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Mutations within the human *GLYT2* (*SLC6A5*) gene associated with hyperekplexia

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In newborns, symptoms of the hereditary neuromotor disorder hyperekplexia (startle disease, stiff baby syndrome, OMIM149400) include exaggerated startle responses and generalized muscle stiffness, which both gradually subside during the first months of life [1]. The disease has been shown to be caused by mutations in genes encoding subunits of the inhibitory postsynaptic glycine receptor (GlyR), *GLRA1* [2–4] and *GLRB* [5]. Orthologous disease alleles exist in mutant mouse lines [6–8], giving rise to phenotypes resembling the human disorder. In about 50% of the hyperekplexia patients, however, genetic defects in GlyR genes have not been found [9]. This suggested that additional genes might be affected in this hereditary disorder.

The efficacy of glycine-mediated synaptic inhibition is determined not only by the properties and number of postsynaptic GlyRs, but also depends on the activity of glycine

transporters that mediate the uptake of synaptically released glycine into glia cells and inhibitory nerve terminals, respectively [10,11]. Previously, we have shown that mice deficient for the neuronal glycine transporter 2 (GlyT2) develop a complex neuromotor phenotype postnatally that mimicks human hyperekplexia [12]. We therefore had suggested that some forms of hyperekplexia might result from mutations in the human *GLYT2* gene [11,12]. Here, we report the identification of *GLYT2* mutations in three different patient families, in which members were diagnosed with hyperekplexia. One of the mutations found is shown to result in a non-functional truncated GlyT2 protein that is retained intracellularly. Our findings identify the *GLYT2* gene as a candidate disease gene in humans.

Materials and methods

Human subjects. All patients who contributed to this genetic study were diagnosed clinically for hyperekplexia during infancy and displayed hallmark features of startle disease, including episodic muscular hypertonia and hyperreflexia [1]. Patients found to carry mutations of GlyR

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genes known to result in hyperekplexia [13] were excluded from further analysis. Consistent with the guidelines of the local committee on ethics, informed consent was obtained for all individuals participating.

DNA isolation and sequencing. DNA from hyperekplexia patients was isolated from peripheral blood, and short genomic fragments containing the respective exons of the *GLYT2* gene were amplified by PCR as described [14]. DNA amplimers were purified and subjected to sequencing using a capillary sequence analyzer (Megabase, Amersham Biosciences, UK). Electropherograms were analyzed with the software sequencer (Gene Codes Corp., Ann Arbor, USA) and compared to the genomic reference sequence (www.ensembl.org, ENSG00000165970). Mutations identified, and predicted genotypes were verified by MALDI-TOF mass spectrometry-based short-nucleotide polymorphism analysis [14].

Recombinant expression and cell culture. Expression constructs for human GlyT2 [15] were kindly provided by J.A. Morrow (Organon, Newhouse, UK). After subcloning into the pcDNA3.1 vector (Invitrogen; San Diego, CA), the respective mutations were introduced by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were verified by sequencing.

Human embryonic kidney (HEK) 293 cells were grown in DME medium with Glutamax (L-alanyl-L-glutamine) supplemented with 10% fetal calf serum (all reagents from Invitrogen, Carlsbad, CA) at 37 °C in a humidified 5% CO₂ atmosphere. Cells were seeded on the day prior to transfection into 6-well plates, and transfection was performed at 50–80% confluency using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer.

Immunocytochemistry. HEK293 cells were seeded onto poly-D-lysine-coated glass coverslips at a density of 1.5×10^5 cells per coverslip. Cells were transfected with plasmids coding for wild-type (WT) or mutant GlyT2. 24–48 h after transfection, the cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by incubation with 100 mM glycine in PBS for another 10 min. Cells were permeabilized, and unspecific binding was blocked by incubation with 2.5% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 in PBS for 15 min. The fixed cells were incubated for 1 h with primary antibodies as indicated, and for 15 min with fluorophore-conjugated secondary antibodies (Alexa488- or Alexa546-coupled anti-rabbit and anti-guinea pig IgG; Mobic, Goettingen, Germany) in blocking buffer. The coverslips were washed and mounted using Aquamount embedding medium (Polysciences, Warrington, UK). Immunostainings were analyzed at 63× magnification, using a AxioImager Microscope (Zeiss, Goettingen, Germany), equipped with an Apotome to acquire optical sections. All pictures are single optical sections averaged three times to reduce noise.

