

# Genotype-phenotype correlations in hyperekplexia: apnoeas, learning difficulties and speech delay

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**Congenital hyperekplexia is a rare, potentially treatable neuromotor disorder. Three major genes of effect are known, and all three affect glycinergic neurotransmission. Two genes encode for subunits of the postsynaptic inhibitory glycine receptor, *GLRA1* encoding the  $\alpha_1$  subunit and *GLRB* encoding the  $\beta$  subunit. The third, *SLC6A5*, encodes the cognate presynaptic glycine transporter 2. Ninety-seven individuals had a clinical diagnosis of hyperekplexia confirmed by genetic testing: 61 cases had mutations in *GLRA1*, 24 cases in *SLC6A5* and 12 in *GLRB*. Detailed retrospective clinical analysis ascertained that all gene-positive cases present in the neonatal period (occasionally prenatally) and that clonazepam is the treatment of choice (95% found it to be efficacious). We confirm that hyperekplexia is predominantly a recessive condition but dominant cases are seen (16%). We found no genetic evidence for 'major' or 'minor' forms of hyperekplexia on a population basis. Thirty-five gene-negative cases were studied for comparison, their cardinal feature was presentation after the first month of life ( $P < 0.001$ ). In addition to the characteristic 'stiffness, startles and stumbles' of hyperekplexia, apnoea attacks (50 of 89) and delayed development (47 of 92) were frequently reported. Patients with *SLC6A5* mutations were significantly more likely to have had recurrent infantile apnoeas (RR1.9;  $P < 0.005$ ) than those with *GLRA1* mutations. Patients with *GLRB* and *SLC6A5* mutations were more likely to have developmental delay (RR1.5  $P < 0.01$ ; RR1.9  $P < 0.03$ ) than those with *GLRA1* mutations; 92% of *GLRB* cases reported a mild to severe delay in speech acquisition. Molecular modelling of pathogenic mutations demonstrates specific patterns of protein disruption that can be used to predict phenotype severity. The developmental delay in hyperekplexia, and speech acquisition in particular, may represent failure of developmental neural networks or subtle neurogenic migration defects in the absence of presynaptic glycine release. We recommend early genetic testing for symptomatic neonates and possibly preconception counselling for those at risk for *GLRB* and *SLC6A5* mutations, because of the more challenging phenotype.**

**Keywords:** hyperekplexia; genetics; phenotype; correlation; treatment options

**Abbreviation:** GlyR = glycine receptor; GlyT = glycine transport

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## Introduction

Hyperekplexia is a neurogenetic condition characterized by hyper-tonia that is predominantly seen in the trunk or lower limbs and easily-provoked startle responses to tactile or acoustic stimuli (Harvey *et al.*, 2008; de Koning-Tijssen and Rees, 2009; Thomas *et al.*, 2010). The generalized stiffness can be prominent enough to permit infants to be rotated between the vertical and horizontal plane with barely a change in posture (Suhren *et al.*, 1966). These startle attacks can be prolonged, with many minutes of axial rigidity. It is a rare condition with <100 published cases confirmed by genetic testing since the first gene of effect (*GLRA1*) was described (Shiang *et al.*, 1993). Surprisingly, for such a clinically recognizable condition, classical (congenital) hyperekplexia was first described as late as 1958 by Kirstein and Silfverskiöld, with exaggerated hypnagogic jerks in addition to sudden falls. Additional features were linked to this unidentified hereditary condition, including epilepsy in some affected family members and sensitivity to alcohol and phenobarbitone, which attenuated the startle response (Kok and Bruyn, 1962; Suhren *et al.*, 1966; Gastaut and Villeneuve, 1967). Periodic limb movements in sleep and a characteristic head-retraction response complete the phenotype (Bakker *et al.*, 2009). Onset of symptoms after infancy should prompt investigations for an acquired cause—pontine injury (Bakker *et al.*, 2006; Davies *et al.*, 2010) or rarely, anti-GlyR antibodies (Hutchinson *et al.*, 2008; Mas *et al.*, 2011). This is an important distinction, as treatment for classic hyperekplexia is based on medication to combat hypertonia and startle rather than true disease modification.

Hyperekplexia is predominantly caused by mutations in the genes encoding the postsynaptic inhibitory glycine receptor (GlyR; *GLRA1* and *GLRB*) and presynaptic glycine transport (GlyT2; *SLC6A5*) (Shiang *et al.*, 1993; Rees *et al.*, 1994, 2006; Chung *et al.*, 2010, 2013; Carta *et al.*, 2012; James *et al.*, 2013). There is rare (<1% of cases), but recognized genetic heterogeneity through association with mutations in gephyrin (*GPHN*; Rees *et al.*, 2003), and collybistin (*ARHGEP9*; Harvey *et al.*, 2004). The phenotypes associated with these mutations are more devastating: homozygous deletions or missense mutations in *GPHN* (also associated with molybdenum co-factor deficiency) are characterized by untreatable seizures and neonatal death. GlyRs are heteropentameric ( $2\alpha_1:3\beta$ ) inhibitory ligand-gated ion channels that facilitate fast chloride-mediated responses predominantly in the brainstem and spinal cord (Grudzinska *et al.*, 2005; Yang *et al.*, 2012). By contrast, GlyT2 is a sodium and chloride dependent transporter, which is involved in the reuptake of glycine from the synaptic cleft into glycinergic neurons, thereby maintaining the pool of glycine for presynaptic vesicular replenishment. Hyperekplexia has some notable landmarks: (i) the description of mutations in *GLRA1* by Shiang *et al.* (1993) was the first channelopathy associated with ligand-gated ion channels; (ii) mutations in *SLC6A5* (Rees *et al.*, 2002) defined the first neurological disorder conclusively linked to a defect in presynaptic transporter for a classical fast-acting neurotransmitter; and (iii) the first demonstration of gain-of-function, tonic activation of the GlyR channel as a novel disease mechanism in ligand-gated ion channels (Chung *et al.*, 2010). Similar phenotypes in mice, cattle and dogs have been described with mutations

in GlyR  $\alpha_1$  subunit,  $\beta$  subunit and GlyT2 genes (Gomez *et al.*, 2003; Harvey *et al.*, 2008). These have helped to guide the choice of candidates for human gene screening.

