assays

To establish whether the GlyR β subunit mutants impaired cell-surface expression of GlyRs, we carried out cell-surface biotinylation assays for heteromeric α1β GlyRs (Chung et al., 2010), detecting β-subunit expression using a specific antibody. Note that in the absence of the GlyR α1 subunit, the GlyR β subunit is not expressed at the cell surface in HEK293 cells, but is retained in the rough endoplasmic reticulum (Kirsch et al., 1995). The M177R, L285R and W310C mutant polypeptides were all detected at equivalent levels to the wild-type GlyR β subunit in total cell lysates, suggesting that they synthesized correctly and are not prone to rapid degradation (Fig. 6A). However, quantification of the biotinylation of heteromeric cell-surface α1β GlyRs revealed significant decreases in cell-surface expression of α1βL285R and α1βW310C subunit GlyRs (Fig. 6A, B) to 27.3 ± 1.1% for L285R and 21.5 ± 8.3% for W310C of the level of wild-type α1β GlyRs. By contrast, α1βM177R GlyRs had a similar level of cell-surface expression (104 ± 2.2%) when compared to wild-type α1β GlyRs.

Functional analysis using EYFP CI− sensors and electrophysiology

HEK AD293 cells were co-transfected with expression constructs encoding the GlyR α1 subunit together with each GlyR β subunit mutant. To reproduce the heterozygous state, GlyR α1 was co-transfected with wild-type and mutant GlyR β subunits. We initially employed the EYFP assay (Chung et al., 2010; Kruger et al., 2005) to provide...
an indication of the anion influx rate through the recombinant GlyRs. The advantage of this approach is that responses of large numbers of cells can be averaged, thus permitting the reliable quantitation of small changes in the current fluxing capacity of functional GlyRs. Glycine dose–response relationships (Fig. 7A, B) revealed that the maximal fluorescence quench was significantly reduced for α1βL285R, α1ββL285R, α1βW310C and α1ββW310C GlyRs relative to wild-type α1β GlyRs (Fig. 7C), suggesting that mutations L285R and W310C reduce anion flux rates in both the homozygous and heterozygous states. We also found that the reduction of Cl– flux was dependent on the ratio of wild-type GlyR α subunit (the amount was held constant) to mutant GlyR β subunits (Fig. 7D), with significantly reduced Cl– flux observed with increasing levels of βW310C expression. Given that αβ GlyRs exist in an invariant 2α:3β stoichiometry (Yang et al., 2012), we infer that increasing the βW310C:α1 subunit ratio increases the proportion of heteromeric GlyRs, which in turn reduces functional GlyR expression. These data are consistent with our biotinylation assays, and suggest βW310C subunits prevent wild-type GlyR subunits from trafficking to the cell surface. EC50 values for wild-type α1β, α1βL285R and α1βW310C GlyRs were 5 ± 1, 4 ± 1, 13 ± 2 μM, respectively (Table 2), suggesting that the L285R and W310C mutations do not substantially alter the agonist sensitivity of recombinant α1β GlyRs reaching the cell surface.

To test if the mutated β subunits incorporate into functional GlyRs, the pore blocker lindane was applied. Lindane potently inhibits homomeric α subunit GlyRs, but has almost no effect on heteromeric αβ subunit GlyRs (Islam and Lynch, 2012). GlyRs containing βL285R or βW310C subunits exhibited a reduced sensitivity to lindane, indicating

![Image](https://i.imgur.com/3.png)

