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ARTICLE

A novel recessive hyperekplexia allele *GLRA1* (S231R): genotyping by MALDI-TOF mass spectrometry and functional characterisation as a determinant of cellular glycine receptor trafficking

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Hyperekplexia or startle disease (stiff baby syndrome, STHE) is a hereditary neurological disorder characterised by an exaggerated startle response and infantile muscle hypertonia. Several autosomal dominant and recessive forms of the disorder have been associated with point mutations in *GLRA1*, the human gene encoding the α1 subunit of the inhibitory glycine receptor. Here, we describe a recessive point mutation (C1073G) in exon 7 of *GLRA1* leading to an amino acid exchange of serine 231 to arginine in transmembrane region TM1. The mutation was detectable by restriction digest analysis of genomic PCR amplimers by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Genotyping of family members was performed using an allele specific primer extension assay in combination with MALDI-TOF-MS and confirmed by conventional DNA sequencing. These studies demonstrate the broad applicability of MALDI-TOF-MS as a comparative screening tool applicable to the analysis of allelic gene variants. In comparison to the wild type α1 subunit, biochemical, electrophysiological, and confocal microscopy data indicate a reduced integration of functional $\alpha 1^{S231R}$ glycine receptors into the cell surface membrane upon recombinant expression. Apparently, the amino acid exchange S231R influences glycine receptor biogenesis and cellular trafficking by introducing a positive charge into transmembrane region TM1.
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Introduction

Strychnine-sensitive glycine receptors (GlyRs) are important mediators of neuronal inhibition in mammalian brain stem and spinal cord where they contribute to the central control of muscle tone. Due to homology in structural architecture and functional features, GlyRs belong to the ligand gated ion channel superfamily which also includes the γ-aminobutyric acid ($GABA_AR$ and $GABA_cR$), nicotinic acetylcholine (nAChR), and serotonin ($5\text{-}HT_3R$) receptor.[1–5] Members of this family comprise five membrane spanning subunits surrounding a central ion pore delineated by the transmembrane segment TM2 of the monomers. Homologous to the other members of the receptor family, GlyR subunits possess a large extracellular N-terminal domain followed by four transmembrane regions (TM1–4) and a short extracellular C-terminal portion.[6,7] As analysed in

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rodents, the adult GlyR isoform represents a protein complex assembled from three ligand-binding α1 and two structural β subunits.[8] In addition, α2, α3, and α4 subunit genes have been identified.[9–11] Determinants of agonist (glycine, β-alanine, taurine) and antagonist (strychnine) binding have been assigned to the extracellular N-terminal domain of the α subunits.[6] Glycinergic agonist responses also depend on amino acid determinants within the loops linking segments TM1 and TM2 as well as TM2 and TM3.[12,13] Opening of the receptor channel is induced by agonist binding, leading to hyperpolarisation of the cell due to an influx of chloride ions.

Symptoms of the human neurological disorder, hyperekplexia (startle disease, stiff baby syndrome, STHE, OMIM #149400 and *138491), resemble sublethal strychnine intoxication.[14,15] Affected infants display extreme muscle stiffness and pronounced startle reactions which may result in fatal apneic attacks. During the first year of life, muscle tone returns to normal, while excessive startling, which may result in immediate, unprotected falling, may persist throughout life.[15,16] Hyperekplexia is associated with mutations of the human *GLRA1* gene encoding the GlyRα1 subunit.[14,17] *GLRA1* is localised on chromosome 5q31.2. The coding region is distributed over nine exons.[10,18–20] Hyperekplexia mutations cluster in exons 7 and 8, inducing amino acid substitutions within a region ranging from TM1 to the extracellular loop connecting segments TM2 and TM3.[17] While most of the hyperekplexia alleles identified follow a dominant trait,[19,21–27] a recessive variant of the disease has been associated with a T1112A mutation leading to an I244N exchange at the C-terminal border of TM1.[22] In addition to the missense alleles, a *GLRA1* null mutation has been associated with recessive hyperekplexia.[15] In mouse models resembling hyperekplexia, mutations of the GlyR α- and β-subunit genes have been identified.[12]

Molecular analysis of GlyR mutant alleles has largely relied on SSCP or direct sequencing of genomic DNA, both methods being time consuming and expensive. Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) has evolved into a technique widely applicable for accurate mass determination in protein chemistry.[28] Introduction of picolinic acid derivatives as matrices for the analysis of nucleic acids has expanded the advantages of MALDI-TOF-MS into molecular biology and genetic analysis.[29,30] Due to the specific molecular weight of the four DNA nucleobases, the detection of DNA sequence alterations relies on comparative mass analysis. Thus, MALDI-TOF-MS combined with molecular techniques offers an excellent opportunity for mutation detection and genotyping, as well as suitability for high throughput screening.