The mainstay of treatment in hyperekplexia has been clonazepam, an allosteric potentiator of GABA<sub>A</sub> receptors, which is efficacious for patients with *GLRA1*, *GLRB* and *SLC6A5* mutations (Andermann *et al.*, 1980; Ryan *et al.*, 1992; Bakker *et al.*, 2009; Chung *et al.*, 2010, 2013; Carta *et al.*, 2012; James *et al.*, 2013). Although potentially treatable, hyperekplexia is not necessarily a benign condition; there have been reports of an increased incidence of sudden infant deaths in hyperekplexia families and startle attacks when ambulant (combined with a variable hypertonic spastic gait) can lead to sudden falls and subsequent head injuries and fractures (Giacoaia *et al.*, 1994). It remains unclear whether the sudden deaths are all attributable to hypertonic/apnoeic attacks, or whether the children who died prematurely had even inherited the familial mutation(s). As one of the global reference centres for hyperekplexia diagnosis and genetics, we wanted to ascertain whether there may be genotype–phenotype correlations in hyperekplexia.

## Materials and methods

Since 1994, we have received referrals for 230 families where a clinical diagnosis of hyperekplexia has been suspected. Our screening process, referral criteria and review procedure for gene-negative patients are described elsewhere (Harvey *et al.*, 2008; Davies *et al.*, 2010). A strict narrow definition of hyperekplexia was used when identifying the gene-negative cases and cases were not included if they had anything more than non-specific abnormalities at MRI. The mutations were identified through Sanger sequencing of the exons, splice sites and flanking DNA sequence up to 40 bp after the splice site. All samples were screened for large insertions and deletions through MLPA (multiplex ligation-dependent probe amplification) analysis in all three genes. Clinical ascertainment was reviewed and approved by the Local Research Ethics Committee, the South West Wales REC. We have used a range of functional analyses for estimating pathogenicity as previously published (Rees *et al.*, 2002, 2003, 2006; Chung *et al.*, 2010, 2013; Carta *et al.*, 2012). Structural modelling of GlyR  $\alpha_1$  and GlyT2 wild-type and variant proteins was performed using a homology modelling pipeline (<http://membraneproteins.swan.ac.uk/modelling/>), assembled with the Biskit structural bioinformatics platform (Grünberg *et al.*, 2007), which scans the entire Protein Data Bank for candidate homologues, as described in Chung *et al.* (2010). The receptor homology modelling has recently improved with publication of the GluCl homologue for GlyR  $\alpha_1$  providing 50% homology (69% sequence coverage), which permits us to describe novel associations in this study (Hibbs and Gouaux, 2011).

We contacted all referring clinicians of patients with a positive genetic diagnosis using a standard *pro forma* to establish both a standardized quality data set and to acquire a clinical update on the individual cases (Supplementary material). In addition 35 unresolved cases were included for comparison. The *pro formas* included details of reported co-morbidities (such as neonatal apnoeas and seizures) and theoretically possible complications such as anxiety or deafness. Data were enhanced with information from clinic letters and in some cases the authors interviewed and examined the patients. We analysed the data by comparing *GLRA1*, *GLRB* and *SLC6A5* mutations, dominant versus recessive or compound heterozygous inheritance and sporadic versus

familial cases. When comparing characteristics we used a single typical index case from each family. The chi-squared test was used using Yates' correction when needed.

## Results

One hundred and ten cases positive for *GLRA1*, *GLRB* or *SLC6A5* mutations were identified from our database and we received *pro forma* replies for 94 cases (86% return). The sex mix was equal and the cases ranged from 2 years 5 months to 47 years 3 months at time of last contact (mean age 14 years and 4 months). A further 35 gene-negative cases were included for comparison. The cases with no *pro forma* return were often referred >10 years ago and the custodian clinicians (and in some cases, the institutions) were no longer clinically active. The non-repliers were proportionate. Three additional cases were obtained from colleagues in Liege, Belgium. There were 61 cases with *GLRA1* mutations [14 dominant missense (23%), 24 recessive missense (39%), 23 recessive nonsense (38%)]; 24 with *SLC6A5* mutations [eight recessive missense (33%), 14 recessive nonsense (58%) and two not confirmed (8%)]; and 12 *GLRB* mutations [one dominant missense (8%), two recessive missense (17%), nine recessive nonsense (75%)] (Table 1).

### Phenotypic characteristics

Every patient exhibited an exaggerated startle in response to tactile or auditory stimuli; whereas the pattern of hypertonia was surprisingly varied. All *GLRB* cases demonstrated clear hypertonia—compared with *GLRA1* (74%,  $P < 0.05$ ) and *SLC6A5* cases (84%, not significant) with many cases explicitly having normal or reduced tone (Table 1). Every gene-positive case had symptoms from birth, indeed some exhibited startle *in utero* during the last trimester—in contrast, 54% of the gene-negative cases had onset after the first month of life, often into childhood ( $P < 0.001$ ). Recurrent injurious falls were common (>50% of the gene-positive cases) in contrast with the gene-negative group where it was reported in less than a quarter ( $P < 0.001$ ). Seizures were a definite feature in seven cases and probable in another five: all 12 cases were due to recessive mutations. This produces an uncorrected prevalence estimate of epilepsy in hyperekplexia of 7–12%. No children were reported as having cardiac arrhythmias, autonomic abnormalities, metabolic deficiencies or hearing difficulties. There were sporadic reports of ocular apraxia and congenital extraocular eye movement disorders (all *GLRB* cases). We received many reports of hypersalivation and relative failure to thrive. Oro-facial tactile stimulation of feeding appears to be a potent trigger for hypertonic attacks, which can result in failure to thrive.

