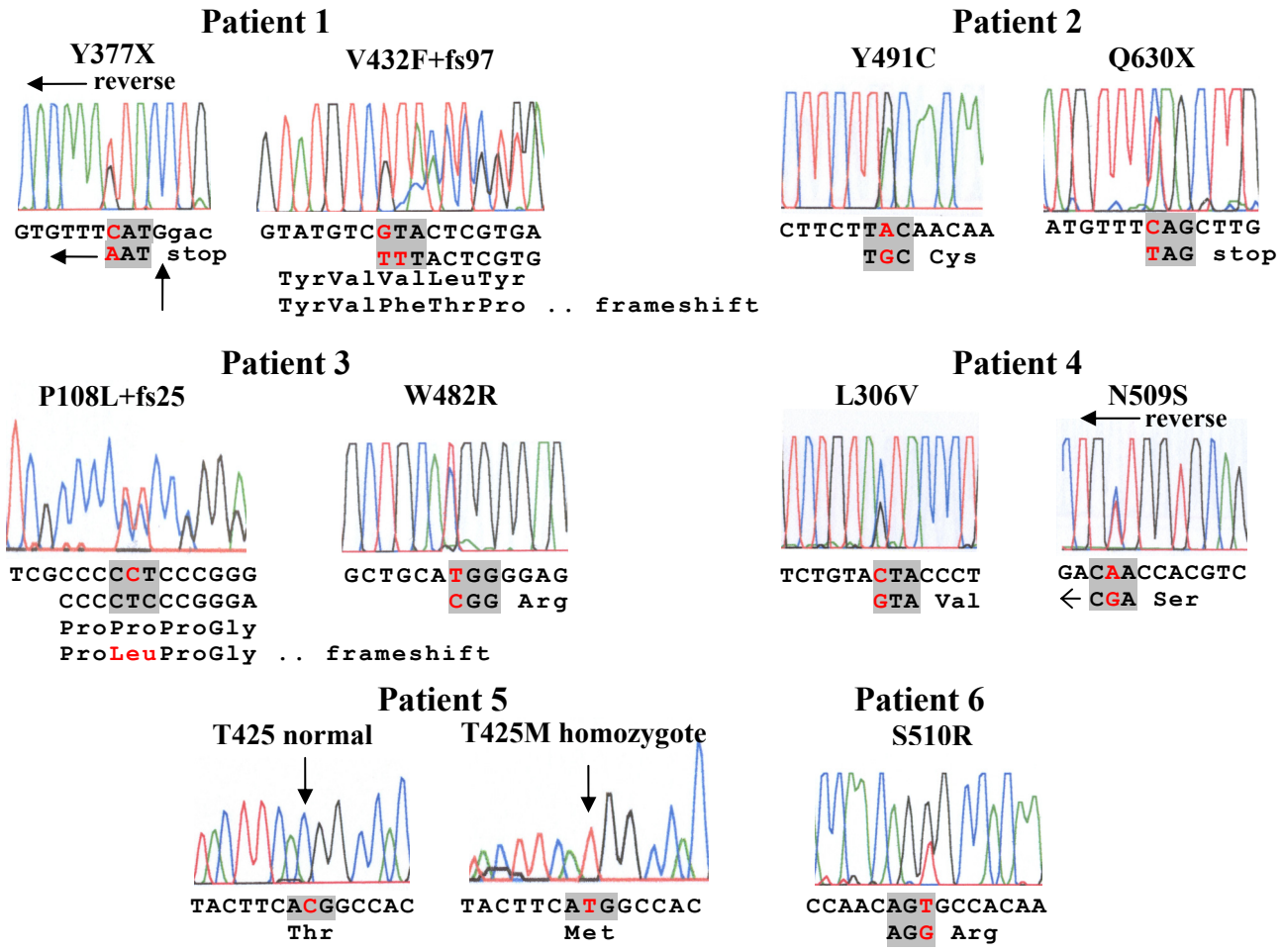
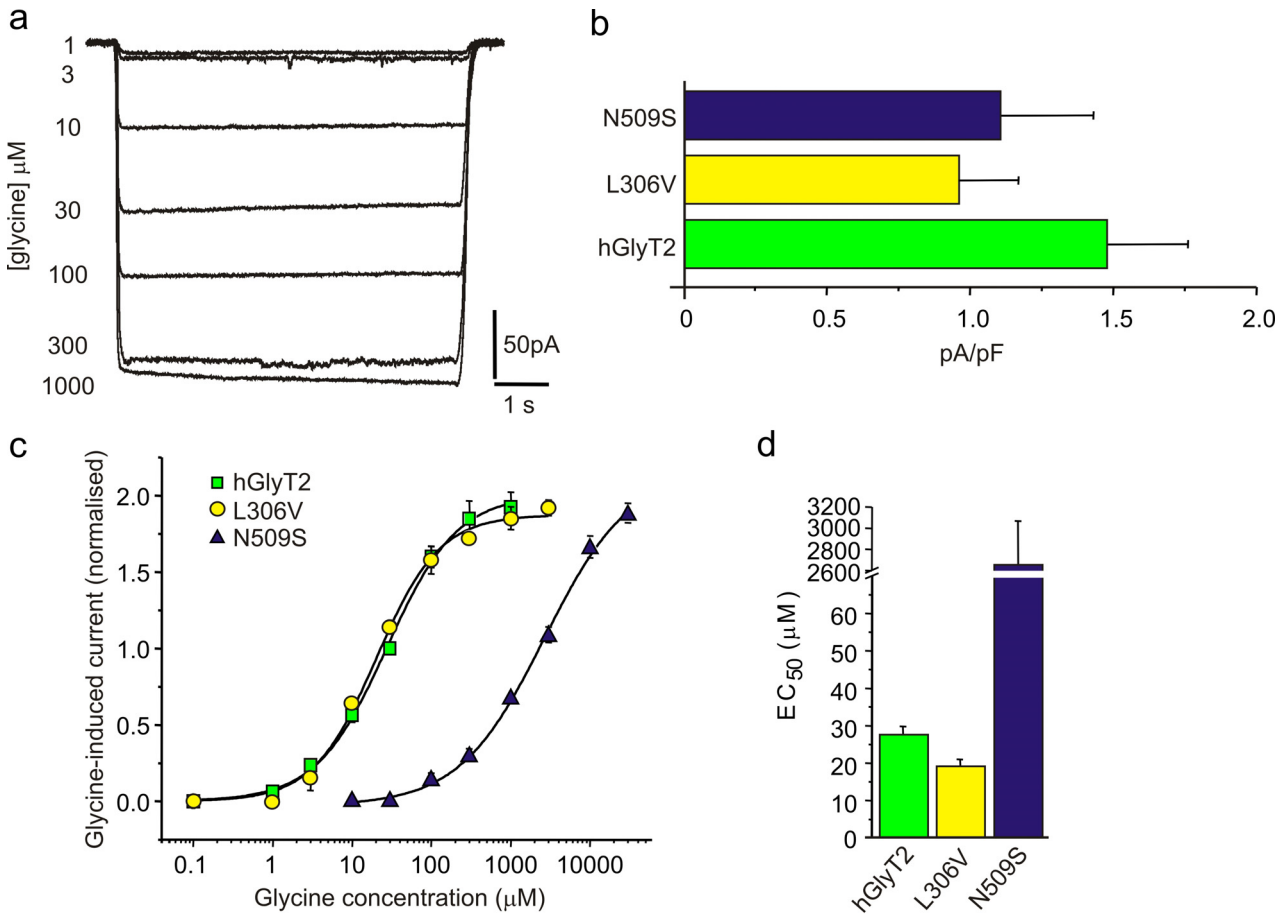