Here, we report a novel *GLRA1* mutant allele C1073G causing recessive hyperekplexia. The missense mutation results in the substitution of serine 231 for arginine in transmembrane region TM1. The mutation was detectable by MALDI-TOF-MS analysis of sequence specific restriction digests. Genotyping of the family was performed by a primer extension assay specific for the novel allele in combination with MALDI-TOF-MS. Recombinant expression studies suggested that alterations of receptor biogenesis and trafficking to the cell surface of $\alpha1^{S231R}$ GlyRs represent the molecular defect of this mutation.

Materials and methods

Patient samples and restriction digest based mutation detection

Chromosomal DNA was extracted from EDTA-blood by means of the RapidPrep™ Macro Genomic DNA Isolation Kit (Pharmacia) according to the manufacturer's instruction. Three overlapping PCR fragments were generated in separate reactions using specific primer pairs. PCR No.1: forward 5′-GTA AAT TCA CCT GCA TTG AGG-3′, reverse 5′-ACA ATG AGC AGG CTG GG-3′ PCR No.2: forward 5′-GGG TTA CTA CCT GAT TCA GAT G-3′, reverse GTG GTG ATG CCT AGG CC; PCR No.3: forward 5′-GAT GCT GCA CCT GCT CG-3′, reverse 5′-CTT GGC AGA GAT GCT CG-3′ (all primers from MWG Biotech). Genomic DNA was denatured at 95°C (5 min) followed by 30 cycles at 94°C (30 s), 55°C (30 s), 72°C (30 s), and 5 min at 72°C using an Applied Biosystems (ABI) – Perkin Elmer GeneAmp 9600 Thermocycler. Each reaction contained 200 ng template DNA, 0.5 μM of forward and reverse primer, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 300 μM dNTPs and 5 U of *Taq* DNA polymerase (Eurobio). PCR amplimers were purified by ethanol precipitation. PCR fragment No. 2, spanning bp 47 to bp 163 of *GLRA1* exon 7, was digested with *Fok*I (2U, NEB) and *Xho*II (1 U, Promega) in buffer C (Promega) in presence of 200 ng/ml acetylated bovine serum albumin at 37°C for 16 h.

Primer extension reaction

Each PCR contained 0.625 μM forward (5′-AGC GGC AGA TGG GTT ACT ACC TGA TT-3′) and reverse primer (5′-TGG TCA TGG TGA GCA CAG TGG TGA T-3′), 50 ng of genomic DNA template, 1 U Expand High-Fidelity in the supplied buffer 2 (Boehringer) and 200 μM dNTPs. The thermal cycling conditions were 94°C for 4 min, 30 cycles at 94°C (60 s), 55°C (30 s), 72°C (30 s), and 72°C (3 min). Subsequently, the samples were treated with 1 U shrimp-alkaline phosphatase and 1 U exonuclease I (Biozym) at 37°C (45 min) followed by heating to 96°C (5 min). The PCR products were used for the primer extension reaction. The extension mix contained 40 pmol forward extension primer 5′-GAT TCA GAT GTA TAT ATT CCC AG-3′, 3 U Thermosequenase (Pharmacia), 400 μM dGTP and 400 μM ddCTP in Thermosequenase reaction buffer. Extension reactions were performed at 94°C (4 min), and 30 cycles at 94°C (10 s), 60°C (10 s) and 72°C (10 s).

MALDI-TOF-MS analysis

Restriction digests and primer extension products were purified using pipette tips with immobilised C18 silica (ZipTips, Millipore). Tips were prewetted with 50% (v/v) acetonitrile and equilibrated in 100 mM triethylammonium acetate buffer, pH 6.8, (TEAA). After addition of TEAA to achieve a final concentration of 10 mM, the oligonucleotides were bound to the C18 material. Immobilised oligonucleotides were washed extensively with 100 mM ammonium acetate, pH 6.7, and eluted with 20% (v/v) acetonitrile. Aliquots of the purified samples were spotted onto matrix crystals of 3-hydroxypicolinic acid on a stainless steel target and air dried.

Mass determinations were performed on a Biflex™ III MALDI-TOF-MS (Bruker Daltonik) equipped with a nitrogen laser (λ=337 nm) and delayed extraction. Laser-desorbed positive ions were analysed following acceleration by either 20 kV in linear or 19 kV in reflectron mode. External calibration was performed by using an oligonucleotide mixture as a weight standard. Usually, 30 individual spectra were averaged to produce one mass spectrum.