### Apnoeas and learning difficulties

Recurrent apnoea attacks are commonly seen in hyperekplexia ranging from a feature of a third of dominant *GLRA1* cases, to 80% of those with *SLC6A5* and 89% *GLRB* mutations (*GLRA1* versus *SLC6A5*  $P < 0.004$ , *GLRA1* versus *GLRB*  $P < 0.03$ ). There is a striking difference between the prevalence of apnoea attacks in recessive *GLRA1* hyperekplexia; missense mutations are associated with 71% of cases and nonsense mutations with 22%

( $P < 0.0002$ ). Delays in gross motor and speech acquisition were commonly reported—with a striking gene of effect pattern seen. Eight of 12 children with *GLRB* mutations reported motor delay and 11 of 12 had delays in speech acquisition (*GLRA1* versus *SLC6A5* RR1.5,  $P < 0.01$ , versus *GLRB* RR1.9,  $P < 0.02$ ). Global developmental delays were predominantly a feature of the recessive hyperekplexias—but observed twice as frequently in *GLRB* and *SLC6A5* (where they were equivalent at >80%) than in recessive *GLRA1*. There is a stark difference between this finding and the reported rate of learning difficulties at 8% in dominant *GLRA1* (the first reported cases).

Twenty-one kindreds had learning difficulties and recurrent apnoea attacks, nine of which had *SLC6A5* mutations and seven with *GLRB* mutations. Ten kindreds had learning difficulty without a previous history of apnoea; all had *GLRA1* mutations,  $P < 0.001$ ; and all, with one exception, were caused by recessive or compound heterozygous mutations. Patients with recessive or compound heterozygote mutations (independent of the gene involved) were also more likely to exhibit developmental delay or learning difficulties ( $P < 0.02$ ), 51% with recessive inheritance compared with 10% dominant.

### Clonazepam and referral pattern

Sixty-five cases had been treated with the GABA<sub>A</sub>R potentiator clonazepam (67%), 96% of these had a satisfactory subjective improvement in either hypertonia, startle or both. As expected, dose-related sedating side-effects limited its utility in some cases. There was no pattern formed by looking at those who had a dramatic or equivocal response to clonazepam. Of the 17 individuals with *SLC6A5* mutations, 14 had been tried on clonazepam and 13 found a sufficient and sustainable benefit from this therapy; similarly all *GLRB* cases responded well to clonazepam (one to nitrazepam).

### Gene-negative cases

The 35 cases without mutations in the three genes of effect had a number of similarities with the genetically proven cases: the majority had exaggerated startle (86%), were hypertonic (69%), and apnoeas and falls were seen albeit at a lower frequency than other cases (23% RR2.4  $P < 0.001$ , 23% RR2.7  $P < 0.001$ ). Importantly, just under half of cases reported that startle symptoms began after the first month of life and not soon after birth; which we suspect may represent a novel sub-phenotype. However, we have not identified a genetic explanation for later-onset cases ( $P < 0.001$ ).

### Structural modelling

#### Modelling of *GLRA1* mutations in glycine receptor homologues

The wealth of *GLRA1* mutations permitted sub-categorization into pathogenic classes. This brought stronger correlations than primarily analysing by phenotypic criteria (Fig. 1). Hypertonia, although not a universal feature of *GLRA1* mutations, can be associated with almost any structural change. Developmental delay is

**Table 1** Distribution of clinical traits dependent on gene mutation and method of inheritance

Gene	Inheritance / mutation	Startle	Hypertonia	Apnoeas	Falls	Delayed development	Epilepsy	Clonazepam	Neonatal onset
GLRA1	Dominant Missense <i>n</i> = 14	14	10	4	9/13	3	0	6/7	14
	Recessive <i>n</i> = 47	47	35	22	30/45	21	3	29/30	47
	Missense <i>n</i> = 24	24	19	17	14/22	11	2	13/14	24
	Nonsense <i>n</i> = 23	23	16	5	9	10	1	16/16	23
GLRA1	<b>Total</b> <i>n</i> = 61	<b>61</b>	<b>45</b>	<b>26</b>	<b>39/58</b>	<b>24</b>	<b>3</b>	<b>35/37</b>	<b>61</b>
SLC6A5	Recessive <i>n</i> = 22	22	14/17	14/18	8/15	12/17	2	16/17	14
	Missense <i>n</i> = 8	8	5/5	5/6	2/4	4/4	1	4/4	8
	Nonsense <i>n</i> = 14	14	9/12	9/12	6/11	8/13	1	12/13	14
	Not confirmed <i>n</i> = 2	2	2	2	0	2	0	2/2	2
	<b>Total</b> <i>n</i> = 24	<b>24</b>	<b>16/19</b>	<b>16/20</b>	<b>8/17</b>	<b>14/19</b>	<b>2</b>	<b>18/19</b>	<b>24</b>
GLRB	Dominant missense <i>n</i> = 11	1	1	1	NA	NA	0	1	1
	Recessive <i>n</i> = 11	11	11	7	7	9	2	11	11
	Missense <i>n</i> = 2	2	2	1	1	1	0	2	2
	Nonsense <i>n</i> = 9	8	9	6	6	8	2	9	8
GLRB	<b>Total</b> <i>n</i> = 12	<b>12</b>	<b>12</b>	<b>8/9</b>	<b>7</b>	<b>9</b>	<b>2</b>	<b>12</b>	<b>12</b>
No mutation	<i>n</i> = 35	30	24	8	8	12	9	NA	19/35
		86%	69%	23%	23%	34%	26%		54%

invariably associated with a conformational change at the cytoplasmic end of M4 with mutations affecting the extracellular domain residues in addition to M2 changes (such as E103K and R65W). Similarly those with delays in speech acquisition not only have this conformational change at M4, but a secondary impact on M2. Of note there is a phenotypic cluster of cases of classic hyperekplexia with hypertonia and no learning difficulties and an excellent response to clonazepam, which have a similar structural profile (M147V, I244N, P250T and R271Q).