Supplementary Figure 1. Sequencing panel representing the pathological hyperekplexia variants detected in *SLC6A5*

Mutations are expressed in their polypeptide nomenclature to maintain consistency with the manuscript and tables. The mutations are indicated in red and the frame of the affected amino acid change is indicated by grey shading and amino acid codes. Most chromatograms are in the forward (i.e. sense) direction, except Y377X and N509S. Mutations were confirmed by cloning PCR products into pCRII-TOPO (Invitrogen) and DNA sequencing to separate the alleles.



Supplementary Figure 2. Electrophysiological analysis of GlyT2 mutants in NG108-15 cells
a, dose-dependent glycine-induced whole-cell current from NG108 cells expressing pRK5myc-hGlyT2. Currents consistently showed rapid onset and offset, and remained stable during glycine application. **b**, maximum whole-cell currents (pA) for hGlyT2 and mutants L306V and N509S were normalised to the surface area of the recorded cell (measured as capacitance, pF) and are plotted as pA/pF. **c**, dose-response (D-R) curves. Currents are plotted against the glycine concentration and normalised to the maximum current. Note the near 1000-fold shift in the curve for N509S. **d,** EC_{50} values (µM) determined from D-R curves are plotted for hGlyT2 (27.4 ± 2.4 µM) and mutants L306V (19.2 ± 1.8 µM) and N509S (2651 ± 417 µM). Data are means ± s.e.m. n = 3-8.