DNA sequencing

Sequence analysis was performed using an Automatic DNA Sequencer (ABI – Perkin Elmer, 377A). The cycle sequencing reaction was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Kit according to the manufacturer's protocol. PCR amplimers were purified by phenol/chloroform extraction or using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Site directed mutagenesis and recombinant expression

Using a PCR-mediated site-directed mutagenesis approach, a single nucleotide exchange at position S231 was introduced into a plasmid (CMV-pRK5) carrying the α1 subunit cDNA of the human glycine receptor (pRK5 – *GLRA1*, wild type).[13] The mutation was verified by direct sequencing. The plasmids carrying *GLRA1* cDNA and the mutated variant, *GLRA1* C1073G (pRK5 – *GLRA1* S231R), were transfected transiently into HEK293 cells (1 μg plasmid DNA/cm^2 dish) using a modified calcium-phosphate precipitation method.[31] A plasmid carrying green fluorescent protein (pCMV-GFP) was co-transfected in all experiments for easy visualisation of transfected cells during electrophysiological recordings and to control for transfection efficiency. For immunoblot analysis, crude cell membranes and cell lysates were prepared from transfected cells on day 3 post-transfection.[26,32] Samples were heated to 95°C for 10 min, separated by SDS – PAGE (30 μg protein/lane) and transferred to a nitrocellulose membrane. GlyR protein was detected using the monoclonal antibody 4a and Cy5-labelled GaM F(ab$_2$) (Dianova). Immunoreactivity was visualised using a phosphoimager (STORM, Molecular Dynamics).

Electrophysiology

Whole cell currents were recorded using the standard patch clamp technique.[33] Signals were acquired using a EPC9 recording system (HEKA Electronics), filtered at 2.3 kHz (−3 dB value, 8-pole Bessel filter), digitised and sampled at 10 kHz using the Pulse data acquisition system (Digidata 1200 interface, HEKA Electronics). Recordings were made at room temperature (22 – 24°C). Patch-electrodes were pulled from filamented borosilicate glass (Sutter, ID: 0.69 mm, OD: 1.2 mm). Final electrode resistances ranged from 3 – 5 MΩ. Series resistances varied from 15 – 25 MΩ and were not compensated. For recording, coverslips with transfected cells were placed into a recording chamber and perfused at 2 ml/min with external bath solution (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES. The intracellular solution (pH 7.4) contained 120 mM CsCl, 20 mM TEACl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES. Agonists were applied using a U-tube system.[34] Fresh stocks of glycine were prepared at the day of recording and diluted to final concentrations with bath solution.

Cell staining and confocal microscopy

HEK293 cells were grown on poly-L-lysine (Sigma) coated glass coverslips (12 mm, Sarstedt) at 40 000 cells/coverslip. At 3 days after transfection (0.25 μg plasmid DNA/coverslip), cells were fixed in ice-cold methanol/acetic acid and stained using the monoclonal antibody 4a followed by Cy3-conjugated secondary antibody (Dianova). Immunofluorescence was examined with a confocal laser scanning microscope (Molecular Dynamics).

Subjects

Patient DE is a 6-year-old son of apparently healthy consanguineous parents of Iranian origin (Figure 1). Three days after birth, DE became conspicuous by nocturnal generalised jerks accompanied by short-windedness and slight trembling. Initially, these states were interpreted as grand mal seizures and he was subjected to anticonvulsive therapy first with clonazepam following a combination of phenobarbitone and carbamazepin. At 2 years of age, he became free of fits. In 1996, the boy was admitted to the North German centre of epilepsy for assessment because of a tendency to fall leading to injuries and fractures. The boy displayed increased muscle proprioceptive reflexes, exaggerated head retraction jerks, myocloni, and atactic gait disturbances. In one incident, a fall occurred caused by a severe startle reaction upon sudden opening of a door during physical examination. Social behaviour was reportedly impaired due to considerable fear of unexpected falling. EEG recordings were normal. Treatment with clonazepam resulted in amelioration of clinical symptoms within days. DE exhibited mild mental retardation. The 8-year-old sister of DE did not exhibit any neurological symptoms. Further relatives were not available for examination.