### Reduced current

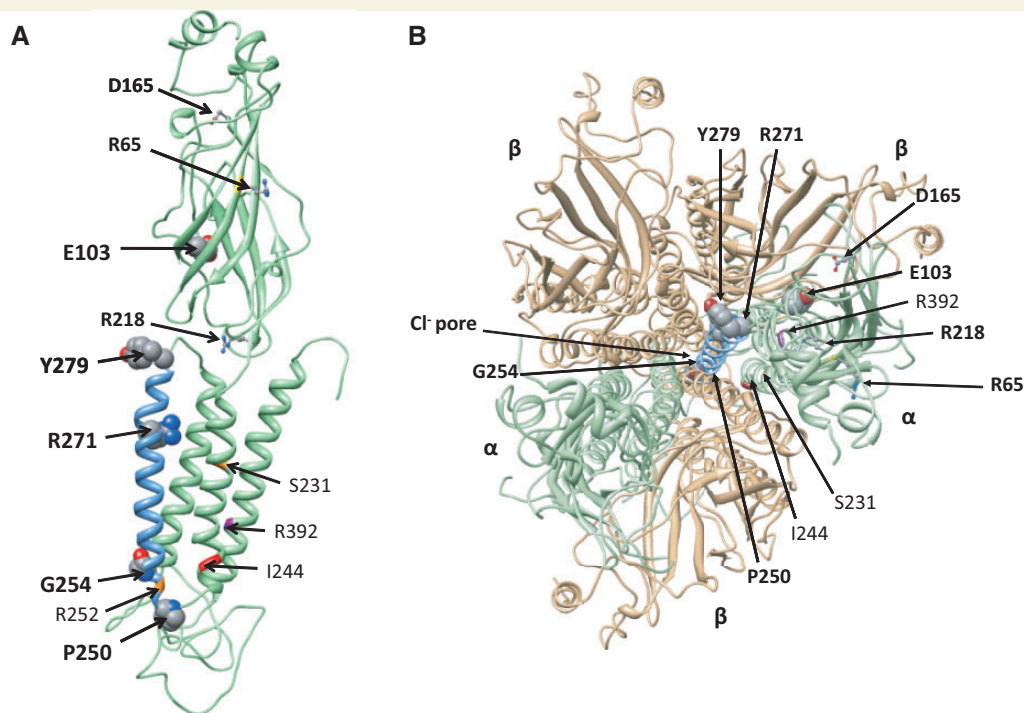
The most frequently occurring mutation (dominant R271Q) is adequately expressed at a cell surface level, but produces a reduced current and cases invariably escape without learning difficulties and without prominent apnoea attacks. Within this class homozygous E103K, compound heterozygous E103K and del634-635CT produce the most severe phenotypes. The narrowing of the channel at M2 is most marked in these cases with a major conformational change at the end of M4 (Fig. 2).

The R65L/del ex 4–7 compound heterozygote, which causes a reduced current, has a particularly benign phenotype with an

excellent response to clonazepam. Although it has the predicted change in orientation of the extracellular end of M1, with a narrowing at the extracellular end of M2 (in keeping with other mutations in the class) these changes are much less dramatic. These minor changes explain why it trafficked so efficiently (identical to wild-type) but as the R65 residue is an important glycine binding determinant the current generated is reduced. R65W, however, (below) is not expressed adequately at a cell surface level.

### Reduced cell-surface expression

The R65W mutation results in drastically reduced number of functional channels and cell-surface receptors (Chung *et al.*, 2010). Mutations that produce channels that are only poorly expressed at the cellular membrane demonstrate more substantial conformational changes with effects on M3 as well as M2 and M4. Examples include the compound heterozygous R65W and homozygous D165G mutations: D165G shows an additional loss of helical structure at the cytoplasmic end of M2. In R65W, the cytoplasmic end of M3 projects towards M2, while in D165G, the helix is extended at the extracellular end of M3 and shortened



**Figure 1** (A) Location of point mutations of *GLRA1* associated with neonatal apnoea, hypertonia learning difficulties and hypotonia. E103K, P250T, G254D, R271Q, Y279C (shown in spacefill) directly affect chloride flux and are mostly found on the pore-forming M2 transmembrane region or closely adjacent loop regions, shown in blue. R65W, D165G (shown in ball and stick) are located in the extracellular domain and involve the loss of a charged residue along with a substantial change in side chain size upon substitution. L291P and R392H (backbone shown in purple) are located on transmembrane regions other than M2 and involve more subtle changes in charge or local secondary structure. I244N (backbone shown in red) is found at the intracellular end and involves no change in sidechain charge. S231N, R252C (backbone shown in orange) are found in transmembrane regions and involve changes in side chain charge or polarity. R65L (backbone shown in yellow) is located at the extracellular end, away from the pore region, and involves changes in residue charge but maintains similar size side chains. (B) The location of *GLRA1* point mutations (where visible) in the GlyR pentamer, viewed from the extracellular side.

at the cytoplasmic end. Both R65W and D165G lead to a conformational change at the cytoplasmic end of M4 (Fig. 2).