Exon	Size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Amplimer (bp)
1	3	CTGCCGGTTTCGGTTTAGTA	GAATCTGCTTTCCCTGTCCC	375
2	546	TAAAAGCTGTTGTGACTTTGTTTT	GACACTGTGCGGGCCGTAAT	629
3	139	GGCCTGCTTGTGGACCTACT	CAGGCGGAAAGAGCGGAAAAG	223
4	132	CCTCCTAGGGCTCTCACTCC	GACAGAGTAAGAAAGGGCCTGA	217
5	174	TAAGATGGAATGAACCCCTGG	AGAATACACACACCTAAAGCAGG	250
6	142	TGCAGAGAGACAAATCTCTGTTTT	CACCTCTGGTCTGCAAATTGA	294
7	133	CCTTCTTTCTATCACTCCCCC	CTGGGTGTCTCACAGCTTTCT	220
8	142	CACTCTGCAGGGCTGCTTCT	CCCCAGGGCTGGTTATAGAT	227
9	104	CAGTCTCCTTCATGGGTCTTG	TTCTTCTGTCCCACTCACCAG	203
10	125	CCAAGCACACCTAATGGAAAA	GGAGCTTGTGACATGAGCCT	227
11	113	GAAGAGCAGCCTTGAGTAGGG	GCATGGGATAGAGACTGATGG	201
12	132	TACCTCCTGGGTGGTACAATTT	CCACCCCAAGCCTGTGCCTA	220
13	100	CAGGACGCATTTGATATTGGT	CATGAATGCCTTACCGACACT	200
14	101	CTCACCTTCCTGCTACTGTGC	ACGTATGCAAGGTGCTGTCTG	201
15	168	GAATAATTCACGCCACCACC	GGAATTGGGAGGGAAAGAAGT	251
16	156	AGGTGCACTACTTCTGTGACCA	AAATGGGAGGAGAGCTATGGAA	286

hGlyT2A-1	5'-TTGGAATTC*GCCACC*ATGGATTGCAGTGCTCCCAAG-3'
hGlyT2A-2	5'-ACCCTCGAGCTAGCACTGAGTGCCCAGTTC-3'

Supplementary Table 1. Oligonucleotide primers for analysis of the human GlyT2 gene (*SLC6A5*) and amplification of human GlyT2 cDNAs

Gene Symbol	Protein	Cohort Number	No. of Gene-Positive Patients	No. of Mutations Detected	Cohort Detection Rate	Mutation Rate in Hyperekplexia
GLRA1	Glycine receptor α1 subunit	103	12	15 (a)	**11%**	**15%**
SLC6A5	Glycine Transporter type-2	83	6	11 (b)	**6%**	**11%**
GLRB	Glycine receptor β subunit	103	1	2 (c)	**1%**	**2%**
GPHN	Gephyrin	90	1	1	**1%**	**1%**
ARGHEF9	Collybistin	90	1	1	**1%**	**1%**

Supplementary Table 2. Patient/mutation detection rates in hyperekplexia candidate genes
From previous genetic screens for this cohort of patients (Rees *et al*., 2001, *Hum*. *Genet*. **109**, 267-270; Rees *et al*., 2002, *Hum*. *Mol*. *Genet*. **11**, 853-860; Rees *et al*., 2003, *J*. *Biol*. *Chem*. **278**, 24688-24696; Harvey *et al*., 2004, *J*. *Neurosci*. **24**, 5816-5826) an impression is emerging in relation to hierarchical testing in hyperekplexia. The number of patients tested in each cohort is different due to the timeline in the analysis, e.g. the unambiguous *GLRA1* and *GLRB* mutation-positive patients were not included in the *SLC6A5* mutation screen, which also included new *GLRA1*-negative samples from the USA and the Netherlands. The mutation detection numbers are greater than the number of gene-positive individuals due to the phenomenon of compound heterozygosity in *GLRA1* (a); *SLC6A5* (b) and *GLRB* (c).

