GLRB is the third major gene of effect in hyperekplexia

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Glycinergic neurotransmission is a major inhibitory influence in the CNS and its disruption triggers a paediatric and adult startle disorder, hyperekplexia. The postsynaptic $\alpha_1$-subunit ($GLRA1$) of the inhibitory glycine receptor (GlyR) and the cognate presynaptic glycine transporter ($SLC6A5$/$GlyT2$) are well-established genes of effect in hyperekplexia. Nevertheless, 52% of cases (117 from 232) remain gene negative and unexplained. Ligand-gated heteropentameric GlyRs form chloride ion channels that contain the $\alpha_1$ and $\beta$-subunits ($GLRB$) in a $2\alpha_1:3\beta$ configuration and they form the predominant population of GlyRs in the postnatal and adult human brain, brainstem and spinal cord. We screened $GLRB$ through 117 $GLRA1$- and $SLC6A5$-negative hyperekplexia patients using a multiplex-polymerase chain reaction and Sanger sequencing approach. The screening identified recessive and dominant $GLRB$ variants in 12 unrelated hyperekplexia probands. This primarily yielded homozygous null mutations, with nonsense ($n = 3$), small indel ($n = 1$), a large 95 kb deletion ($n = 1$), frameshifts ($n = 1$) and one recurrent splicing variant found in four cases. A further three cases were found with two homozygous and one dominant $GLRB$ missense mutations. We provide strong evidence for the pathogenicity of $GLRB$ mutations using splicing assays, deletion mapping, cell-surface biotinylation, expression studies and molecular modelling. This study describes the definitive assignment of $GLRB$ as the...
third major gene for hyperekplexia and impacts on the genetic stratification and biological causation of this neonatal/paediatric disorder. Driven principally by consanguineous homozygosity of GLRB mutations, the study reveals long-term additive phenotypic outcomes for affected cases such as severe apnoea attacks, learning difficulties and developmental delay.

INTRODUCTION

Hyperekplexia is a severe paroxysmal neuromotor disorder (OMIM 149400) that exhibits both dominant and recessive inheritance of mutations in pre- and postsynaptic glycineric genes (1–4). Although first described only 50 years ago, the molecular genetics that underpins classical hereditary hyperekplexia is better described than most common disorders. Hyperekplexia typically presents soon after birth or in the first week of life, although prenatal interuterine startle episodes are sometimes reported (5). Neonates present with hypertonia and an exaggerated startle response on tactile or auditory stimuli. The generalized hypertonia, which attenuates in sleep, is exacerbated by handling and can trigger recurrent neonatal apnoeas. Startle-induced falls without loss of consciousness and social anxiety in adults are all characteristic of genetically confirmed hyperekplexia. Symptoms for some modulate over time and can be ameliorated with clonazepam and alcohol; however, some patients continue to experience symptoms in adulthood.

Hyperekplexia can be described as a glycine synaptopathy with known mutations residing in the major constituent parts of the inhibitory glycineric system (3). The most frequent mutations are found in the postsynaptic α1 subunit of the inhibitory glycine receptor (GLRA1), where a compendium of mutational findings is observed (1,4,6,7). In the postnatal and adult CNS, GLRA1 forms with the glycine receptor β subunit (GLRB) to form postsynaptic heteropentameric glycine receptor (GlyR) ion channels with a 2α;3β subunit stoichiometry (8,9). These heteropentameric GlyRks are targeted and clustered in the postsynaptic density by the pleiotropic subsynaptic protein, gephyrin (GPHN) via a direct protein–protein interaction with the GlyR β subunit (10–12). A second major gene of effect is the cognate presynaptic Na+/Cl−-dependent glycine transporter (SLC6A5), mutations in which are principally inherited in a recessive or compound heterozygous mode (2,13). Both GLRA1 and SLC6A5 exhibit missense, nonsense, splice-site and frameshift mutations that result in either receptor dysfunction/depletion or compromised glycine reuptake from the synaptic cleft. Hyperekplexia is historically the first disorder of the CNS, which had penetrant manifestations in isolated Korean and Arabic families (19,20) supported our decision to systematically re-examine the well-defined hyperekplexia cohort collected over 20 years (n = 232), 52% of which remains gene negative for GLRA1 and SLC6A5 (n = 117 cases). GPHN and Collybistin (ARHGEF9) have also been associated with isolated hyperekplexia reports (21,22), but they are now regarded as atypical cases and phenocopies of early neonatal hypertonia and excessive startle. ARHGEF9 has strong association with catastrophic degenerative epileptic encephalopathy, mimicking early neonatal hyperekplexia-like symptoms (21,23–26). Meanwhile, GPHN rearrangements and mutations are now linked to molybdenum co-factor deficiency and leukaemia breakpoint disruptions (27–33). Screening of both genes in classical hyperekplexia cases has remained historically negative, and both genes are no longer considered viable candidate genes. In this study, we provide strong evidence for GLRB being a third major gene-of-effect by describing deleterious GLRB mutations in 11 recessive cases and 1 dominant case. The functional consequence of each GLRB mutation was examined, and phenotype studies revealed a significant association with neonatal apnoeas, learning difficulties and developmental delay, thereby providing the basis for further genetic and clinical stratification of hyperekplexia.