### Receptor trafficking deficits

A failure to traffic the receptor to the membrane produces a moderate to severe phenotype. The cytoplasmic ends of M3 and M4 appear to be critical in trafficking defects; such as in the homozygous R252C mutation. Along with a narrower channel at the extracellular end of M2, this also produces a significant conformational change at the cytoplasmic end of M4. A more dramatic example of the misalignment of M2 and the significant extension and reorientation of the cytoplasmic ends of M3 and M4 is the homozygous G254D (Fig. 2A). These trafficking mutants all have recurrent neonatal apnoeas.

## Modelling of *SLC6A5* mutations in glycine transporter 2 homologues

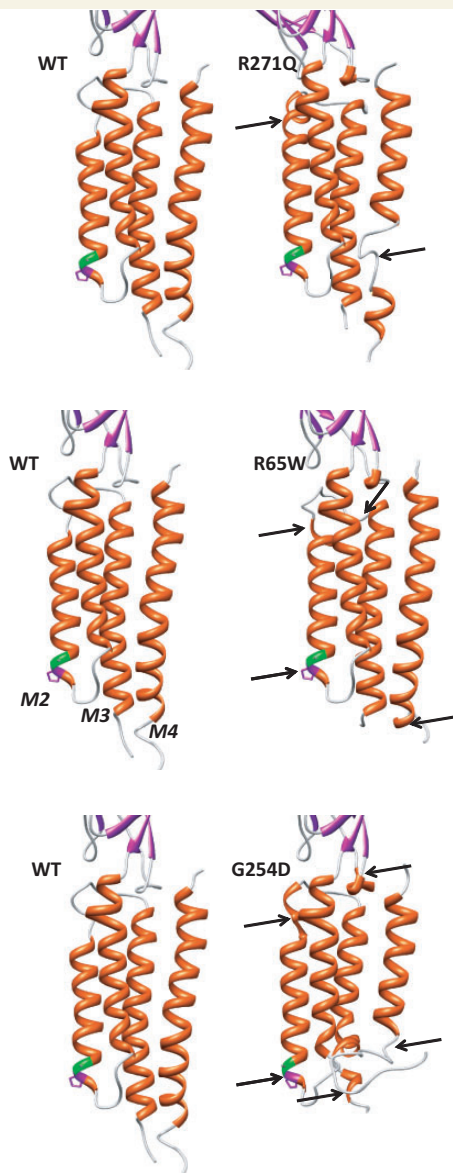
### A common effect at T578

There are fewer *SLC6A5* variants and yet a clear pattern emerges between changes in molecular structure and phenotype. In the

pathogenic hyperekplexia variants, the position and orientation of the T578 side chain is notably different to that of wild-type GlyT2, which markedly changes the predicted interactions with one of the two Na<sup>+</sup> ions and the glycine ligand. This change occurs with all the variants (except the truncated variants where the residue is not encoded). In all but the S256R variant, T578 is predicted to form an alternative threonine side chain oxygen interaction with the glycine ligand H2 atom (such as in P243T, Y705C and E248K, Fig. 3). The side chain oxygen of T578 of the P243T and Y705C variants is predicted to be within the interaction range of Na<sup>+</sup>1 in addition to the glycine ligand. In contrast to the normal protein, other parts of the T578 side chain are within 3 Å of Na<sup>+</sup>1 in all the variants and so may impede the passage of this Na<sup>+</sup> ion. L574 is introduced into the Na<sup>+</sup>1 binding site in all variants, with predicted spatial overlap. More notably D577 is also introduced into the Na<sup>+</sup>1 binding site of all variants where it is coded in the protein, except for E248K (Fig. 3), which interestingly, is associated with a milder phenotype with no learning difficulties.

### *SLC6A5* compound heterozygosity

The analysis of compound heterozygote variants reveals a similar pattern with regard to the changes in position of key residues



**Figure 2** Molecular modelling of GlyR  $\alpha 1$  variants. *Left column:* wild-type (WT); *top right:* R271Q (adequate expression, reduced current); *middle right:* R65W (poor cellular expression); *bottom right:* G254D (trafficking defects). R271Q displays extension of M2 helix at extracellular end, which leads to narrowing of the chloride channel; and conformational changes at the cytoplasmic end of M4. R65W displaying minor extension of M2 helix at extracellular end, the cytoplasmic end of M3 projected more towards M2, which results in further misalignment of M2 at the cytoplasmic end, along with conformational changes at the cytoplasmic end of M4. G254D displays extension of M2 helix at extracellular end; helix extension and conformational changes at cytoplasmic end of M3; along with conformational changes at the cytoplasmic end of M4, resulting in substantial shortening of the helix. There is a trend of increasing aggregated structural change, progressing from modest changes for mutations that do not affect expression but do affect current, tolerated in terms of incorporation into the pentameric structure; to substantial changes for mutations that result in much reduced cellular expression, less tolerated; to profound changes that result in trafficking defects, not tolerated.

being consistent with more severe phenotypes. The S256R/Y656H combination displays introduction of T578 to interaction range of  $\text{Na}^+1$ .

These changes are consistent with those of the single mutations associated with neonatal apnoeas. D577 is not introduced to the binding site of  $\text{Na}^+1$  of S256R/Y656H, similar to the E248K variant; neither case is associated with learning difficulties. The F547S/Y656H combination shows all of the above changes, as well as the introduction of D577 to the binding site of  $\text{Na}^+1$  and is likewise associated with a panoply of startle, falls, learning difficulty, neonatal apnoeas and impaired motor development.