Sequence change	Exon number	Predicted consequence	Frequency in Study Cohort	Frequency in Population
T→G at -176	5' UTR, Exon 1	No effect	0.07	0.06
A→G at -33	5' UTR, Exon 1	No effect	0.09	0.08
C266A	Exon 2	Non-Synonymous A89E	0.012	0.015
G304A	Exon 2	Non-Synonymous G102S	0.39	0.42
C336T	Exon 2	Synonymous S112S	0.24	0.22
C352T	Exon 2	Synonymous L118L	0.26	0.23
C371T	Exon 2	Non-Synonymous S124F	0.285	0.255
A951G	Exon 5	Synonymous T317T	0.10	0.09
IVS6 +45 C→T	Exon 6	No effect	0.05	0.07
G1371C	Exon 8	Non-Synonymous K457N	0.12	0.15
G1386T	Exon 8	Synonymous T462T	0.03	0.04
G1387A	Exon 8	Non-Synonymous D463N	0.20	0.21
IVS14 -3 C→A	Exon 15	No effect	0.09	0.11
G2103A	Exon 15	Non-Synonymous E701E	0.45	0.49

Supplementary Table 3. Single nucleotide polymorphisms detected in the coding regions of the human GlyT2 gene (*SLC6A5*)

Several common synonymous and non-synonymous variations were detected and their frequency in cohort and controls are reflected by the number of chromosomes analysed. For the pathogenic *SLC6A5* changes a greater number of control chromosomes were used. Some of the above non-synonymous changes have been reported by others (e.g. D463N; Evans *et al*., 1999, *FEBS Lett.* **463**, 301-306; G102S; Gallagher *et al*., 1999, *Mol. Brain Res.* **70**, 101-115) and, like A89E, are currently considered to be inert.

Supplementary Note: Detailed clinical reports for affected *SLC6A5* patients and relatives

Patient 1 (Canada; compound heterozygote for Y377X and V432F+fs97)
The proband was the product of an unremarkable pregnancy to a woman of Northern European origin and her Italian/Yugoslavian partner. Foetal movements were described as unusually jerky throughout the pregnancy. The proband was born at 40 weeks gestation. She had Apgars of 8 and 9 at 1 and 5 minutes. At 90 minutes of age she suffered a generalized tonic-clonic seizure of 50 minutes duration and was successfully treated with phenobarbital. An EEG demonstrated 'non-specific excessive partial sharp waves over the right hemisphere', but no clear evidence of epilepsy. A brain CT scan and MRI were normal. Between seizures 'twitching' was often noted, reflexes were brisk and her startle response was exaggerated. The diagnosis of hyperekplexia was made and she was treated with clonazepam from 10 days of age with marked improvement. During her first year she had episodes of spontaneous stiffening lasting as long as 10 minutes and associated with cyanosis. These could be minimized by picking her up and sometimes aborted by flexing her legs against her body in a specific fashion. The head retraction reflex (HHR) was strongly positive. Over the years the episodes of stiffening diminished. Tripping would precipitate whole body stiffening that prevented her from bracing her fall. At the age of 4 years, she was still requiring 0.08 to 0.09 mg clonazepam administered every 6 hours. Family history revealed that the mother reported significant sleep myoclonus characterized by stiff jerks of her entire body on a nightly basis, but had no episodes of infantile stiffening or seizures as well as a normal startle response.

Patient 2 (USA; compound heterozygote for Y491C and Q630X)
During pregnancy, the mother of the proband was hospitalized on a number of occasions for hyperemesis in the first trimester. She received both oxygen and steroid therapy which manifested in a case of gestational diabetes. She later developed pre-eclampsia and was put on bed rest. She was induced at 38 weeks of gestation. The proband, a boy, was delivered with a tight nuchal cord around his neck. He was noted to be experiencing some respiratory grunting but was resolved when treated with a saline bolus. Within 12 hours of birth, the proband was diagnosed as having a massive neonatal stroke which was not confirmed by CT testing. He suffered 47 respiratory arrests in an eight-week period. These episodes were resolved with positive pressure ventilation but caused bradycardia. Treatment with caffeine, phenobarbital and clonazepam did not resolve the apnoea. He also presented with hypertonia and exaggerated startle response to tactile stimuli, resulting in the diagnosis of hyperekplexia. He was tested for the common startle mutations in *GLRA1* but none were found. By nine months of age, the startle response resolved spontaneously and his tone became hypotonic. The proband also was diagnosed with a heart murmur, a large hiatal hernia and gastroesophageal reflux. By five years of age, his symptoms had resolved. His parents are unaffected.