RESULTS

Missense, nonsense and splice-site GLRB mutations in hyperekplexia

Multiplex-polymerase chain reaction (PCR) sequencing of GLRB in 117 GLRA1- and SLC6A5-negative patients revealed 8 novel and 1 recurrent variants in 12 index cases (Table 1). Many of the variations displayed homozygous or compound heterozygous inheritance underlying a spectrum of missense, nonsense, deletion and splice-site mutations (Figs 1 and 2). There is one example of a single dominant missense allele that is also found in the homozygous state in another case (Table 1). All GLRB variants were excluded from 200 control DNA samples and were not present in any single nucleotide polymorphism (SNP) databases [SNP database (dbSNP) or 1000 genomes], and in 10 out of 12 cases, the alleles were inherited from consanguineous parental transmission. Phylogenetic alignment demonstrated that the missense variants changed highly conserved amino acids (Fig. 1B). Molecular modelling indicated likely structural defects in GlyR β subunits and heteropentameric models (see below).

Case 2 is the only example of compound heterozygosity in the cohort and joins case 1 (14) that is included only for clinical correlation purposes. Specifically, case 2 carries a
maternally inherited in-frame 3 bp deletion (c.849-852 ΔCCT) causing the removal of a serine residue (p.ΔS262) from the first transmembrane domain (TM1) of the GlyR β subunit (Fig. 1A and C). This is in addition to a de novo stop codon mutation (p.R190X) that originates on the paternally inherited GLRB allele of case 2. A homozygous splice site variant (g.IVS5+5 G>A) was detected in three unrelated cases from India (cases 8, 9 and 10) and one from the UK (case 7) and is a recurrent genotype (15) (Fig. 2A–C). A further three cases of Indo Chinese origin (cases 3, 3* and 11) were homozygous for premature stop codon mutations (p.E24X and p.R450X). Case 6 revealed a complex homozygous 2 bp duplication and 1 bp insertion (g.8_9 dupTT, 8 insA) causing a homozygous frameshift mutation and early premature stop truncation (p.F-19I fs3X) in the signal peptide region of the GlyR β subunit (Fig. 1A). Case 4 displayed a large homozygous deletion encompassing GLRB exons 1–8 and was detected by multiple ligation probe amplification (MLPA) and failure of PCR amplification (Fig. 2D). Two homozygous missense variants were detected with p.P169L in case 5 located in the GlyR β subunit N-terminus and close to residues important for glycine binding and p.Y470C in case 12 situated in the TM4 domain of the GlyR β subunit (Fig. 1A–C). Finally, the only single-hit case of dominant inheritance was observed in a 9-month-old infant with classic hyperekplexia (case 13), who was heterozygous for the p.Y470C variant. Both the homozygous and homozygous Y470C cases have classical hyperekplexia phenotypes, but are not old enough for paediatric neurologists to evaluate developmental milestones (Table 1). A maternal sample was available for case 12 and was confirmed heterozygous for p.Y470C, but has no adult features of hyperekplexia or recollection of infantile/childhood disorders.

Genotype correlation with phenotypic stratification and development

In keeping with hyperekplexia associated with GLRA1 and SLC6A5 mutations, every child demonstrated pathogenic startle, neonatal hypertonia with apnoea attacks and symptoms that were diagnosed within the first week of life (Table 1). These startle episodes are often triggered by tactile or auditory stimuli, but are occasionally spontaneous (such as siblings 3 and 3*). They do not habituate, and the face appears to be an area where tactile stimulation is particularly likely to provoke a reaction. This in turn can lead to problems with weaning and swallowing. The startle episodes can involve myoclonic speed movements of arms, legs and head (predominantly arms) in an upward and outward direction. They can exacerbate the generalized hypertonia that, when focussed on the torso, can lead to prolonged apnoic episodes or a characteristic staccato cry. All cases demonstrated improvement over time; however, the exaggerated startle response persisted in childhood. Of note (cases 2, 5, 6 and 13), these triggered startle episodes were recognized prenatally as ‘recurrent hiccup’s in utero during the last trimester. We can also confirm that clonazepam appears to be as efficacious in GLRB hyperekplexia as in GLRA1 and SLC6A5: 12 cases had taken it and all had a satisfactory or dramatic response from it—although 2 cases stated that the effect wore off over months to years. One case had a satisfactory response from nitrazepam (Table 1). Reflecting the age of the children at last clinical contact, 6 out of 11 reported prominent falls, and these are probably a representation of the startle response once the child is ambulant. The falls are commonly triggered by auditory stimuli and are frequently injurious as the child is unable to protect itself fully when falling stiffly on the floor.

The inheritance pattern is—thus far—autosomal recessive (in keeping with SLC6A5 and the majority of GLRA1 cases). The ethnicities represented are of interest—with 5 out of 11 kindreds of Indian origin and only 3 Caucasian—originating from North-West Europe and the first kindred with Chinese ethnicity. It is predicted that hyperekplexia is recognized clinically in India, and only a tiny minority get referred for genetic testing; however, the disproportionate number of Indian ethnicities is in contrast to previous series. Ten of the children had late motor milestones (not walking at 18 months). Seven cases had learning difficulties (ranging from mild to severe), and two were reported to have generalized tonic clonic seizures in addition to the non-epileptic startle attacks. None had any identified abnormality of brain imaging (seven tested).

There were no reports of dysmorphic features; however, three children (cases 4, 7 and an untested sibling) had congenital dysconjugate gaze disorders. This is of clinical interest because Al-Owain et al. previously identified esotropia, an eye misalignment disorder, as a clinical feature in a Saudi family with a homozygous missense mutation (p.M177R) in GLRB (20).