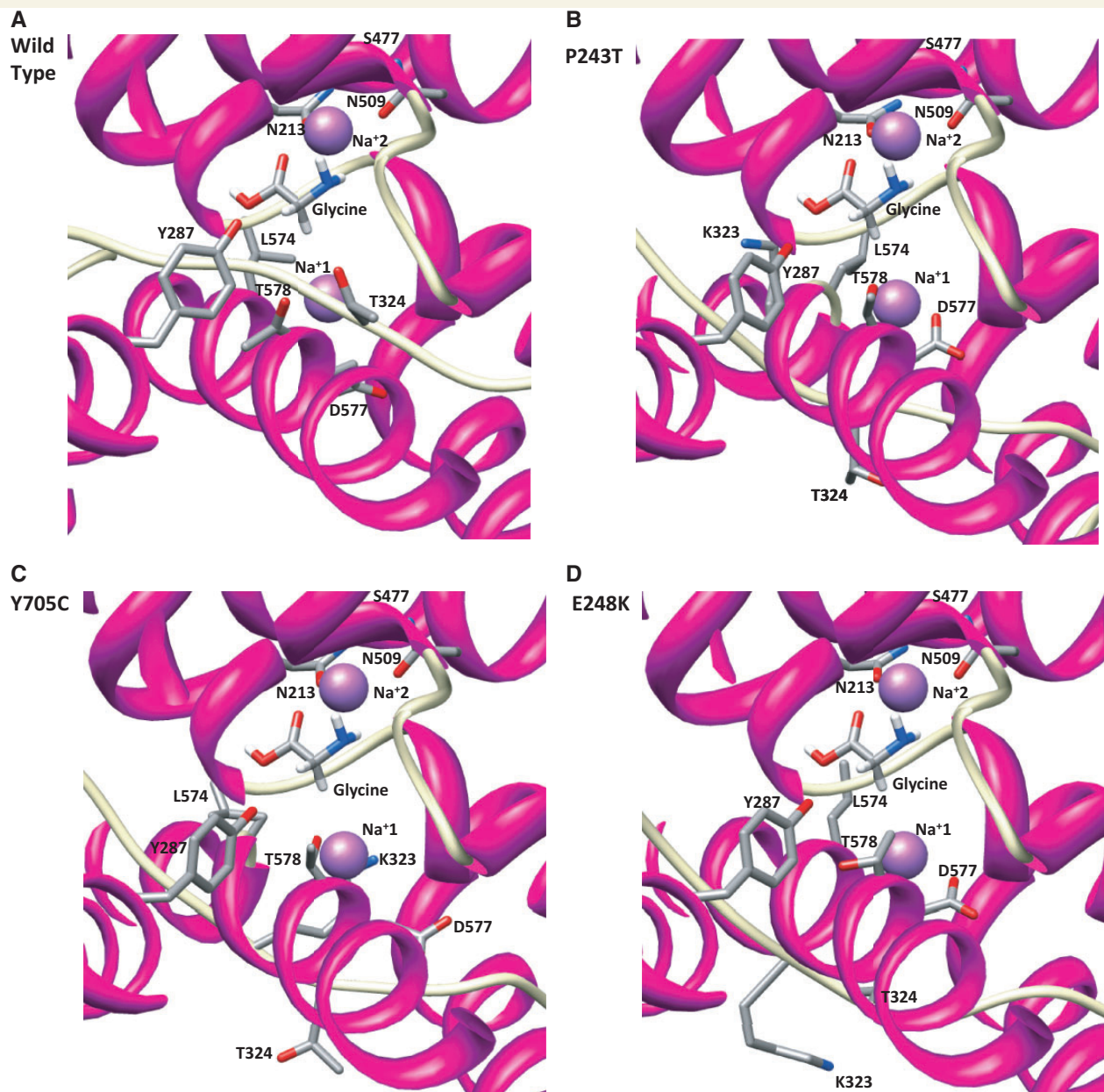
No molecular modelling is presented for *GLRB* as the vast majority of mutations are deletions, nonsense or splice site changes. Molecular modelling of missense mutations including P169L and Y470C has been recently described (Chung *et al.*, 2013).

## Discussion

This is the first description of how the hyperekplexia genotypes affect the associated clinical phenotypes. We report definitive indications for gene-specific phenotypic differences and an association of recessive inheritance with increased risk of learning difficulties and developmental delay, particularly speech acquisition. We also highlight the risk of severe recurrent neonatal apnoeas with hyperekplexia (particularly associated with mutations in *GLRB* and *SLC6A5*), which has hitherto not been recognized. The inclusion of gene negative cases identified startle at birth and falls as the cardinal features of classic hyperekplexia. Furthermore the scale of this cohort permits molecular modelling to explain phenotypic variation between pathogenic mechanisms and partially within each class. We also acknowledge some limitations to our study: it has proved impossible for us to examine each patient individually and so we are dependent on the information provided by the referring clinicians. Patients of differing ages are being compared: some are too young for certain features (learning difficulties or falls for example) to exhibit themselves. Similarly, there is a recall bias from older patients as their neonatal apnoeas may be forgotten, under-reported or ignored. It is very probable that a lack of familiarity with this condition leads to under-reporting and misdiagnosis (or an over-diagnosis of seizures). Furthermore we are more likely to be referred cases when there is greater diagnostic doubt and a more complicated phenotype. Despite this being the largest description of hyperekplexia families and cases by a significant margin, it is quite possible that we are unable to recognize or prove the existence of rarer but relevant co-morbidities and characteristics.