Patient 3a and b (Australia; compound heterozygote for P108L+fs25 and W482R)
The eldest brother was born after a pregnancy complicated by pre-eclampsia. He had some neonatal episodes consisting of hypertonia and convulsive features considered to be seizures and treated as such. After settling over time the young infant was described as very tense and prone to trembling and episodes of stiffening. In childhood he was prone to episodes of generalised stiffness resulting in falls that were provoked by startle. At 5 years of age he was otherwise healthy with retention of the stereotypical nose-tap response. The younger brother was also born by Caesarean section after a pregnancy complicated with pre-eclampsia. At 6 hours of life, he began having hypertonic episodes with respiratory obstruction that were treated as seizures. At 13 weeks, he was a very tense, stiff baby who became very rigid when bathed, and some episodes were associated with cyanosis. The

generalised hypertonia and prominent nose-tap response were markedly improved with small doses of clonazepam. Treatment was discontinued during infancy without further symptoms.

Patient 4 (The Netherlands; compound heterozygote for L306V and N509S)
This 5-year-old girl was born after an uncomplicated pregnancy by vacuum extraction. Despite the presence of meconium-stained amniotic fluid, she had Apgars of 9 and 10 at 1 and 5 minutes. Seven hours post-partum she had her first period of cyanosis. These episodes of cyanosis occurred several times a day during the following days. She was initially treated with luminal lidocaine and clonazepam. During the first days of life periods of intermittent hypertonia and excessive startle responses to unexpected stimuli became evident. After the startle reflexes the stiffness increased and was accompanied by apnoea. Clonazepam was effective for the stiffness and the excessive startle. On neurological examination a head retraction reflex could be elicited. Motor milestones were slightly delayed but caught up. The startle attacks and the stiffness reduced in frequency during the first years of life. The family history for startling and stiffness was negative. MRI and EEG were unremarkable.

Patient 5 (The Netherlands; homozygote for T425M)
This 9-year-old boy was seen at the out-patient clinic at the age of 2 years. He was the first born of consanguineous parents. The pregnancy was unremarkable. During delivery there was presence of meconium-stained amniotic fluid, but his Apgar scores were 7 and 9 at 1 and 5 minutes. During the first days of life episodes of intermittent generalised stiffness were noted. At the first day of life a generalised tonic clonic seizure was observed lasting 1½ minutes. Treatment with phenobarbital was therefore started. An EEG during a seizure showed bilateral synchronous epileptic activity. During the hospital stay attacks of stiffness with jerks and cyanosis with duration of 1-3 minutes were observed. Between these attacks myoclonus was noted. Over the years the child suffered from episodes of stiffness and startling. During these episodes, he frequently turned blue and lost consciousness for short periods. These attacks were interpreted as breath-holding spells. On neurological examination this patient showed excessive startle responses with a bilateral pyramidal syndrome. He had persistent delays in cognitive and motor development. While the parents were unaffected, a daughter of a nephew of the mother was reported as having startle attacks.

Patient 6 (UK; heterozygote for S510R)
This first-born male, previously reported by Stephenson (1992, *Lancet* **340**: 430-431), developed severe convulsions at age 40 hours, up to six per day, most often precipitated by bathing. After immersion in warm water he would have rapid quivering of his limbs, an interrupted cry with fast grunting, and then silence with intense stiffening in a semi-flexed posture, leading to deep cyanosis, profound syncope with isoelectric EEG and junctional bradycardia, non-epileptic spasms with forcible urination, and a grey moribund appearance. Surface EMG during episodes showed repetitive giant compound muscle potentials (Pascotto & Coppola, 1992, *Epilepsia* **33**, 817-820) in the 'clonic' phase that became more closely spaced in the 'tonic' phase. Between these triggered attacks of gross hypertonia, he behaved appropriately, with normal muscle tone. Nose-tapping in the first months of life elicited excessive startle with no habituation. Clonazepam was only given for 48 hours during which time life-threatening syncopes continued: daily baths were then discontinued. After a finding of low CSF GABA (14nmol/l) vigabatrin (0.5g daily from age 7 months) led to marked improvement, insofar as he was then able to have a bath happily for the first time, but in retrospect this may have been spontaneous improvement, as at age 14 months, when he was on no medication, daily baths did not provoke stiffenings. The abnormal nose-tap response gradually waned and was minimal age 10 years. Aged 14 he no longer had as any excessive startle and does

not react to nose-tap. Although his father has no history of abnormal tone or startle, and neither parent reacts to nose-tap or sudden noise, it is now known that the mother was hospitalised in the first four months of neonatal life and has a permanent scar on her ankle which was caused by IV entry into the saphenous vein. Although the reason for this hospitalisation remains unclear, and all clinical notes have been destroyed or lost, the time-course of illness and apparent recovery are consistent with neonatal hyperekplexia caused by *SLC6A5* mutations. In summary, this boy had severe life-threatening neonatal hyperekplexia but between attacks was not a stiff baby. There was spontaneous remission in infancy with no residual tendency to startle.