Splice assay confirms the removal of Exon 5

A homozygous splice-site mutation IVS5+5G>A was identified in four samples (cases 7–10). Previously, a splicing assay on a heterozygous IVS5+5G>A genotype was performed using mini-gene cassettes containing exons 4 and 5 (14) (Fig. 2A). In this study, it was decided to repeat this analysis using patient-specific long-range PCR to capture genomic variation contexts within the 3.5 kb fragment (exons 4–6) and subsequent cloning into a splicing vector. The genomic DNA encompassing exons 4–6 was directly amplified from all four samples with IVS5+5G>A mutation. The exon-trapping assay revealed that the homozygous mutation causes the skipping of exon 5 in transcripts from all four affected samples when compared with the wild type (Fig. 2B). Sequence analysis of the RT-PCR products confirmed the removal of exon 5 and the joining of exons 4–6 of the GLRB transcript (Fig. 2C). In addition, the skipping results in a frameshift mutation generating a truncated protein, p.S176Rfs6X.

Defining the breakpoints of a large 95 kb GLRB deletion

During the multiplex-PCR screening, case 4 failed to generate PCR amplification products for all GLRB exons with exception to exon 9. The presence of a homozygous deletion was validated during MLPA analysis (Fig. 2D), confirming that exons 1–8 had been deleted on both alleles. To further define the extent of the deletion and capture the fusion breakpoint, a multiplex PCR analysis upstream of exon 1 and 5’ UTR regions and within intron 8 was used. PCR products
reappeared 4.3 kb upstream from the exon 1 ‘ATG’ start site (Fig. 2E and F) and 17 kb 3′ of exon 8, revealing a deletion of 95.975 kb. Sequence analysis of PCR products using flanking 5′ and 3′ primers to the breakpoint revealed an identical break-point junction in case 4 and the heterozygous parents (Fig. 2G). Sequence search and comparison tool (BLAST) analysis of the regions surrounding the deletion breakpoint revealed that the rearrangement has occurred within LINE-1 and Mer-8 repeat structures. This is similar to the genomic environment encountered with the recurrent large deletion in GLRA1 (34) and LINE-1 element rearrangements in glrb (spa), generating the spastic mouse model of hyperekplexia (16).

**Functional imaging of live cells reveals reduced maximal GlyR currents**

HEK AD293 cells were co-transfected with either wild-type (WT) or mutated β subunits together with the WT α1

| Case | Origin | Age (in 2012) | Ethnicity | Mode of inheritance | Genotype | Class of mutation | Protein mutation | Onset | Stiff | Startle |
|------|--------|--------------|-----------|--------------------|----------|-------------------|-----------------|-------|------|--------|
| 1°   | Belgium | 15           | Caucasian | Compound Heterozygote | c.G920A (P) IVS5+5G>A (M) | Missense Splice site | p.G229D Loss of exon 5 | 3 h after birth | +     | +     |
| 2    | Germany | 4            | Caucasian | Compound Heterozygote | c.C634T (P) c.849-852 delCT (M) | Nonsense in-frame deletion | p.R190X p.S262 | In utero | +     | +     |
| 3    | Japan   | 25           | Chinese   | Recessive          | c.G136T (H) | Nonsense            | p.E24X          | 18 h after birth | +     | +     |
| 3°   | Japan   | 24           | Chinese   | Recessive          | c.G136T (H) | Nonsense            | p.E24X          | Just after birth | +     | +     |
| 4    | India   | 6            | Indian    | Recessive          | Δex1-8 (H) | Large deletion      | Null            | First week of life | +     | +     |
| 5    | Germany | 3            | Turkish   | Recessive          | c.C572T (H) | Missense           | p.P169L         | In utero | +     | +     |
| 6    | India   | 4            | Indian    | Recessive          | c.8_9 dup(TT), 8_9 ins(A) (H) | Frameshift | p.F-191 fs3X | In utero | +     | +     |
| 7    | UK      | 10           | NR        | Recessive          | IVS5+5G>A (H) | Splice site | Loss of exon 5 and p.S176R fs6X | NR | +     | NR     |
| 8    | India   | 3            | Indian    | Recessive          | IVS5+5G>A (H) | Splice site | Loss of exon 5 and p.S176R fs6X | First week of life | +     | +     |
| 9    | India   | 15           | Indian    | Recessive          | IVS5+5G>A (H) | Splice site | Loss of exon 5 and p.S176R fs6X | First week of life | +     | +     |
| 10   | India   | 7            | Indian    | Recessive          | IVS5+5G>A (H) | Splice site | Loss of exon 5 and p.S176R fs6X | First week of life | +     | +     |
| 11   | Jordan  | 2            | Arabic    | Recessive          | c.G1415A (H) | Nonsense     | p.R450X         | At birth | +     | +     |
| 12   | Jordan  | 9 months°b  | Arabic    | Recessive          | c.A1475C (H) | Missense     | p.Y470C         | 2 weeks | +     | +     |
| 13   | UK      | 10 months°b | Caucasian | Heterozygote dominant | c.A1475C | Missense     | p.Y470C         | In utero | +     | +     |

Each case was diagnosed by neurologists in various international centres and updated for the purpose of this comparison. Case 1 was included for phenotype of consanguineous scenarios. Only cases 1, 2 and 13 are non-consanguineous, and they have compound heterozygosity or a plausible dominant mutation. Developmental delay and are generally responsive to clonazepam.

M, maternal; P, paternal; H, homozygous; NR, not recorded.

°Amino acid signal peptide subtracted.

°Too young for full phenotypic evaluation.

°Nitrazepam therapy used.

°Presented for phenotypic comparison only.