**Fig. 4.** Mutation W310C disrupts hydrophobic stacking of membrane-spanning domains M1–M3. (A) Cutaway view from the extracellular side of the GlyR β subunit showing how the side-chain of residue W310 in M3 contributes to a hydrophobic stack of aromatic residues contributed by membrane-spanning domains M1, M3 and M4. Hydrophobic side-chains of residues predicted to be involved in this stack are colored according to membrane-spanning domain: M1: red; M3: yellow and M4: blue. (B) Mutation W310C results in the loss of the aromatic, hydrophobic side chain of tryptophan and replacement with the shorter, reactive cysteine sulphydryl side chain. (C–F) Top and side views showing the hydrophobicity of individual residues in the stack by coloring the atom surfaces. Hydrophobic surfaces are depicted in orange, hydrophilic in blue and white indicates neutral polarity. The W310C substitution is predicted to disrupt the hydrophobic stack, altering the tertiary fold of the membrane-spanning domains and introduce an empty space (F, circled with a dashed line). Disulfide bond formation is unlikely, since this is rare within the membrane or an intracellular environment and the only adjacent sulfur atom (C314 in M3) is 6.4 Å away from C310 sulfur.
that receptors incorporating these mutant subunits are functionally expressed at the cell surface (Fig. 7E). Since artificial mutations at the 9′ position in GABA<sub>A</sub>C and GABA<sub>B</sub>R subunits result in spontaneously-opening channels (Chang and Weiss, 1998, 1999), we also investigated whether α<sub>1</sub>β<sub>L285R</sub> GlyRs showed this property, by quantifying the fluorescence change in the absence of glycine. Both α<sub>1</sub>β<sub>L285R</sub> and α<sub>1</sub>β<sub>W310C</sub> GlyRs showed a significant increase in quench relative to heteromeric α<sub>1</sub>β<sub>GlyRs</sub>, which is suggestive of spontaneous activity (Fig. 7F). Even in the absence of glycine, the quench observed was >20%. By contrast, the quench observed in the absence of glycine for α<sub>1</sub>β<sub>GlyRs</sub> was only slightly higher than for wild-type GlyRs, suggesting that these GlyRs do not show spontaneous gating. Averaged results obtained using the EYFP Cl<sup>−</sup> flux assay for L285R and W310C are summarized in Table 2.

Electrophysiology was subsequently used to analyze the effects of the L285R and W310C mutations on GlyR function in greater detail. Dose–response relationships confirmed that the agonist sensitivity for GlyRs containing either mutated subunit did not change relative to wild-type α<sub>1</sub>β<sub>GlyRs</sub>. As in the EYFP assays, the presence of the GlyR β subunit was confirmed by applying lindane (Fig. 8A). Spontaneous activity of α<sub>1</sub>β<sub>L285R</sub>–containing GlyRs could be inhibited by the pore-blocker picrotoxin (Fig. 8C). However, due to the resistance of heteromeric α<sub>1</sub>β<sub>GlyRs</sub> to picrotoxin (Pribilla et al., 1994), this inhibition was typically only about 100 pA. Single-channel recordings in the absence of glycine confirmed the spontaneous activity of α<sub>1</sub>β<sub>L285R</sub> GlyRs (Fig. 8D). Averaged over four patches, mean open probability in the absence of applied glycine was 0.04±0.01. The maximal unitary conductance level (denoted by γ2 in Fig. 8D) was 49.4±0.7 pS with an additional sub-conductance level (γ1) at 23.9±0.3 pS (both n=4 patches). The dominant 49 pS level corresponds closely to that observed in wild-type α<sub>1</sub>β<sub>GlyRs</sub> (44 pS) (Bormann et al., 1993), indicating that the L285R substitution does not affect single-channel conductance. To investigate the properties of α<sub>1</sub>β<sub>L285R</sub> GlyRs, whole-cell recordings were made from >200 EGFP-expressing cells that had been transfected with both α<sub>1</sub> and β<sub>L285R</sub> subunits in a 1:10 molar ratio. Only 20 of these cells expressed glycine-gated currents, with only two of the 20 expressing lindane-insensitive GlyRs, suggesting a predominance of α<sub>1</sub>β<sub>L285R</sub> GlyRs in the cell membrane. In both of these cells, the maximal current amplitude was significantly reduced relative to wild-type GlyRs (Fig. 8E), suggesting a reduction in either maximum open probability, single-channel conductance, and/or the number of functional channels expressed in the plasma membrane. Based on the biotinylation results, we can conclude that the reduced current amplitude is at least partly caused by reduced cell-surface expression. The properties of whole-cell currents mediated by α<sub>1</sub>β<sub>L285R</sub> and α<sub>1</sub>β<sub>L285R</sub> GlyRs are summarized in Table 3.