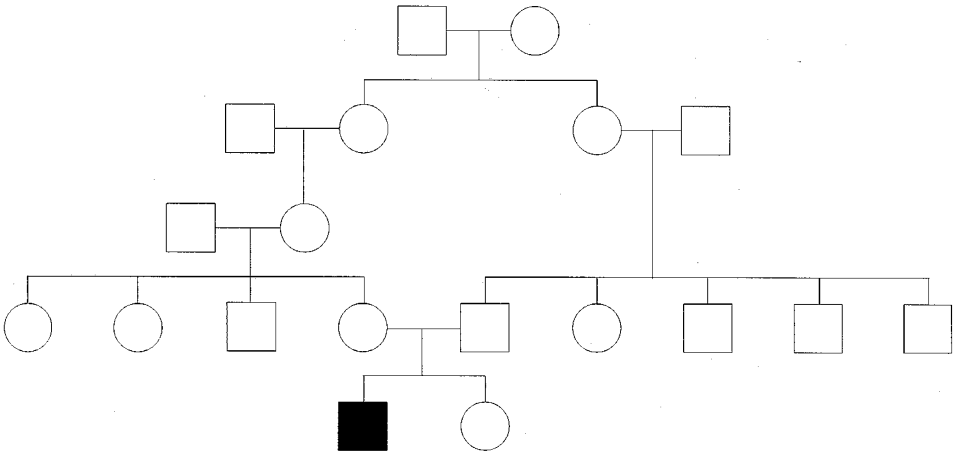


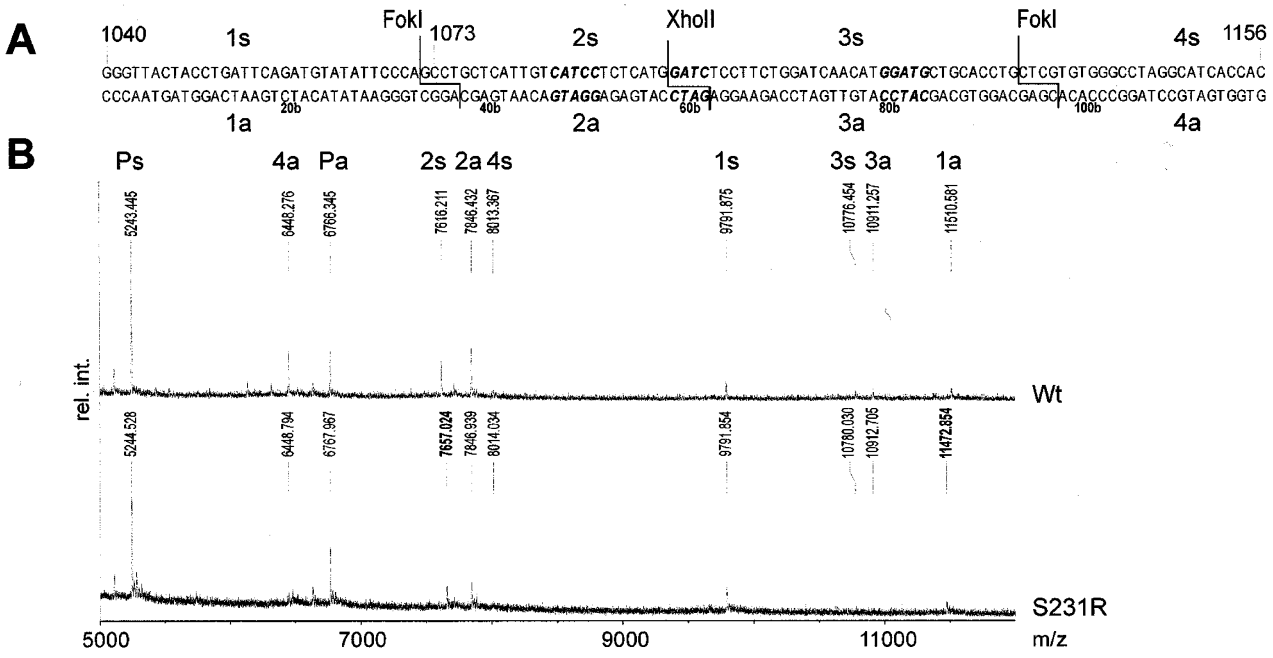
Figure 1 Pedigree of the family of DE. The filled symbol represents patient DE; birth order was altered to preclude identification of participating subjects.

Results

Hyperekplexia or startle disease (STHE) is a neurological disorder characterised by infantile muscle hypertonia and exaggerated startle responses.[19] Dominant and recessive forms of this disorder have been identified, most of which are caused by point mutations of the *GLRA1* gene.[5] Suffering from an apparently recessive form of hyperekplexia, patient DE is a 6-year-old son of consanguineous parents of Iranian origin (Figure 1). To assess the genetic basis of this hyperekplexia variant, we focused on exon 7 of *GLRA1* (exon numbering according to[26]), the most probable region for a mutation, for the novel MALDI-TOF-MS based DNA screening approach.

Overlapping amplimers of 120 bp were generated in three separate PCRs completely covering exon 7 (nucleotide positions 994–1208), each providing mutation information on DNA stretches of approximately 80 bp. Purified PCR products were digested with restriction enzymes to generate a defined set of oligonucleotides. For example, PCR fragment no. 2 was digested with *Fok*I and *Xho*II, producing four sense and four antisense fragments of defined masses (Figure 2A). A control individual referred to as wild type produced an identical restriction digest mass pattern (Figure 2B, upper spectrum). All expected peaks as well as the remaining PCR primers were detected with a maximal mass difference of less than 3 Da from theoretical values (Figure 2C). In the MALDI-TOF-MS spectrum of patient DE, all wild type peaks were recovered except for the two peaks representing fragments 2s and 1a. In comparison to the control DNA mass pattern, two additional peaks had appeared with a mass difference of about 40 Da, compatible with a point mutation in the 4 bp overlapping segments of fragment 2s and 1a (nt 1072–1075; sense: GCCT; antisense: AGGC). As the nucleotides cytosine and guanosine possess a mass difference of 40 Da, the increase in molecular mass of fragment 1a and corresponding decrease of fragment 2s of about 40 Da as compared to the wild type mass signals indicated a C to G exchange at nucleotide position 1073 or 1074. It should be noted that the restriction enzym *Fok*I cleaves DNA 13 bases upstream of its recognition sequence CATCC. For this reason, *Fok*I may cut DNA even when the cleavage site is mutated.