°Siblings.
| Triggered | Falls Neonatal apnoeas | Response to clonazepam | Epilepsy | Squint | MR brain | Improvement over time | Learning difficulties | Motor milestones |
|-----------|------------------------|------------------------|----------|--------|----------|-----------------------|----------------------|---------------------|
| +         | +                      | +                      | No       | NR     | NR       | Yes. Startles to tactile stimuli at age six | No                   | No delay            |
| +         | –                      | Yes                    | ++       | No     | No       | Normal                | Yes                  | Mild learning difficulties — secondary to language delay, Bayley scale 42 | Walked at 19 months |
| +         | –                      | Yes                    | +c       | No, but ocular apraxia | No | NR       | Yes. Startles to tactile stimuli at age seven | Severe learning difficulties | Walked at 2 years |
| +         | –                      | Yes                    | ++       | No, but ocular apraxia | No | NR       | Yes. Startles to tactile stimuli; falls prominent. | Severe learning difficulties | Walked at 18 months |
| +         | +                      | Yes                    | +        | No     | Normal   | Normal                | Yes                  | Mild learning difficulties | Walked at 18 months |
| +         | +                      | Yes                    | +        | No     | No       | CT normal             | Yes. Reduction in startle, spastic gait | Mild learning difficulties | Mild motor delay |
| +         | –                      | Yes                    | Yes      | No     | Normal   | Yes. Tactile stimuli still trigger startles and falls | No                   | Delayed             |
| +         | +                      | NR                     | Yes      | No     | Normal   | Yes. Startle still present | Mild learning difficulties | Delayed             |
| +         | +                      | NR                     | Yes      | No     | Normal   | Yes. Startle still present, mild stiffness | Mild learning difficulties | Delayed             |
| +         | +                      | NR                     | No       | No     | Normal   | At 2.5 years still needs clonazepam and phenobarbital; discontinuation leads to tonic attacks and apnoea recurrence | Yes | Walked at 2 years |
| +         | NR                     | Yes                    | +        | No     | No       | CT normal             | Resolution of apnoeas and tonic attacks with clonazepam | No | No delay |
| +         | NR                     | Yes                    | +        | No     | NR       | Normal               | NR                   | NR                  |

comparison reasons only and is already published (14). The table shows that the majority of cases are homozygous for GLRB mutations and this is largely because The phenotypic data suggest that, beyond the core phenotype, GLRB-affected hyperekplexia cases are associated with learning difficulties, neonatal apnoeas.

subunit. The yellow fluorescent protein (YFP) assay provides an indication of the chloride influx rate (and hence the functional integrity) of the resultant recombinant channels (35). This approach allows for responses to large cell numbers to be averaged, permitting the reliable quantitation of small changes in the expression levels of functional GlyRs. The glycine sensitivity of the receptors on the cell surface was only slightly altered as indicated by the absence of a significant EC$_{50}$ shift (Fig. 3A and B). However, the maximal fluorescence quench was significantly reduced for receptors containing mutated $\beta$ subunits relative to WT subunits, indicating a reduced chloride influx rate (Fig. 3C). For all heteromeric GlyR constructs, the effects were observed independently of the WT GlyR $\beta$ subunit expression. The pore blocker, lindane was applied to test whether the mutated $\beta$ subunits were incorporated into functional GlyRs in the plasma membrane. Lindane inhibits homomeric $\alpha$ subunit GlyRs, but has no effect on heteromeric $\alpha$$\beta$ subunit GlyRs (36). Receptors...
Figure 1. Representation of the case-specific GLRB mutations in this study. (A) Trio pedigrees, mutation panels of GLRB missense and nonsense mutations. Each nuclear family structure is presented along with the sequencing profile reflecting the mutational finding. Case 2 is presented as a compound heterozygote case, whereas the remaining homozygous mutations have a corresponding WT comparator sequence. (B) Missense mutations in cases 5 (p.P169L) and cases 12/13 (p.Y470C) were phylogenetically aligned with GlyR β subunit orthologues showing a highly conserved profile. (C) GlyR β subunit sequence showing hyper-ekplexia mutations. The amino acid sequence is 475 amino acids long after subtraction of the signal peptide region. The novel GLRB mutations in this study are labelled in bold black font. The historical p.G229D mutation (14) is labelled in red, and recent mutations from Lee et al. (19) and Al-Owain et al. (20) are shown in blue. The coloured boxes reflect regions that are transmembrane domains as defined by alignments with C. elegans GluCl (38) with TM1 in red; TM2 in orange; TM3 in blue and TM4 in purple. The underlined region between TM3 and TM4 defines the gephyrin-binding motif within the GlyR β subunit.
resulting from the co-expression of α1 WT and a mutated β subunit exhibited only a slightly reduced sensitivity to lindane, indicating that either the heteromeric GlyRs with mutated β subunits are not functional or that mutated subunits are not expressed at the cell surface (Fig. 4A).

**Cell-surface biotinylation assays demonstrate trafficking outcomes**

Hyperekplexia GlyR β mutants, p.P169L and p.ΔS262, p.R450X and p.Y470C were further investigated for alterations in surface expression. Biotinylation of cell-surface proteins revealed that the reduction in the number of functional heteromeric GlyRs channels observed in the lindane assay is because of the decreased cell-surface expression of mutant GlyR β subunits (Fig. 4B). The whole-cell expression of the mutants was comparable to that of the WT GlyR β subunit. However, the cell-surface expression levels were significantly decreased for all four mutants (<30% of wild type) (Fig. 4C). Similarly, trafficking defects were the major mechanism under- rescuing GlyR α1 mutations in a previous study (4).