Cell surface biotinylation and Western blotting. Ca. 48 h after transfection, HEK293 cells were washed twice with PBS and incubated for 30 min at 4 °C with 1.5 mg/ml sulfo-succinimidobiotin (sulfo-NHS-biotin; Pierce, Rockford, IL) freshly dissolved in cold PBS. The reagent solution was then removed, and excess biotinylating reagent was quenched by incubating the cells for 10 min with cold PBS containing 100 mM lysine. Cells were lysed in solubilization buffer [25 mM Tris, pH 7.5, containing 150 mM NaCl, 1.0% (v/v) Triton X-100, 1 mM EDTA, 5 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Products, Hertfordshire, UK)] for 30 min at 4 °C with constant shaking. Lysates were centrifuged at 20,000g for 20 min at 4 °C, and the protein concentration in the supernatants was determined using a Bio-Rad (Hercules, CA) DC Protein Assay kit. Monomeric avidin beads (175 µl; Pierce) were added to the lysates (200 µg protein). After 1 h at 4 °C, the beads were washed four times with solubilization buffer and incubated with 60 µl of 2× loading buffer [100 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, and 0.2% (w/v) bromophenol blue] for 30 min at 37 °C. After centrifugation, the resulting supernatant was collected as the “biotinylated” fraction. Aliquots of the lysate (15 µg protein) and biotinylated fractions (30 µl) were separated by SDS-PAGE, transferred onto a Hybond-C Extra membrane (Amersham Biosciences, UK), and probed with a polyclonal antibody against the N-terminal region of the GlyT2 protein (1:1000) [12]. After washing, bound Igs were visualized with horseradish peroxidase-conjugated secondary antibodies using the ECL plus detection system (Amersham Biosciences).

[³H]Glycine uptake. Experiments were performed as described previously [16]. In brief, HEK293 cells were transfected with expression plasmids encoding WT or mutant GlyT2. One day after transfection, cells were trypsinized, and 1×10^5 cells were seeded onto poly-D-lysine-coated 48-well plates. Twenty-four to 48 h later, each well was washed once with 0.5 ml KHP buffer (Krebs-Hepes buffer; Hepes 10 mM, NaCl 120 mM, KCl 3 mM, CaCl₂ 2 mM, MgCl₂ 2 mM, and glucose 20 mM, final pH 7.3). Cells were incubated with 60 nM [³H]glycine (30 Ci mMol⁻¹, Perkin-Elmer Life and Analytical Sciences, Inc, Boston, MA) and various concentrations of unlabeled glycine in a final volume of 100 µl for 4 min at room temperature. Next, uptake buffer was aspirated, and the cells were washed twice with ice-cold buffer and lysed in 250 µl of 1% (w/v) SDS. Radioactivity in the lysates was measured by liquid scintillation counting. Non-specific uptake was defined as uptake determined in parallel with non-transfected HEK293 cells.

Results and discussion

To examine the potential involvement of *GLYT2* mutant alleles in hyperekplexia, we focussed on 12 patients who had been tested negative for disease causing GlyR subunit genotypes (data not shown). All of these patients displayed symptoms indistinguishable from hyperekplexia associated with GlyR mutations.