To specify the position of the mutation, genomic DNA sequencing of *GLRA1* exon 7 was performed. We thus identified a C to G transversion at position 1073 of the open reading frame leading to a non conservative amino acid substitution of serine 231 for arginine in transmembrane region TM1 of the GlyR α1 subunit. Based on this information, patient DE and his family were genotyped for the novel GLRA1 allele by site-specific primer extension reactions and MALDI-TOF-MS analysis of the extension products (Figure 3). The forward extension primer used for the determination of nucleotide position 1073 produced a peak corresponding to a molecular mass of 6421 Da. The extension primer was designed to anneal to the target DNA directly in front of the mutation site. In case of the wild type sequence, incorporation of a ddCTP was found to terminate the primer extension reaction, thereby extending the primer to a mass of 6694 Da. On the other hand, when a C to G exchange was present, the primer was elongated by a dGTP and terminated in the next extension step by incorporation of the following ddCTP, producing a mass of 7023 Da (Figure 3A). In contrast to the wild type allele (6694 Da), patient DE was found to be homozygous for the mutation with a single mass peak of 7023 Da detectable (Figure 3B). All non-symptomatic members of the family analysed were found to be heterozygous for the allele as indicated by the presence of both peaks of 6694 and 7023 Da, respectively (Figure 3B). The unextended primers remaining from the extension reaction served as an internal calibration standard. All of the MALDI-TOF-MS based genotyping results were confirmed



DNA fragment	wild type calculated mass [Da]	wild type measured mass [Da]	mutant S231R calculated mass [Da]	mutant S231R measured mass [Da]
primer sense	5243.452	5243.445	5243.452	5244.528
4 antisense	6448.241	6448.276	6448.241	6448.794
primer antisense	6766.461	6766.345	6766.461	6767.967
2 sense	7615.906	7616.211	**7655.930**	**7657.024**
2 antisense	7845.095	7846.432	7845.095	7846.939
4 sense	8013.167	8013.367	8013.167	8014.034
1 sense	9790.430	9791.875	9790.430	9791.854
3 sense	10778.962	10776.454	10778.962	10780.030
3 antisense	10911.064	10911.257	10911.064	10912.705
1 antisense	11511.483	11510.581	**11471.458**	**11472.854**

Figure 2 Restriction based mutation detection of a *GLRA1* mutant allele by MALDI-TOF-MS. (**A**) Sequence of wild type DNA fragment of GLRA1 exon7 No.2 (bp 1040–1156) including the restriction sites for *Fok*I and *Xho*II. While *Xho*II possesses recognition and cleavage sites at an identical position, *Fok*I cuts 13 bp upstream of its recognition sequence CATCC. Recognition sequences are shown in bold italic. The resulting theoretical fragments are numbered from 1s (sense) to 4s and 1a (antisense) to 4a, respectively. (**B**) MALDI-TOF-MS spectra of *GLRA1* exon 7 PCR No.2 (bp 1040–1156) digested with *Fok*I and *Xho*II: Samples from control individual (top) and patient DE (bottom). The informative peaks are labelled by 1s to 4s, 1a to 4a and PCR primers by Ps and Pa. The peaks of 7657.024 Da and 11472.854 Da displaying the *GLRA1*(C1073G) allele are presented in bold. (**C**) Calculated and measured masses of DNA fragments from *GLRA1* (exon 7, no. 2, bp 47–163) wild type and allele C1073G after restriction digest. Fragment 2 sense (C to G exchange, Δm=+40.024 Da) and fragment 1 antisense (G to C exchange, Δm=−40.024 Da) cover the mutation S231R. (**D**) Sequencing results of DNA fragments from *GLRA1* (exon 7): Reverse sequences (nt 1075–1071) of wild type DNA (left panel) and DNA heterozygous for allele C1073G (right panel) are depicted.

by conventional DNA sequencing. The wild type and the heterozygous DNA for the C1073G allele are depicted in Figure 2D. This sequence motif has not been found in more than 130 unrelated individuals, excluding this exchange to be a common population polymorphism.