**Molecular modelling predicts protein-damaging effects of GLRB mutations**

We next sought to model the molecular mechanisms by which the mutations affected GlyR function (4,15,37). WT and mutant subunit monomers and α/β pentamers were modelled based on homology with the GluCl (38) template (Fig. 5). p.P169L alters highly conserved central proline residue of the neurotransmitter-gated ion-channel signature Cys-loop, CPLDLTLFPMDTQRC (Fig. 5B). The p.P169L mutation also leads to minor changes elsewhere in the conformation of the extracellular domain and also minor changes in the orientation of TM2 (Fig. 5B). Thus, the p.P169L mutation shows a cumulative impact on ion channel conformation, demonstrating a widening of the channel. The distance across the pore between A274 of the opposing GlyR β subunits is measured at 7.14 and 6.89 Å, respectively. The substitution also results in the introduction of contacts between L169 and Y252 (2.95 Å) and L307 (3.15 Å), which do not exist for the WT GlyR β subunit. This results in the forcing of Y252 towards the pore (Fig. 5C). In the WT protein, S262 maintains hydrogen bonds (shown as blue lines), longitudinal packing, lead to wider orientation changes to the transmembrane regions and constriction of the channel at positions 273 and 274.

Lastly, the p.Y470C mutation does not introduce new disulphide bridging because of the peripheral location of Y470 and the absence of any proximal sulphhydryl groups from adjacent regions. There is no notable change in the immediate contacts of C470 when compared with Y470, but the mutation leads to local loss of helical structure at the end of TM4 near C470 and a change in the orientation of TM4 and consequent impacts on the orientation of all four transmembrane regions towards their cytoplasmic ends (arrows). This leads to a consequent projection of S273 and A274, which leads to the predicted constriction of the pore. The extracellular domain remains largely unaffected. The pentameric arrangement of the channel incorporating homozygous Y470C mutation reveals a notable narrowing of the chloride pore (Fig. 5D). The distance across the pore between A274 of the opposing β subunits is measured at 5.86 and 5.67 Å, respectively, a reduction of over 0.6 Å when compared with the WT pentamer.

**DISCUSSION**

This study reveals that GLRB is the third major gene-of-effect in hyperekplexia and that cases have a very high incidence of learning difficulties and developmental delay. Despite isolated descriptions in 2002 and 2012 (14,19,20), our greater compendium of mutations unequivocally confirms that GLRB joins GLRA1 and SLC6A5 to make a trinity of diagnostic genes for this paroxysmal neuromotor disorder. Using a cohort screening approach of 117 cases, we also confirm that GLRB-mediated hyperekplexia is predominantly inherited through recessive or compound heterozygote transmission, very similar to SLC6A5-mediated hyperekplexia and something similar to GLRA1-mediated hyperekplexia (2–4,13). Complex homozygous GLRB genotypes have emerged, including in-frame deletions, a two-hit duplication/insertion event, large repeat-mediated genomic deletions and splice-site changes (cases 7, 8, 9 and 10). They clearly differ from the gel migration of WT controls and display a size difference that reflects the approximate contribution of exon 5 to transcripts. To confirm this, the gel bands were sequenced and the results, seen in C, confirm the removal of exon 5 from the PCR products resulting in a fusion of the end of exon 4 with the start of exon 6. (D–G) A large 97.5 kb deletion in GLRB encompassing the promoter, 5'UTR and exons 1–8. MLPA analysis of case 4 displayed a diploid knockdown of probe signal covering exons 1–8 which was confirmed by the inability to PCR amplify this region of GLRB, with only exon 9 being responsive to MLPA probes and PCR amplification. This placed the 3’ breakpoint in the intron 8 region; however, it was uncertain how far the 5’ breakpoint had extended until new PCR amplifiers (red, yellow and blue) were placed in a stepwise manner leading into distal 5’ regions. The gel illustration shows how the WT sample has all three PCR bands (as expected) while the DNA from case 4 displayed a 217 bp PCR band corresponding to the most distal 5’ PCR. Sequencing primers placed in the 5’ and 3’ distal ends of the homozygous deletion produced a fusion product that was patient specific, and sequencing revealed the breakpoints that occurred 4.35 kb upstream from the GLRB ‘ATG’ start site and within GLRB intron 8, thus confirming a homozygous 95.7 kb deletion.

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**Figure 2.** Splice-site mutations and large deletions in GLRB. (A–C) Patient-derived splice-site assay confirms loss of exon 5 from GLRB IVS5+5G>A transcripts. A shows the exon arrangement of exon 5 surrounded by flanking exons 4 and 6. Overlaid are the transcript webs based on WT and aberrant IVS5 splicing. B demonstrates the RT-PCR products from four patients with homozygous IVS5+5G>A changes (cases 7, 8, 9 and 10). They clearly differ from the gel migration of WT controls and display a size difference that reflects the approximate contribution of exon 5 to transcripts. To confirm this, the gel bands were sequenced and the results, seen in C, confirm the removal of exon 5 from the PCR products resulting in a fusion of the end of exon 4 with the start of exon 6. (D–G) A large 97.5 kb deletion in GLRB encompassing the promoter, 5'UTR and exons 1–8. MLPA analysis of case 4 displayed a diploid knockdown of probe signal covering exons 1–8 which was confirmed by the inability to PCR amplify this region of GLRB, with only exon 9 being responsive to MLPA probes and PCR amplification. This placed the 3’ breakpoint in the intron 8 region; however, it was uncertain how far the 5’ breakpoint had extended until new PCR amplifiers (red, yellow and blue) were placed in a stepwise manner leading into distal 5’ regions. The gel illustration shows how the WT sample has all three PCR bands (as expected) while the DNA from case 4 displayed a 217 bp PCR band corresponding to the most distal 5’ PCR. Sequencing primers placed in the 5’ and 3’ distal ends of the homozygous deletion produced a fusion product that was patient specific, and sequencing revealed the breakpoints that occurred 4.35 kb upstream from the GLRB ‘ATG’ start site and within GLRB intron 8, thus confirming a homozygous 95.7 kb deletion.
mutations resulting in exon skipping—in addition to more qualitative missense mutations that have compromised cell-surface expression. From our extended cohort of 232 hyperekplexia cases, we know that 35% have GLRA1 mutations, 13% have SLC6A5 mutations and now 6% (13/232) have GLRB mutations. Collectively, this means that 46% of hyperekplexia cases referred to our centre are without a genetic explanation, and the search for new loci has taken a new urgency in the wake of next-generation sequencing.