The M177R mutant resulted in both a reduction of maximal fluorescence quench (Fig. 9A, B) and a rightward shift in the EC<sub>50</sub> value for glycine (Fig. 9A) from 17±5 μM for wild-type α<sub>1</sub>β<sub>GlyRs</sub> to 45±14 μM for α<sub>1</sub>β<sub>M177R</sub> GlyRs and 64±13 μM for α<sub>1</sub>β<sub>L285R</sub> GlyRs (Table 4). Heteromeric α<sub>1</sub>β<sub>M177R</sub> GlyRs exhibited a slightly reduced sensitivity to lindane (Fig. 9C). However, the reduced glycine sensitivity indicates that receptors incorporating the β<sub>M177R</sub> subunit are expressed at the cell surface. Using patch-clamp electrophysiology, we confirmed...
that the EC50 value of $\alpha_1\beta^{M177R}$ GlyRs was significantly increased from $39 \pm 4$ μM for wild-type $\alpha_1\beta$ GlyRs to $162 \pm 3$ μM for $\alpha_1\beta^{M177R}$ GlyRs (Fig. 9D, E), and that peak current magnitudes relative to wild-type $\alpha_1\beta$ GlyRs compared to wild-type $\alpha_1\beta$ GlyRs. It is tempting to speculate that mutations such as W310C in the large extracellular domain (Y128C) with a major role in glycine recognition, might form the genetic basis for hyperekplexia.

Discussion

This study reports the identification and functional characterization of novel mutations in the GlyR β subunit gene (GLRB) that reveal new pathogenic mechanisms underlying startle disease/hyperekplexia. We identified two new missense variants in GLRB causing L285R and W310C substitutions in membrane spanning domains M2 and M3. Using molecular modeling, cell-surface biotinylation assays, an EYFP-based anion flux assay and patch clamp electrophysiology, we were able to establish the likely pathogenic mechanisms for L285R and W310C, as well as a third mutation M177R, which reduces peak current magnitude by reducing the cell-surface expression of $\alpha_1\beta^{M177R}$ GlyRs. Despite these reductions, incorporation of $\alpha_1\beta^{M177R}$ homomers even in the presence of a ten-fold excess of $\alpha_1\beta^{W310C}$ GlyRs compared to wild-type $\alpha_1\beta$ GlyRs.

On first impressions, W310C also appeared to be a classical recessive hyperekplexia mutation, interfering with the formation of cell-surface GlyRs, rather than affecting glycine sensitivity. However, detailed analysis revealed both novel pathogenic mechanisms and mode of inheritance for this missense mutation. Molecular modeling revealed that W310 is a key residue involved in a hydrophobic stack formed by aromatic residues in M1, M3 and M4 that determines GlyR subunit stoichiometry (Haeger et al., 2010). The W310C substitution in M3 is predicted to act by destabilizing intramembrane packing of $\alpha$-helices, a result that is in accord with our functional studies, which show compromised cell-surface expression of $\alpha_1\beta^{W310C}$ and $\alpha_1\beta^{M177R}$ GlyRs and the formation of a significant proportion of $\alpha_1\beta^{W310C}$ GlyRs. However, we cannot rule out the possibility that reductions in channel open probability or single-channel conductance may also have contributed to the reduced current-carrying capacity of these mutant receptors. The inheritance of this mutation is of significant clinical interest, since mild startle symptoms were reported in both parents. This mutation is likely to represent a case of incomplete dominance, i.e. a mutation that has an intermediate effect in heterozygous carriers. This mechanism is supported by our functional data, since the maximal quench was significantly reduced for both $\alpha_1\beta^{W310C}$ and $\alpha_1\beta^{M177R}$ GlyRs compared to wild-type $\alpha_1\beta$ GlyRs. It is tempting to speculate that mutations such as W310C in GLRB might form the genetic basis for ‘minor startle’, an excessive startle response in the absence of hypertonia. This phenomenon has been reported (Tijssen et al., 1995, 1996, 2002) in several hyperekplexia studies, but even though ‘major’ and ‘minor’ startle can occur together in the same family, the genetic basis remains unresolved and it has been assumed by some to be a ‘normal but pronounced startle response’. Our study suggests that certain GlyR mutations may show incomplete dominance, providing a potential genetic explanation for mild startle in heterozygous carriers. This mild startle is predicted to be more common for GLRB mutations, since the likelihood of a heterozygous carrier being able to synthesize fully wild-type GlyRs with a 2$\alpha_1\beta$ stoichiometry when carrying a defective GLRA1 allele is 1 in 4, versus 1 in 8 for a defective GLRA1 allele.

The remaining GLRB substitution we studied in detail was M177R, which both molecular modeling and functional studies suggest does not disrupt cell-surface trafficking, but interferes indirectly with agonist binding, by disrupting a local $\beta$-sheet fold in the large extracellular domain. M177 is located just five residues away from F182, which participates in a cation-n interaction with glycine (Pless et al., 2008). Thus, the pathogenic mechanism for M177R resembles that of a previously reported mutation G229D, which also displayed a reduced EC50 for glycine (Rees et al., 2002).