The human *GLYT2* gene (*SLC6A5*, OMIM604159) is located on the short arm of chromosome 11 (Chr 11p15.1–11p15.2) [15]. Its 16 exons are distributed over a region of about 21.4 Mb, with the first exon containing only the site for initiation of translation. DNA sequence analysis of all protein coding exons and splice junctions of *GLYT2* revealed coding mutations in three of the patients analyzed. First, patient #104-3 was heterozygous for adenosine at cDNA position 266 (266C → A) (Figs. 1A and 2B), which results in the amino acid substitution A89E (Fig. 2A). Furthermore, a single nucleotide exchange at coding position 2299 (2299C → T) within exon 16 (Figs. 1B and 2B) was identified in patient #89-3 that would lead to a G767R exchange (Fig. 2A). Interestingly, this patient was also heterozygous for I244T of the *GLRA1* gene. This *GLRA1* mutation, however, has been characterized as recessive, and thus cannot be causal for the hyperekplexia syndrome in this patient [17]. Finally, sequencing of exon 7 from index patient #96-3, a 4-month-old child, revealed a new heterozygous mutation at coding position 1130, where a cytosine to adenosine substitution (1130C → A) results in the generation of a stop codon (Figs. 1C and 2B), and thus to a predicted truncation (Y377X) of the human GlyT2 protein within extracellular loop 2 (EL2) (Fig. 2A). Notably, both parents (#96-1 and #96-2, see Fig. 1D) carried the same mutation heterozygously (Fig. 1E), although they had no medical records describing hyperekplexia-like symptoms. Furthermore, the patient's uncle (#96-4) was homozygous for the substitution 1130C → A, consistent with a *GLYT2* null genotype (Fig. 1E). Although he had erroneously been diagnosed with epilepsy shortly after birth, he had displayed all hallmark symptoms of hyperekplexia during the first year of his life. Subsequently, the patient recovered and now shows no detectable neuromotor impairments.