For most of our genotypes, the functional consequence is the creation of GLRB-null alleles generated from homozygous stop codons (p.E24X, p.R190X and p.R450X), a large 95 kb deletion (Δexons 1–8), complex signal peptide frameshifts (p.F-19I fs3X) and four instances of IVS5+5 G>A (+ p.S176Rfs6X) resulting in the selective removal of exon 5 in the majority of GLRB transcripts and a frameshift. For each of these null cases, the biological consequences begin as the fetal α2 homopentameric GlyRs are replaced in a programmed developmental switch with heteropentameric α1β GlyRs in the third trimester. The consequences include: (i) the unavailability of α1β, or indeed α2β and α3β, heteropentameric GlyRs in the CNS, (ii) the absence of gephyrin-mediated clustering and anchoring by virtue of the lack of GlyR β subunit–gephyrin interactions, (iii) severely compromised glycineergic neurotransmission in the brain stem and spinal cord consistent with spastic (glrb<sup>−/−</sup>) mouse model and (iv) a hyperekplexia phenotype (including apnoeas), but with clinical features that outlast infancy such as learning difficulties and developmental delay.

It would be fascinating to observe if any biological contingency is mediated by α<sub>1</sub>_homopentameric compensation or upregulation of gamma aminobutyric acid (GABA)ergic neurotransmission in response to a null GlyR (α1β, α2β, α3β) environment. It is also unknown how this may also affect developing neural networks during embryogenesis, fetal development, infancy and childhood. Whatever the effect is, we can exclude global brain malformations as indicated by normal MRI, and only a minority have co-morbid epilepsy. However, we cannot exclude that GLRB-null status may have subtle effects on neural networks (39) or result in the reduction in glycineergic specification (40) or cause compromised activity-driven synapse formation (41). GlyRs have a role in most brain regions, including the cortex, hippocampus, amygdala and co-localize with GABA receptors at inhibitory interneurons, despite the reputation of predominant localization in the brainstem and spinal cord (42–45).

It is clear from our phenotypic observations that a GLRB-null status has clinical outcomes that persist after infancy, despite the hypertonia resolving and the exaggerated startle attenuating. There is a degree of developmental catch up seen—particularly for those who do not have co-morbid learning difficulties. For those with long-term effects, it is generally unknown whether this is a direct effect of fetal and neonatal neural development or a window of physical brain damage from repeated neonatal apnoeas and severe hyper-
tonia. A further limitation to proving a definitive phenotype correlation is based on the influence of consanguineous inheritance in many of our GLRB cases. In these cases, homozygosity across haplotype blocks will conspire with the genome-wide loading of deleterious gene variation to modulate the presented phenotype. However, it is noteworthy that non-consanguineous compound heterozygous cases (cases 1 and 2) have very similar presentation and developmental outcomes.

Our functional characterization revealed that the pathogenic effects of all mutations were because of a failure of the GlyR β subunit to reach the cell surface. This in turn led to a reduction in the overall level of functional expression of α₁ subunit-containing GlyRs (Fig. 3C). Molecular modelling revealed that p.P169L, p.ΔS262 and p.Y470C all induced non-specific alterations in the β-subunit structure that compromised either the assembly, trafficking or surface stability of heteromeric GlyRs. It has recently been suggested that the profound absence of GlyR β subunit hyperekplexia mutations that work by affecting GlyR gating is due to the β subunit having only a minimal role in the gating process (15). The findings of the present study strongly support this mechanism by providing evidence only for β subunit mutations that disrupt the expression, not the gating, of heteromeric GlyRs.

What do animal models tell us? spastic (glrb<sup>hpa</sup>) is a spontaneously occurring homozygous LINE-1 insertion mutant mouse with aberrant splicing of glrb, which severely reduces expression of the glrb mRNA and causes aberrant splicing of remaining transcripts, causing an 80% reduction in functional GlyRs (16,17). The animals show symptoms in response to handling, but this behaviour can also occur spontaneously and consists of rapid tremor, stiffness of posture and difficulty in righting when placed on the back. For the null human cases, this model is the most analogous situation from a molecular and phenotypic point of view. It is, therefore, interesting that glrb<sup>hpa</sup> mice have two separate symptomatic fates, where some littersmates recover from the spastic phenotype, whereas others become severely affected by a degenerative excitotoxic mechanism (46). The recovering littersmates have a much higher GlyR α₁ subunit expression profile, whereas degenerative littersmates have a preferential loss of glycinergic/GABA interneurons and much lower α₁ subunit expression. It would be interesting to assess whether similar compensatory expression of the GlyR α₁ subunit occurs in human cases or whether the developmental profile is generated by the loss of inhibitory influences on brain development and a failure to initiate neuronal remodelling. There is also the unquantified effect of glycine on excitatory NMDA glutamate receptors and how that may modulate the phenotype. Bandoneon is a subunit mutants (p. P169L, p. ΔS262, p. R450X and p. Y470C) were expressed at similar levels in whole-cell protein expression when compared with WT GlyR (total). However, all four mutations revealed markedly reduced cell-surface protein expression (surface) when compared with WT GlyRs. One-hundred micrograms of protein lysates were loaded into each lane. (C) Quantification of cell-surface and whole-cell expression of the GlyR β subunit mutants using ImageJ software, with values expressed as a percentage of the WT GlyR β subunit expression. *P-values were calculated relative to the WT GlyR β subunit expression using an unpaired t-test; ***P < 0.001.