Our ongoing genetic screening program has recently revealed numerous recessive mutations in the genes encoding the GlyR α1 subunit (GLRA1) and GlyT2 gene (SLC6A5). However, one condition is why so few GLRB mutations have been reported in startle disease. In part, this may be historical: GLRA1 was the first startle disease gene to be discovered in 1993 (Shiang et al., 1993) and mutations in this gene explain the majority of cases of dominantly inherited hyperekplexia. By contrast, mutations in GLRB and SLC6A5 were first reported in 2002 and 2006, respectively (Rees et al., 2002, 2006). This ‘head start’ for GLRA1 is reflected in the NCBI gene-testing registry. Of the clinical laboratories world-wide offering screening for startle disease, seven screen for GLRA1 mutations, three offer screening for GLRB and only...
one offers screening for SLC6A5 mutations. However, this testing does not reflect the true prevalence of disease alleles — for example, we recently reported twenty new pathogenic sequence variants for GlyT2 in 17 index cases (Carta et al., 2012), as well as a new dominant GlyT2 mutation found in multiple families (Giménez et al., 2012). These studies certainly suggest that SLC6A5 is a major startle disease gene. Methodology may also play a role: initial attempts at screening GLRB used single-strand conformation polymorphism (SSCP) or bi-directional di-deoxy fingerprinting (ddF) methods (Milani et al., 1998; Rees et al., 2002), which have variable sensitivity for mutation detection. By contrast, our study and two other recent reports (Al-Owain et al., 2012; Lee et al., 2012) have used Sanger DNA sequencing — currently the method of choice for mutation detection — although this is likely to be superseded in time by targeted next-generation sequencing panels (Lemke et al., 2012). The extended clinical phenotype in these cases, encompassing breathing difficulties/severe neonatal apnea episodes, bradycardia and developmental delay is likely to be explained by the loss of multiple synaptic GlyR

**Table 2**

Properties of human GlyRs containing βL285R and βW310C subunits using the EYFP assay. EC50, Hill coefficients (nH), the maximal fluorescence changes (ΔFmax), the maximal percentages of quenched cells (cellQuench) and the inhibition of 10 μM glycine currents by 100 μM lindane (LINinh) are represented. p-Values were calculated relative to GlyR α1β subunit heteromers except for inhibition by lindane, which was compared to GlyR α1 subunit homomers by unpaired t-test.

| EYFP assay          | EC50 (μM) | nH    | ΔFmax (FU) | cellsQuench (%) | LINinh (%) | n |
|---------------------|-----------|-------|------------|-----------------|------------|---|
| α1                  | 8±1       | 1.7±0.1 | 44±1       | 94±2            | 111±4      | 12|
| α1β                 | 5±1       | 3.9±2.2 | 44±2       | 96±2            | 38±2       | 12|
| α1βL285R            | 4±1       | 0.9±0.1 | 19±2       | 86±7            | 6±7        | 12|
| α1βW310C            | 4±1       | 0.9±0.1 | 19±2       | 83±5            | 14±7       | 12|
| α1βL285R            | 13±2      | 4.3±1.8 | 32±3       | 81±5            | 51±16      | 12|
| α1βW310C            | 11±1***   | 5.1±1.5 | 32±1**     | 90±1            | 32±12***   | 12|

*p <0.05.

**p <0.01.

***p <0.001.
GlyRs is included as a control. (B) Normalized glycine dose–response curves for the GlyRs shown in panel A. (C) Inhibition of leak currents in αβ GlyRs by unpaired patch-clamp electrophysiology. EC50 values, Hill coefficients (nH) and the maximal currents (Imax) are represented. p-Values were calculated relative to the wild-type GlyRs with gephyrin-binding motif are capable of significantly impairing GlyR β-subunit function is less sensitive to hyperekplexia-mimicking mutations introduced into the M2–M3 loop of the GlyR β-subunit than the α-subunit. This suggests that the GlyR α1, M2–M3 loop dominates the β-subunit in gating heteromeric αβ GlyRs and in turn that it is perhaps unlikely that a set of equivalent dominant mutations in the M2–M3 linker (Fig. 1A) will be found in GLRB. This is consistent with our finding that GlyRs containing the L285R mutant exhibit an EC50 that is similar to wild-type, confirming that the β-subunit is not the major mediator of signal transduction in heteromeric GlyRs. Rather, the GLRB mutations found to date appear to cluster near key glycine binding residues (M177R, G229D) or are found in membrane-spanning domains M1–M3 (Fig. 1B), where they disrupt receptor trafficking and affect ion-channel function (L285R) or hydrophobic side-chain stacking (W310C).