For functional analysis, the mutation C1073G was introduced by site directed mutagenesis into a plasmid carrying the complete *GLRA1* cDNA. Wild type and mutated plasmid were transiently transfected into HEK293 cells. Crude cell membranes and cell lysates were analysed by immunoblotting using the monoclonal antibody 4a (mAb4a) which recognises an N-terminal extracellular epitope of GlyRα1 (aa 96–105,[35], Figure 4A). A marked reduction of receptor expression was observed in membrane fractions from cells transfected with the mutant plasmid (GlyRα1^{S231R}) compared to cells carrying GlyRα1 (Figure 4A, lanes 1 and 2). In contrast, overall receptor expression was indistinguishable for whole cell lysates (Figure 4A, lanes 3 and 4), indicating that surface accumulation of GlyRα1 was affected by the mutation.

For further functional characterisation of mutant α1^{S231R} GlyRs, whole cell glycinergic currents were recorded from transfected cells.[34] Cells carrying GlyRα1^{S231R} exhibited a strong reduction of the maximal current (I_{max}) as compared to cells transfected with the wild type construct (Figure 4B),

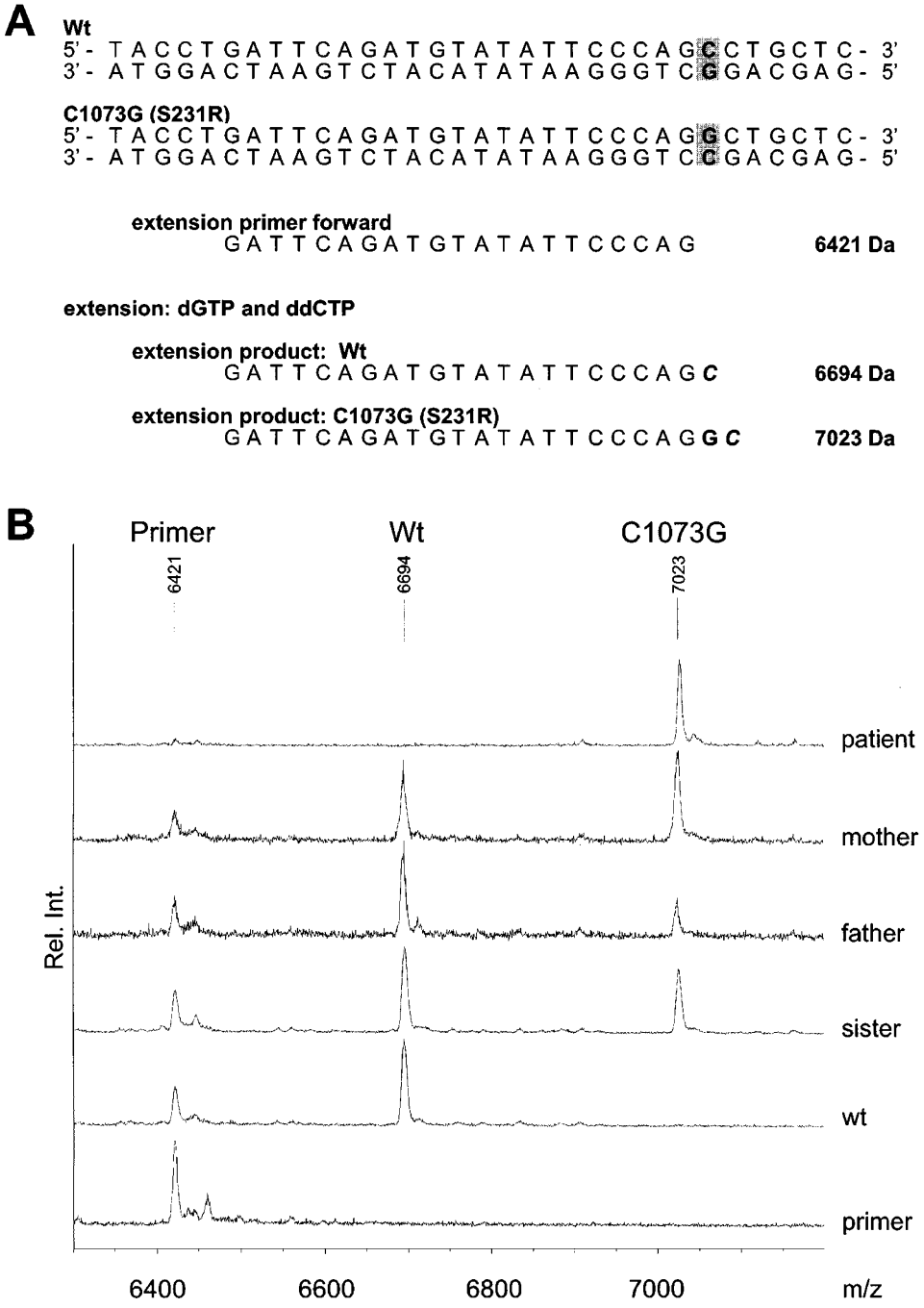


Figure 3 MALDI-TOF-MS based genotyping of allele *GLRA1* C1073G. (**A**) Schematic design of the primer extension reaction for allele *GLRA1* C1073G. (**B**) MALDI-TOF-MS spectra of patient DE and his sister, mother and father, a healthy control person, and the primer without extension reaction (from top to bottom). The unextended primer possesses a molecular mass of 6421 Da. The wild type allele is represented by the molecular mass of 6694 Da, whereas the mass of 7023 Da is indicative for the point mutation C1073G.

consistent with an altered accumulation of functional mutant GlyRs on the cell surface.