Figure 4. Expression level of GlyR β subunit mutations. (A) Inhibition of glycine EC<sub>50</sub> currents by 100 μM lindane. The level of incorporation of β subunits into functional GlyR receptors membrane was analysed using lindane that inhibits homomeric α GlyRs, but not heteromeric αβ GlyRs. The inhibition of glycine currents is represented as a percentage of reduction in the control current magnitude. (B) Cell-surface biotinylation assays on heteromeric α₁β GlyRs expressed in HEK293 cells revealed that all GlyR β subunits are expressed at similar levels in whole-cell protein expression when compared with WT GlyR (total). However, all four mutations revealed markedly reduced cell-surface protein expression (surface) when compared with WT GlyRs. One-hundred micrograms of protein lysates were loaded into each lane. (C) Quantification of cell-surface and whole-cell expression of the GlyR β subunit mutants using ImageJ software, with values expressed as a percentage of the WT GlyR β subunit expression. *P-values were calculated relative to the WT GlyR β subunit expression using an unpaired t-test; ***P < 0.001.
zebrafish model with missense mutations in glrb with touch-induced uncoordinated contraction of trunk muscles (18). This may represent a better model for high-throughput drug trials in startle phenotypes, although realistically rare disorders rely on mainstream drugs targeted at more common disorders (anti-epileptic drugs), rather than orphan drugs aimed at true disease modification.

Why was there an underreporting of GLRB mutations in the literature? This can be attributed to several methodological developments in molecular genetics. In previous studies (7,14), our laboratory concentrated heavily on heteroduplex platforms such as single-strand conformation polymorphism, dideoxy fingerprinting and denaturing HPLC that would retrospectively and inadvertently select against recessive variants and present a selection bias towards dominant and compound heterozygote mutations. It became apparent following Sanger sequencing gene-negative cases and using Mutation SurveyorTM software that more recessive GLRA1 and SLC6A5 mutations were being detected (4,13). On this basis, a complete resequencing of GLRB was justified resulting in the findings presented in this study. What is the future for hyperekplexia research? As we approach a level of genetic and phenotypic stratification, we can better design studies based on the characterization of the symptoms, interventions, neurophysiological and neuropsychological features. In particular, how can we prevent the developmental consequences of defective glycineric neurotransmission and how can this be targeted at early life? There is also the gene-negative status of many cases in our cohort (n = 105), where the audit of clinical progression and application of genome or exome sequencing is the next logical step. This may highlight new gene networks or even restrict all genetic forms of hyperekplexia to within the glycineric proteome.

In conclusion, this study confirms that GLRB mutations cause hyperekplexia in 6% of the patient population and that GlyR deficiency or dysfunction is the fundamental basis of the disorder. This further differentiates hyperekplexia into a hierarchical genetic framework and aids the diagnostic stratification required to develop specific neonatal and paediatric intervention strategies and downstream genetic counselling approaches concerning recurrent risk. There remains a substantial population of gene-negative cases in this cohort, and these are now primed for copy number variation analysis and next-generation sequencing scrutiny, and it will be fascinating to see if any other gene

Figure 5. Structural modelling of GlyR β subunit mutants. (A) WT GlyR β subunit monomer (left) showing the position of the cys-loop in orange (red arrow), along with the positions of the key pore-bordering residues S273 (dark red) and A274 (grey); and in pentameric arrangement viewed from the extracellular side (right), showing α3 subunits in green and β subunits in blue and the pore forming residues in spacefill, P250 (purple), A251 (grey) on α3 subunits and S273 (dark red) and A274 (grey) on β subunits. The distance across the pore between A274 of the opposing β subunits is measured at 6.54 and 6.28 Å, respectively. (B) P169 is located within the cys-loop (left), and pentameric form (2α/3β) of WT α1 and β subunit P169L mutant shows conformational changes in the extracellular domain and the orientation of TM2. (C) AS262 leads to the loss of helical structure at point of deletion (red arrow, TM1 domain) and subsequent (slight) change in the orientation of the channel lining TM2 helix. (D) Superposition of GlyR β C470 (pink) on the WT Y470 form (left). S273 in purple (β C470) subunit or in brick red (WT) and A274 in white (C470 variant) or in grey (wild type). Y470 causes a loss of helical structure from S472 to L475 in the extracellular part of TM4, which may affect the local membrane topology. The pentameric arrangement of GlyR with the β subunit Y470C mutant leads to a narrowing of the pore region (right).
networks/proteomes are implicated in this pathology of the startle reflexes and related disorders.

MATERIALS AND METHODS

Clinical ascertainment and assessment

Our method of case ascertainment, screening and phenotype selection is described elsewhere (5,47), and ethical approval for the project was granted by the Local Research Ethics Committee and the South West Wales Regional Ethics Committee. Clinical data capture was via a structured research questionnaire, and a review of medical notes was available (see Supplementary Material, Information S1). This information was transferred into a hyperekplexia database that contained the details of 232 cases that we have ascertained over 20 years. This database was used to compile the clinical presentation and disorder profiles of the GLRB cases in conjunction with clinical updates from all referring centres.