## Recessive versus dominant inheritance

Hyperekplexia was initially misrepresented as demonstrating solely autosomal dominant inheritance, because these families were easier to identify and study, which exacerbated an ascertainment bias. Autosomal dominant inheritance accounts for 16% of the cases in this study. This may be an underestimate; we are more likely to receive requests for a genetic diagnosis when the clinical pattern is ambiguous, such as a lack of family history. However, it



**Figure 3** Disruption of  $\text{Na}^+$  and glycine binding sites in severe and mild GlyT2 hyperekplexia variants. Wild-type GlyT2 (**A**) showing the glycine ligand bound by T578. In the hyperekplexia variants, T578 is predicted to form an alternative threonine side chain oxygen interaction with the glycine ligand hydrogen atom (such as in P243T, Y705C and E248K) (**B–D**). The side chain oxygen of T578 of the P243T and Y705C variants is predicted to be within interaction range of  $\text{Na}^+$ 1 as well as the glycine ligand. In contrast to the normal protein, other parts of the T578 sidechain are within 3 Å of  $\text{Na}^+$ 1 in all the variants (other than the truncations) and so may also impede the passage of this  $\text{Na}^+$  ion. L574 is introduced into the  $\text{Na}^+$ 1 binding site in all variants, with predicted spatial overlap. D577 is also introduced into the  $\text{Na}^+$ 1 binding site of all variants where encoded, except E248K (**D**). E248K is associated with a milder phenotype with no learning difficulties.

is important to recognize the importance of recessive inheritance in the context of genetic counselling. In addition consanguineous families may also harbour other undetected recessive mutations that may explain the extended phenotypes.

Parental sampling has identified hyperekplexia as a highly penetrant condition, although occasionally incomplete penetrance has been reported (Zoons *et al.*, 2012). There is a small degree of

variability within families, for example, a family from the UK with autosomal dominant inheritance due to the common *GLRA1* R271Q mutation exhibited mild learning difficulties, until the present generation, where an affected individual completed mainstream schooling. However, this may represent the success of early diagnosis and prompt treatment with clonazepam (rather than chlordiazepoxide which her ancestors used;

Lindahl, 2005). Of the 12 pairs of siblings who share the same genotype, there appears to be an extensive degree of homology. When the core features of hypertonia, startle, falls, learning difficulty, response to drugs and apnoea are compared there is 4% discordance, driven mainly by the ambiguity due to variable questionnaire entries. There is heterogeneity, however, derived from the variation in the physical characteristics during development (worst in the first year) and over the course of a day (improved in sleep, with alcohol and benzodiazepines—provoked by external stimuli). However, we cannot exclude other considerations such as *in vivo* pathophysiological mechanisms, incomplete genetic penetrance or the occurrence of digenic or multigenic cases.

## Hyperekplexia and apnoeas

Neonatal apnoeas have been recognized in hyperekplexia following a case report (Kurczynski *et al.*, 1983) and were estimated to occur in 10% of patients (Giacoia *et al.*, 1994). Apnoeas occurred in 56% of all patients in our study, but in >80% of cases with *GLRB/SLC6A5* mutations. These events are often associated with the triggered tonic, abdominal splinting attacks and can be life-threatening. A patient who was compound heterozygous for *SLC6A5* mutations (Y491C and Q630X) suffered 47 respiratory arrests in an 8-week period (Rees *et al.*, 2006). The explanation as to why apnoeas were regarded as a feature of 'sporadic' hyperekplexia (Bakker *et al.*, 2006, 2009) lies within the inheritance patterns; patients with *GLRB/SLC6A5* mutations are significantly more likely to describe serious neonatal cyanotic attacks and the majority of *GLRB/SLC6A5* mutations are recessive—presenting sporadically. The same trend applies for *GLRA1* mutations where sporadic index cases have been linked to recessive mutations (Rees *et al.*, 2002; Chung *et al.*, 2010).

## Learning difficulties and developmental delay

Forty per cent of people with *GLRA1* mutations had neither neonatal apnoeas nor delayed development, in contrast to cases with *SLC6A5/GLRB* mutations where just one person had neither comorbidity. It has been incorrectly stated that the cognitive profile of people with hereditary hyperekplexia is unaffected or mildly impaired—although recognizing that 'sporadic' cases were more complex (Zhou *et al.*, 2002); and late motor milestones have been previously described (Tsai *et al.*, 2004) in a family with recessive *GLRA1* hyperekplexia. MRI is ubiquitously normal in classical hyperekplexia. It is likely that the apnoea attacks do not directly cause cognitive impairment: 40% of the cases with learning difficulty or developmental delay had no previous apnoea attacks (all had *GLRA1* mutations). The developmental delay demonstrated in some patients may represent subtle alterations in neuronal migration and targeting within the context of an environment where glycine is depleted from presynaptic terminals, or incomplete neural networks due to the lack of GlyR activation during brain development. The lack of GlyR activation during embryonic and infantile brain development will produce direct widespread glycinergic consequences and indirect effects on other neural networks

such as gamma-aminobutyric acid (GABA) and *N*-methyl-D-aspartic acid (NMDA).

The phenomenon of last trimester intrauterine startle first described by Leventer *et al.* (1995) is confirmed in this study. It suggests that the glycinergic synaptopathy is apparent months before birth—during the time of greatest neurocortical development and the developmental switch from GlyR alpha-2 homopentamer receptors to GlyR  $\alpha_1\beta$  heteropentameric receptors. In the neonatal brain many inhibitory synapses initially are mixed GABAergic and glycinergic, which changes during CNS maturation. Castaldo *et al.* (2004) suggested that the glycinergic synapse and GlyRs may well participate in cognitive development. GlyRs have been described in septal cholinergic neurons, which play a major role in learning and memory by means of their connections with the hippocampus and cerebral cortex, and their function can be modulated by psychotropic medication (Laube *et al.*, 2002). Our finding that speech acquisition is preferentially affected implicates glycinergic pathways in expressive language. However, many patients reported later developmental 'catch-up', suggesting a sophisticated compensatory mechanism that plausibly may involve upregulation of inhibitory GABA, or downregulation of antagonistic glutamate systems.