Confocal microscopy of transfected HEK293 cells immunostained with mAb4a was performed to analyse the subcellular distribution of wild type and mutant receptors. Consistent with recording data, cells transfected with wild type GlyR exhibited strong immunoreactivity at the circumference of the cell, indicating a predominant localisation in

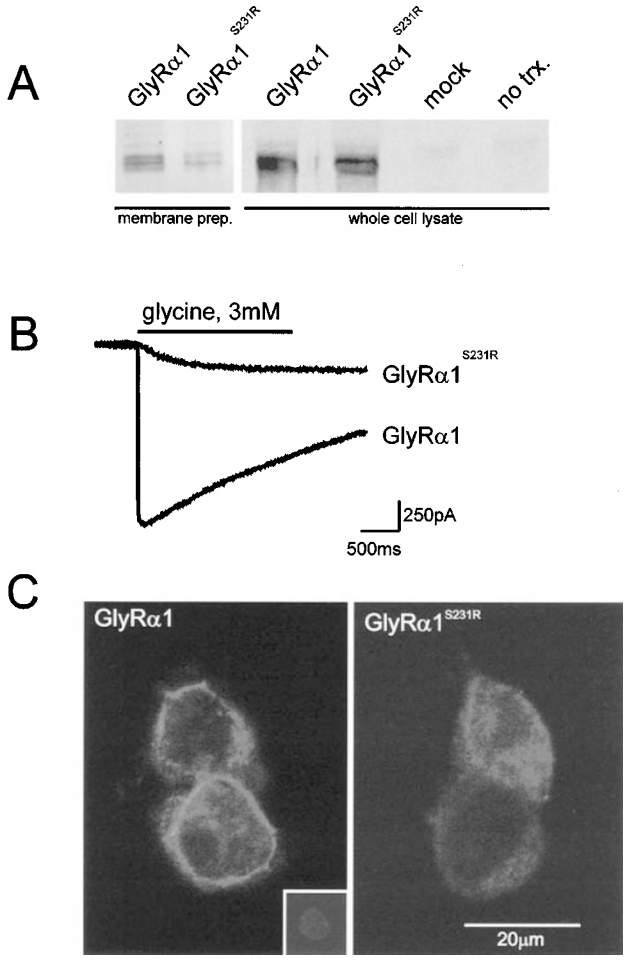


Figure 4 Functional characterisation of the GlyRα1^{S231R}. (**A**) Immunochemical detection of recombinant GlyRα1 and GlyRα1^{S231R} receptors in membrane fractions and in whole cell lysates. Crude membrane fractions and whole cell lysates of HEK293 cells transfected with GlyRα1 or GlyRα1^{S231R} were separated by PAGE and GlyRs visualised by immunoblotting using the GlyR-specific monoclonal antibody mAb4a. In membrane fractions, the signal at 45 kD was weaker with mutant receptors than with wild type receptors (left panel). In contrast, intensities of immune signals were comparable for both receptors in whole-cell lysate preparations (right panel). Mock treated cells transfected with CMV-GFP and untreated cells (not exposed to plasmid DNA) served as controls. (**B**) Patch-clamp analysis of HEK293 cells expressing GlyRα1 or GlyRα1^{S231R}. Glycine-induced whole cell currents were recorded from HEK293 cells 48–72 h following transfection using a saturating concentration of agonist (3 mM glycine). Cells transfected with the mutant plasmid displayed a strong reduction in the maximum current, I_{max} (I_{max} S231R: 69.9±23.3 pA, range: 14–219 pA, n=9 cells; I_{max} wild type: 6.3±1.6 nA, range: 1.8–12 nA, n=7 cells; $P<0.001$, t-test). (**C**) Confocal microscopy of cells expressing GlyRα1 or GlyRα1^{S231R}. Confocal laser scanning microscopy of transfected HEK293 cells immunostained with mAb 4a was performed to analyse subcellular distribution of wild type and mutant receptors. In cells transfected with wild type GlyR, mAb4a immunoreactivity was detected at the circumference of the cell (left panel, insert: control staining without primary antibody). In contrast, in cells transfected with mutant α1^{S231R}, the antigen was localised to intracellular compartments mostly (right panel).

the plasma membrane (Figure 4C, left panel). In cells transfected with the mutant α1^{S231R}, most of the receptor antigen was intracellular (Figure 4C, right panel).