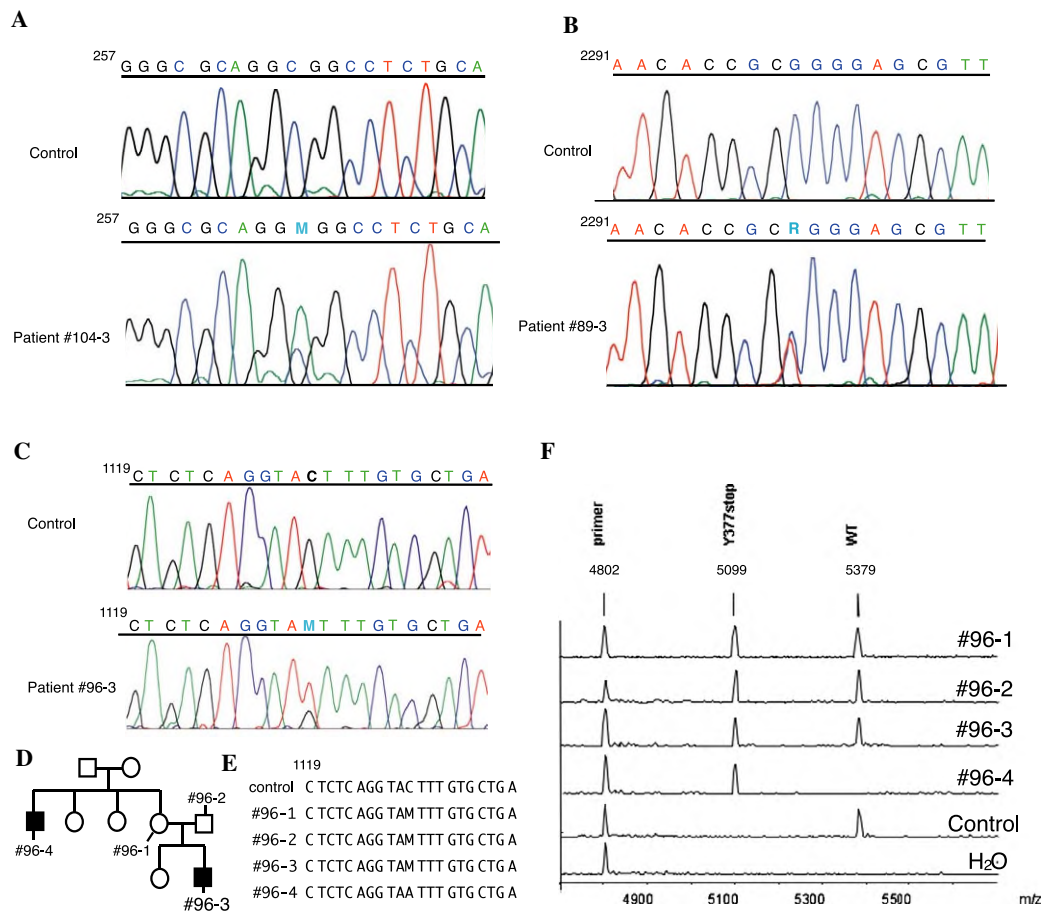


Fig. 1. Mutations found in the *GLYT2* genes of hyperekplexia patients. (A) Partial sequences of exon 2 of a control sample (upper sequence) and patient #104-3 (lower sequence). Here, a C to A substitution was identified (266C → A). (B) Partial sequences of exon 16 from control and patient #89-3 DNA, respectively. Note the heterozygous mutation C → T at position 2299 of the coding region. (C) Identification of a mutation in exon 7 of hyperekplexia patient #96-3. Partial sequences of exon 7 of a control person and the patient reveal the heterozygous single nucleotide exchange 1130C → A. (D) Pedigree of family #96. Males and females are represented by squares and circles, respectively. Shaded symbols represent individuals who are or were affected during early childhood. (E) Sequence comparison of the patient's closest relatives. Not only the patient but also both parents (#96-1 and #96-2) were found to carry 1130C → A substitutions within exon 7 heterozygously. Additionally, the patient's uncle (#96-4) was identified as an affected homozygous carrier. (F) Verification of the sequencing results of family #96 by mass spectrometry-based short-nucleotide polymorphism analysis.

The mutation and genotypes predicted on the basis of the DNA electropherograms were confirmed by MS-based SNP analysis [14]. As shown in Fig. 1F, the mutation was only found in the patients' DNA and/or their closest relatives, but not in control samples. Although two of the mutations found here (A89E and G767R) have been reported previously as rare *GLYT2* alleles ([18] and rs16906628), the accumulation of mutations in this gene observed here supports the hypothesis that defects in *GLYT2* cause the disease phenotype. In addition, comparison of the GlyT2 polypeptide sequences from different vertebrate species revealed that the regions in which the mutations were found show high evolutionary conservation (Fig. 2C). This suggests that these regions of GlyT2 are essential for transporter function.

To investigate the functional consequences of the *GLYT2* mutations found here, expression constructs for the WT and mutant GlyT2 proteins were generated. After transfection into HEK293 cells, transport activities of the recombinant proteins were analyzed by [³H]glycine uptake

measurements (Fig. 3A). Both, the A89E and G767R mutations did not significantly change transport characteristics. In contrast, the stop mutation Y377X caused a complete loss of transport activity. Thus, this *GLYT2* allele apparently produces a non-functional protein.

To examine expression of the mutant GlyT2 proteins directly, lysates prepared from transfected HEK293 cells were subjected to Western-blot analysis. WT GlyT2 as well as the A89E and the G767R mutant proteins gave rise to two major immunoreactive bands of about 100 and 75 kDa (Fig. 3B), which correspond to the glycosylated and core-glycosylated forms of the mammalian GlyT2 protein, respectively [19]. In addition, a minor immunoreactive band of about 170 kDa was seen, which might correspond to dimers of the 75 kDa species [20]. In contrast, expression of the Y377X construct resulted in a single immunoreactive band of 47 kDa, which is in agreement with the calculated mass of a GlyT2 polypeptide truncated in EL2 (Fig. 3B). We also analyzed the ratio of intracellular versus plasma membrane GlyT2 by cell surface labeling. Consistent with