Molecular genetics

We tested 117 hyperekplexia cases for variation in GLRB. All cases had previously been tested for GLRA1 and SLC6A5 variation (Sanger sequencing) as part of our research-based screening programme. Multiplex-PCR (Qiagen, UK) was adopted to rapidly amplify GLRB coding regions and splice sites, and PCR amplimers were Sanger sequenced with ABI™ capillary technology (Foster City, USA). Population studies of newly identified GLRB variants were performed using panels of commercial controls (Sigma, UK) using modalities like LightScanner™ high-resolution melting (Idaho Technology, USA) or restriction fragment length polymorphism analysis. Bioinformatic databases were scrutinized [dbSNP, a prediction website to see if amino acid substitution affects protein function, Polyphen and Grantham scores] to indicate protein-damaging consequences or to establish phylogenetic conservation. In addition to Sanger sequencing, all DNA samples were screened for large deletions or insertions in GLRB, GLRA1 and SLC6A5 using a MLPA DNA detection kit, following the manufacturer’s protocol (MRC-Holland, UK).

Deletion breakpoint analysis

To determine the breakpoint of the large homozygous deletion involving exons 1–8, 12 sets of primer pairs were designed to represent 1–2 kb steps within regions adjacent to the 5′UTR and the intronic regions between exons 8 and 9. Multiplex PCR results of the affected case were compared with control profiles to locate the boundaries of the homozygous deleted regions. Based on the multiplex PCR results and analysis, the junctional PCR results were captured using the following primer set: forward 5′ TCCTTCAGATAGCATTAGCTACAG 3′ and reverse 5′ TCCTTCAGATAGCATTAGCTACAG 3′. The exact breakpoint was determined by Sanger sequencing analysis of the junctional PCR product.

Exon splicing assay

The aberrant splicing of IVS5+5G>A was described previously (14) using an in vitro minigene system containing exons 4 and 5 of GLRB. In the present study, this was repeated using a direct patient-based genomic PCR approach to capture the homozygous variant context. The genomic region encompassing exons 4–6 of GLRB (3.3 kb) was PCR amplified in affected cases (cases 7–10) and a control DNA using primer sets containing Xhol/XbaI restriction sites: forward primer (5′-TCCTTCAGATAGCATTAGCTACAGTC-3′), reverse primer (5′-ATATCTAGAATGGGAGATGG 3′). PCR products were digested with Xhol and XbaI and cloned into an exon trapping (pET) vector (MoBiTec, Germany). pET vectors were then transfected into HEK293 cells using a magnetofection method in combination with CombiMag (OZ Biosciences, UK) and Lipofectamine 2000 (Invitrogen, UK). Following 24 h, total RNAs were isolated using a RNeasy kit (Qiagen, UK) based on the manufacturer’s protocol. One microgram of total RNA was reverse transcribed using SuperScript III kit (Invitrogen, UK) and PCR amplified using primer sets supplied with the Exontrap assay kit. PCR products were analysed on 2% (w/v) agarose gels and purified PCR products were sequenced.

Mutagenesis and expression of constructs

Full-length human GlyR α1 and β subunits were cloned into the vector pRK5 and the pRES2-EGFP plasmid vector (Clontech, USA), respectively (4). Mutations were introduced into pRES2-GlyR β using the QuikChange site-directed mutagenesis kit (Stratagene, UK). All expression constructs were validated by sequencing the entire open-reading region. WT or mutant GlyR β subunits were transiently expressed in human embryonic kidney (HEK293) cells using the magnetofection method or a calcium phosphate technique.

Fluorescence-based imaging

Cells were imaged with an automated fluorescence-based screening system using YFP-152L fluorescence quench as an indicator of anion influx rate (35). Briefly, HEK AD293 cells were transfected with the plasmid DNAs for WT and mutated GlyRs (at a ratio of α1:1β) and with the DNA for YFP as described in the Results section, plated into 384-well plates and used for experiments 24–32 h later. Around 30 min before experiments were commenced, cells were superfused by Ringer’s 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). 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 and > 200 cells per well. To determine the glycine dose–response curve, an empirical three parameter Hill equation was fitted by a non-linear least squares algorithm using the SigmaPlot 11.0 software.

Cell-surface biotinylation

Between 24 and 32 h after transfection, surface expression of GlyR β subunits in HEK293 cells was investigated using a cell membrane-impermeable reagent Sulfo-NHS-LC-Biotin.
(Pierce Biotechnology) as previously described (4). GlyR α and β subunit expression constructs were transfected at a DNA ratio of 1:10 to maximize the formation of heteromeric GlyRs (9,48). Proteins in whole-cell lysates or cell-surface fractions were analysed by western blotting with an antibody against the GlyR β subunit (1:90; Millipore and 1:100; Santa Cruz, USA). An anti β-actin antibody (1:5000; AbCam, UK) was used as a control to confirm that intracellular proteins were not labelled with biotin. The intensity of the immunoreactivity signal was quantified with the ImageJ software (http://rsb.info.nih.gov/ij). Datasets were analysed using GraphPad Prism (version 3.02, GraphPad Software Inc.) and expressed as mean ± SEM. Statistical significance was determined by Student’s t-test.

Bioinformatics and structural modelling

Structural modelling of GlyRs was carried out using a homology modelling pipeline built with the Biskit structural bioinformatics platform, which scans the entire Protein Data Bank (PDB) for candidate homologs (37). The best match attained for GlyR subunits was based on 44% (GlyR α) and 39% (GlyR β) identity with the protein sequence of the Cae-norhabditis elegans glutamate-gated chloride channel (GluCl; PDB:3RHW) (38). The pipeline workflow incorporates the NCBI tools platform, including the BLAST program for similarity searching of sequence databases. T-COFFEE was used for alignment of the test sequence with the PDB template, and homology models were generated over 10 iterations of the MODELLER program (4). All models were visualized using the molecular graphics program Chimera.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. The authors declare that they have had no conflicts of interest arising from the publication of this paper.

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