Discussion

As allelic variants of the human GLRA1 gene underlie the neurological disorder hyperekplexia (reviewed in:[14,17]), genetic screening for hyperekplexia alleles has become a highly informative tool supporting the clinical diagnosis of hypertonic disorders. However, a routine screening for candidate alleles essentially depends on both, a comprehensive knowledge of disease associated genetic polymorphisms as well as efficient and reliable detection systems. Single stranded conformation polymorphism (SSCP) analysis has been widely used to screen patients suffering from different hereditary disorders for allelic gene variants.[25] As this analysis relies on electrophoretic mobility shifts of two species of partially refolded single stranded DNA, changing temperature conditions or even minor sequence alterations may affect informative DNA secondary structures. Although compared to DNA sequencing, SSCP possesses low detection rates (*ca.* 70%), it represents a first approach for genetic characterisation due to its simplicity and low costs. In contrast, direct DNA sequencing using the dideoxy nucleotide technique is most reliable, yet time consuming and expensive. Moreover, determination of heterozygous alleles still remains a difficult task with simultaneous bands influencing the mutual intensities on the sequencing gel.

MALDI-TOF-MS offers a direct approach to DNA analysis of defined sequences. The combination of PCR, restriction digests and analysis by MALDI-TOF-MS, should be a suitable way to detect unknown mutations. While mass differences between mutant and reference spectra are indicative of alterations of nucleotide sequences, reliability of MALDI-TOF-MS analysis is further enhanced by the fact that both, the DNA sense and antisense strand generated by PCR and digest, are measured simultaneously. Except for an inversion, an altered DNA sequence will result in a mass shift of a peak or an appearance of a novel peak. The occurrence of a second complementary shift or peak is sufficient for the unambiguous detection of a genetic variant. As the human genome project progresses, more and more attention is paid to the genetic heterogeneity of individuals and populations.[36] It is expected that single nucleotide polymorphisms (SNP) emerge with a frequency of at least 1 : 1000. MALDI-TOF-MS analysis of SNPs or point mutations offers the potential for reliable high-throughput screening. Here, the application

of a primer extension assay to determine the *GLRA1* (C1073G) mutation in a patient and his family has been successful. Both alleles were reliably distinguishable as the peaks representing the alleles had a mass difference of about 300 Da. This analysis shows that MALDI-TOF-MS is applicable to detect unknown mutations, and for a fast and reliable analysis of known genetic variations. A note of caution, however appears warranted: Due to suppression effects of DNA species differing over a broad range in molecular mass, detectability of restriction fragment patterns is restricted to digests producing a limited number of informative fragments.

Most of the *GLRA1* point mutations known are associated with dominant forms of hyperekplexia, suggesting that the incorporation of mutated subunits into the final pentameric complex either leads to gain of function effects or to non functional GlyRs. Previously, the recessive mutant allele *GLRA1* (I244N) has been identified,[22] decreasing apparent affinities for glycine, taurine and β-alanine by a factor of 10 to 15 as compared to the wild type.[37] While mutant GlyR$\alpha1^{I244N}$ receptors desensitise significantly faster than the wild type receptors, the same study provided strong evidence for an impaired expression or stability of the mutant subunit. The novel point mutation *GLRA1* (S231R) likewise causes a recessive form of hyperekplexia. Our biochemical and electrophysiological data suggest that the allele *GLRA1* (S231R) produces functional GlyRs. Prediction of transmembrane topology of the nicotinic acetylcholine receptor family places the mutant site within TM1 of the GlyR $\alpha1$ subunit.[12] Accordingly, introduction of volume and/or positive charge would destabilise nascent receptor subunits and interfere with intracellular routing to the plasma membrane. Based on protease accessibility studies combined with mass spectrometry, however, Leite *et al*[38] recently published an alternative topological model of the GlyR placing amino acid position 231 within a β-sheet structure starting with Y227. Accordingly, flanking N-terminal amino acids might interact with the mouth of the channel. This would be in agreement with studies on the acetylcholine receptor where homologous residues block channel activity in the closed state.[39] In this scenario, residue S231 would be located within the membrane, close to the extracellular surface. Thus, the mutation GlyR$\alpha1^{S231R}$ may prohibit correct protein folding as the positively charged residue R231 remains near the membrane. In either case, altered intracellular trafficking of nascent $\alpha1^{S231R}$ GlyRs is supported by a dramatic reduction of receptor insertion into plasma membrane and altered channel functionality. These observations suggest that the recessive phenotype may be a consequence of a dramatic loss of functional receptor in the homozygous state.

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