## Gene-negative cases

Hindsight allows us to reappraise previously published gene-negative patients. Shiang *et al.* (1995) described 17 patients: 11 had definite autosomal dominant inheritance and uncomplicated hyperekplexia without learning difficulty, whereas five of the six gene-negative patients had developmental delay. We now know that one had compound *SLC6A5* mutations (Carta *et al.*, 2012) and another has a recessive mutation in *GLRB* (Chung *et al.*, 2013). The 35 gene-negative cases form a looser grouping with exaggerated startle co-presenting with occasional hypertonia, falls and apnoea. It leads us to speculate that the gene-negatives fall into the following categories: (i) genuine hyperekplexia where intragenic DNA variations in *GLRA1*, *GLRB* and *SCL6A5* remain undetected (ENCODE consortium *et al.*, 2012); (ii) classical hyperekplexia with genes yet undiscovered; (iii) neonatal/early-infancy acquired hyperekplexia that is a phenocopy of genetic hyperekplexia e.g. autoantibody driven hypertonia and startle such as described by Hutchinson *et al.* (2008) with autoantibodies against GlyR or GlyTs; and (iv) other undiagnosed or unrecognized neuromotor disorders presenting as mimics, sharing common features such as excessive startle or hypertonia.

## Modelling explains pathogenic class differences

The large number of *GLRA1* cases identified and the delineation of their functional consequences permits a degree of classification by pathogenic mechanism. Broadly there are four groups of equal size, with mutations causing: (i) adequate cell-surface expression but reduced current; (ii) limited cell-surface expression of poorly-functioning channels; (iii) trafficking defects; and (iv) prematurely truncated GlyR subunits (Table 2). The first class has the least complicated hyperekplexia (learning difficulties and apnoeas are



**Table 2** Molecular modelling of the glycine receptor  $\alpha_1$  subunit demonstrates that mutation position and pattern of protein disruption can predict phenotype

Transmembrane domain	Reduced current	Reduced cellular expression	Trafficking deficits
M2	Extension of helix at extracellular end. Narrowing of the channel at extracellular or cytoplasmic end	Narrowing of the channel at the extracellular end	Narrowing of the channel at the extracellular end
M3		Extended helix at cytoplasmic end	Marked conformational changes at cytoplasmic end
M4	Conformational change at the cytoplasmic end accompanying M2 changes	Significant conformational change at the cytoplasmic end	Cytoplasmic end critical – conformational changes typically extreme
Phenotype	Mild	Severe	Moderate to severe

Reduced current: mutations are adequately cell-surface expressed but have impaired function *in situ* (by various mechanisms i.e. ligand-binding TM2 pore dynamics) when compared with wild-type.

Reduced cellular expression: mutations reduce the number of receptors at the cell-surface (insertion dynamics, remain in endoplasmic reticulum), they may also not work adequately at the cell-surface.

Trafficking deficits: mutations have a greatly reduced number of receptors at the cell-surface.

more scarce) and the other three are similar in severity. The fifth class is rarer, producing a tonic-opening leaky channel (Chung *et al.*, 2010).

It has been suggested that mutations that lead to a complete lack of function may be more likely to exhibit a severe phenotype with learning difficulties, such as R218Q, or the nonsense mutation Y202X in *GLRA1* (Table 2; Brune *et al.*, 1996; Castaldo *et al.*, 2004). Y202X does have an extended phenotype in keeping with mutations in the truncated class, but surprisingly R218Q has a much attenuated phenotype. Modelling of R218Q predicts a narrowing at extracellular end of M2, with an extended end to cytoplasmic M3 and a major conformational change to the cytoplasmic end of M4: dramatic changes but not as severe as others within its class.

## Hyperekplexia treatments

A prompt diagnosis is of importance, as the vast majority of cases respond well to clonazepam, which may offer more relief from startling than hypertonia (Tijssen *et al.*, 1997). Physical activity (for hypertonia) and patient education should form part of a treatment regime for anyone with hyperekplexia (such as the 'Hyperekplexia Society' on Facebook). The major non-pharmacological treatment for infants with hyperekplexia is the Vigevano manoeuvre (Vigevano *et al.*, 1989). Understanding that there appears to be a difference in clinical presentation (between *GLRB/SLC6A5* and *GLRA1* hyperekplexia) means that we must recommend both early genetic testing for suspected neonates and pre-conception counselling for couples who have a family history of hyperekplexia.

## Conclusions

This study represents, by far, the largest cohort of hyperekplexia cases reported, supported by 20 years of genetic hierarchical analysis and functional characterization. We firmly establish that the constellation of symptoms caused by failure of glycinergic synaptic transmission can be generated by mutations affecting presynaptic

and postsynaptic proteins. However, mutations in *GLRB* and *SLC6A5* cause repeated apnoea attacks in the majority of cases and most either report developmental delay (with speech delay prominent) or learning difficulties. Sporadic cases of this type are explained by recessive mutations, with consanguinity a risk factor and early treatment may help alleviate the morbidity due to recurrent apnoeas. Hyperekplexia remains a clinical diagnosis, but we argue that prompt genetic analysis will help estimate prognosis, assist in pre-conception counselling and aid the planning of safer neonatal care. For those patients that remain gene-negative we can now begin a process of phenotypic reassessment and further stratification of hyperekplexia in preparation for second generation genetic analysis.

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## Supplementary material

Supplementary material is available at *Brain* online.

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