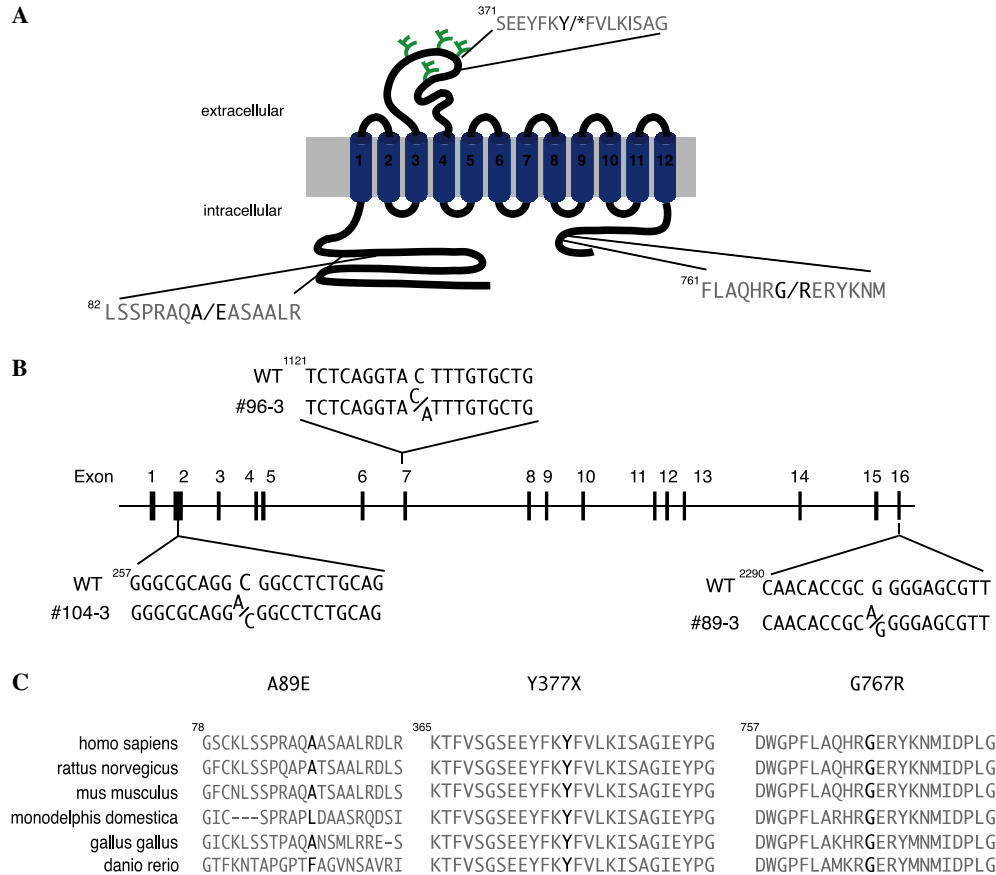


Fig. 2. Schematic representations of GlyT2 protein topology and gene structure indicating positions of the mutations shown in Fig. 1. (A) Positions and predicted partial amino acid sequences of the GlyT2 mutations identified. (B) Exons 2, 7, and 16 harbor hyperekplexia substitutions. Exons are represented as black boxes, and intronic regions as lines. Nucleotide numbers correspond to positions of the published cDNA sequence [15]. (C) Phylogenetic comparison of partial amino acid sequences of the GlyT2 regions containing the different mutations identified in hyperekplexia patients. Positions of amino acid exchanges are indicated in bold. Note that all three regions are highly conserved between vertebrate species.

previous findings [21], surface-biotinylated fractions were highly enriched for the 100 kDa glycosylated band (and putatively dimeric forms of the transporter) in case of the WT, A89E, and G767R transporters, whereas the Y377X fragment was excluded from this fraction (Fig. 3B). Together, these findings demonstrate that the Y377X GlyT2 fragment did not reach the cell surface but was retained intracellularly.