Other potential pathogenic mechanisms for GLRB include protein truncation (via deletion, frameshift, splice site or nonsense mutations) or mutations in the gephyrin-binding site located between M3 and M4 (Fig. 1B). At least in vitro, artificial missense mutations affecting single amino acids, such as F398A in the GlyR β-subunit gephyrin-binding motif are capable of significantly impairing GlyR β–gephyrin interactions (Kim et al., 2006), which would in turn abolish GlyR clustering at synapses. Based on our previous experience with GLRA1 and SLC6A5, we consider it likely that a number of recessive SNVs remain to be discovered in GLRB. Based on the discovery of four new GLRB variants associated with hyperekplexia in this year

Table 3

| Whole-cell patch-clamp electrophysiology | EC50 (µM) | nH | Imax (nA) | n |
|------------------------------------------|----------|----|-----------|---|
| αβ                                      | 39 ± 4   | 2.4 ± 0.2 | 14 ± 3 | 5 |
| αβ1β1208R                                | 49 ± 7   | 1.2 ± 0.1 | 9 ± 4  | 3 |
| αβ1βW310C                                | 28, 58   | 1.7, 4.0  | 0.22, 0.15 | 2 |
| αβ1βM177R                                | 162 ± 3  | 2.2 ± 0.1 | 8 ± 1  | 5 |

**p < 0.001.**
alone (Al-Owain et al., 2012; Lee et al., 2012, this study), we recommend that GLRB should have equal status alongside GLRA1 and SLC6A5 in the molecular genetic diagnosis of startle disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2012.12.001.

Competing interests
All authors have declared that no competing interests exist.

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Table 4
Properties of αβM177R GlyRs using the EYP assay. EC50 values, Hill coefficients (nH), the maximal fluorescence changes (ΔFmax), the maximal percentages of quenched cells (cellsquench) and the inhibition of 100 μM glycine currents by blocking with 100 μM lindane (LINinh) are represented. P-values were calculated relative to GlyR αβ subunit heteromers except for inhibition by lindane, which was compared to GlyR αβ subunit homomers by unpaired t-test.

| GlyR     | EYP assay | EC50 (μM) | nH  | ΔFmax (FU) | cellsquench (%) | LINinh (%) | n  |
|----------|-----------|-----------|-----|------------|-----------------|------------|----|
| α1       | 25 ± 5    | 1.5 ± 0.1 | 46 ± 2 | 97 ± 1 | 92 ± 5 | 12 |
| α2β      | 17 ± 5    | 1.7 ± 0.1 | 50 ± 1 | 96 ± 1 | 42 ± 6*** | 12 |
| α2βM177R | 64 ± 13** | 3.4 ± 1.9 | 29 ± 1*** | 81 ± 2** | 54 ± 8** | 12 |
| α2βM177R | 45 ± 14   | 3.4 ± 1.9 | 31 ± 2** | 84 ± 2** | 60 ± 7** | 12 |

* p<0.05.
** p<0.01.
*** p<0.001.

Fig. 9. Functional characterization of human GlyRs containing βM177R using the EYP assay and electrophysiology. (A) Mean glycine dose–response curves for α1, α1β, α1βM177R and α1ββM177R GlyRs using the EYP assay. The percentage quench is plotted against the applied glycine concentration (μM). Averaged parameters of best fit to dose–response curves are summarized in Table 3. All data points represent the average quench from four experiments comprising three wells each with >200 cells per well. (B) Maximal changes in fluorescence. ΔFmax is the initial fluorescence value minus the final fluorescence value and is represented in fluorescence units. P-values were calculated relative to α1β GlyR heteromers by unpaired t-test: * p<0.05, *** p<0.001. (C) Inhibition by 100 μM lindane. The inhibition of currents activated by 100 μM glycine by 100 μM lindane is represented as a percentage reduction of the control maximal fluorescent quench. P-values were calculated relative to GlyR α1 homomers by unpaired t-test: ** p<0.01, *** p<0.001. (D) Normalized glycine dose–response results using whole-cell patch-clamp electrophysiology for the GlyRs shown in panel E. The normalized maximal currents of cells transfected with the indicated plasmid vectors are plotted against the applied glycine concentration (μM). (E) Sample glycine dose–response traces. Filled bars indicate the applied glycine concentration in μM and non-filled bars the application of 100 μM lindane.
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