The results presented above were confirmed further by immunocytochemistry using antibodies directed against both, the N- and C-terminal domains of GlyT2. In transfected HEK293 cells, WT, A89E, and G787R transporter immunoreactivities all were located at the plasma membrane. In contrast, the Y377X protein was detected in intracellular compartments by the N-terminal antibody only (Fig. 3C). In cotransfection experiments, the Y377X mutant, but neither the WT nor the A89E and G767R variants of GlyT2, colocalized with a DSRred marker protein that is retained in the endoplasmic reticulum (ER). This indicates that the truncated polypeptide accumulated within this subcellular compartment (Fig. 3D).

In conclusion, here we identified three distinct mutant alleles of the human *GLYT2* gene in hyperekplexia

patients. Our results extend the number of possible disease loci for this genetic disorder. As a consequence, the *GLYT2* gene should be investigated, in addition to *GLRA1* and *GLRB*, in genetic analyses of affected individuals. Importantly, *GLYT2* mutations may cause hyperekplexia by different mechanisms. For example, substitution Y377X causes truncation of the GlyT2 protein in its EL2 region, and thereby fully abolishes transport activity. This is consistent with reduced GlyT2 function causing hyperekplexia, as suggested previously from the analysis of GlyT2 deficient mice [12]. In contrast, the two mutations found in the N- and C-terminal domains of GlyT2 did not impair the transport activity or subcellular localization of the recombinant protein generated in HEK293 cells. These substitutions might be pathogenic by affecting transporter trafficking, functional interactions with scaffolding proteins, or modulation by kinases or phosphatases in neurons. Moreover, mutations in a single *GLYT2* allele might be sufficient to cause hyperekplexia in humans, as patient #96-3 had developed hyperekplexia in early childhood although being only heterozygous for the Y377X allele. This contrasts observations with mice carrying only a single func-

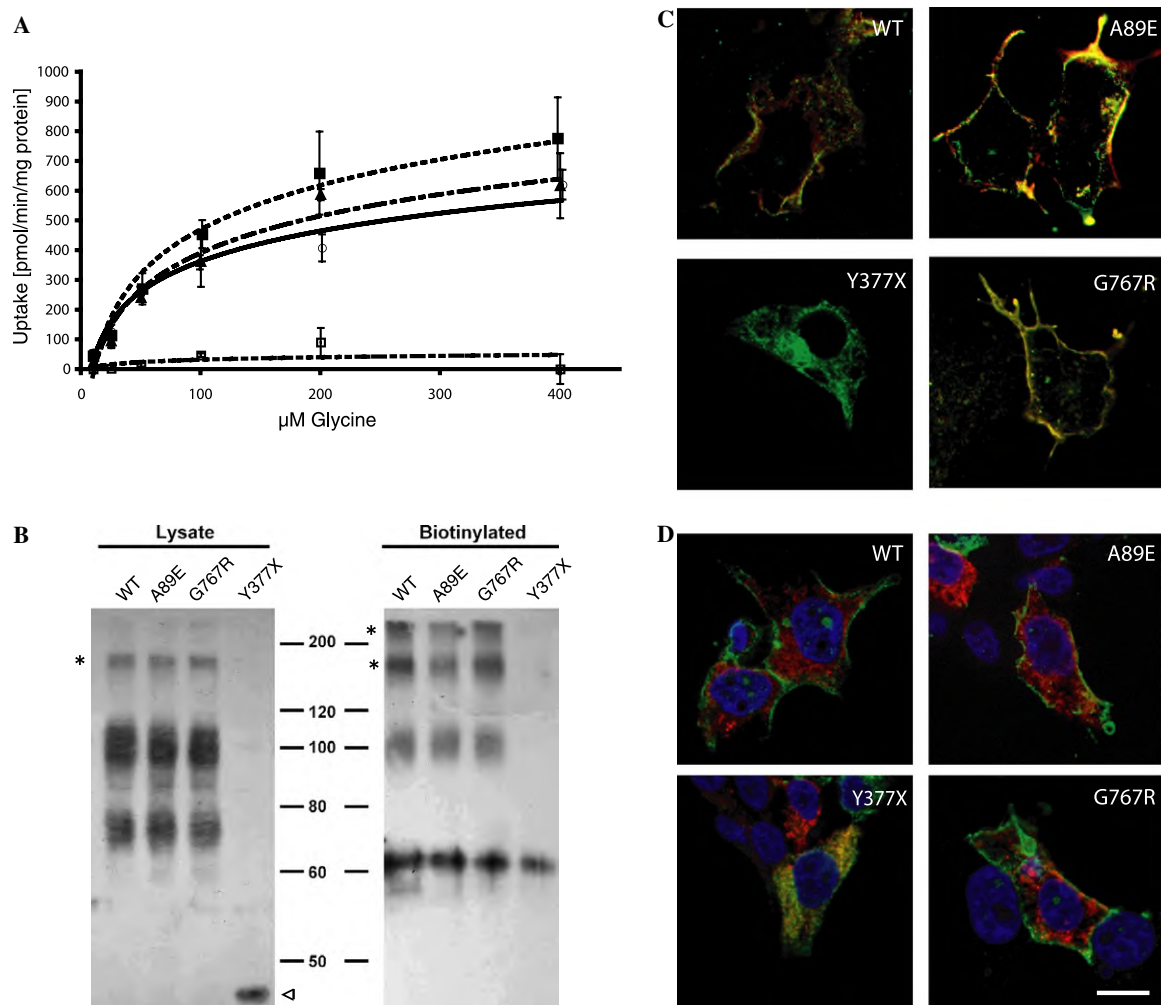


Fig. 3. [3 H]Glycine uptake activity and subcellular distribution of mutant GlyT2 proteins, (A) HEK293 cells were transfected with GlyT2 expression constructs as indicated (m WT, j A89E, h Y377*, s G767R), and GlyT2 specific [3 H]glycine uptake was determined as described [16]. (B) Western-blot analysis of total lysates and fractions of surface-biotinylated proteins prepared from HEK293 cells transfected with the GlyT2 expression constructs indicated. Lysates and surface-biotinylated fractions were prepared 48 h after transfection. The bands labeled with asterisks are likely to correspond to GlyT2 dimers. The 45 kDa Y377X transporter fragment is indicated by a triangle. (C) HEK293 cells were transfected with the GlyT2 expression constructs indicated, and the subcellular localization of the recombinant proteins was visualized by double-immunostaining with antibodies raised against both the N-terminal domain (green) and the C-terminal intracellular tail (Chemicon, CA, red). Note that the Y377X protein was recognized only by the antibody raised against the N-terminal domain. (D) HEK293 cells were cotransfected with the indicated GlyT2 expression constructs and an ER-retained DS-Red marker cDNA (BD-Bioscience, CA, red). GlyT2 protein was visualized by the N-terminal GLYT2 antibody (green). DAPI staining was performed for better orientation (blue). The WT GlyT2 and the A89E and G767R mutant proteins were enriched at the plasma membrane, whereas the Y377X polypeptide colocalized with the DS-Red ER marker intracellularly. Scale bar: 10 μ M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tional *GLYT2* gene; such animals are phenotypically normal [12]. Species differences in *GLYT2* gene expression or dominant-negative effects of the mutant proteins, such as described for the human norepinephrine transporter [22], might explain these divergent observations. In this context, it should be noted that the diagnosis of human hyperekplexia is compromised by the low incidence of this hereditary disorder [1,3], and a high variability of the disease phenotype [4,23]. Furthermore, in many patients typical hyperekplexia symptoms are detectable only transiently during childhood. Together these difficulties in identifying patients suffering from this neurogenetic disease might explain the weak correlation seen here

between reported disease incidence and genotypes in family #96 members. Clearly, further work will be needed to understand how mutations in the *GLYT2* gene impair glycine-mediated inhibition in vivo to cause a disease phenotype.

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