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Point mutation of glycine receptor α1 subunit in the *spasmodic* mouse affects agonist responses

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Abstract

Homozygotic *spasmodic* (*spd/spd*) mice suffer from a motor disorder resembling poisoning by the glycine receptor antagonist strychnine. Here, a point mutation was identified in the glycine receptor α1 subunit gene of the *spasmodic* mouse which predicts an alanine-to-serine exchange at position 52 of the mature polypeptide. Upon expression in *Xenopus laevis* oocytes, $\alpha1^{A52S}$ receptor channels displayed reduced responses to glycine, β-alanine and taurine when compared to recombinant α1 glycine receptors. As glycine receptor content in spinal cord and native molecular weight appeared unaltered, this suggests that the *spasmodic* phenotype results from an altered neurotransmitter sensitivity of the mutant $\alpha1^{A52S}$ subunit.

Key words: Glycine receptor; Post-synaptic inhibition; Mouse mutant; Point mutation; *spasmodic* mouse

1. Introduction

Neuronal inhibition in spinal cord and brainstem is primarily mediated by the amino acid glycine and is effectively antagonized by the convulsant alkaloid strychnine [1,2]. The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel composed of ligand binding α and structural β subunits which are highly homologous to each other [3]. Within the extracellular N-terminal domain of the α subunits, determinants of ligand binding [4–8] and sequence motifs crucial for the oligomerization of the GlyR complex [9] have been identified. Of the four hydrophobic segments (M1–M4) predicted to span the post-synaptic membrane, segment M2 is thought to form the inner wall of the receptor channel [3,10]. Differential expression of distinct variants of the ligand binding subunits, α1–α4 [4,11–16], contributes to a developmental and regional heterogeneity of the GlyR [2,17]. The receptor isoform prevailing in adult rodent spinal cord, $GlyR_A$ [17], is a pentameric assembly of α1 and β subunits [2].

As exemplified by sublethal strychnine intoxication, GlyR dysfunction results in muscle rigidity and hyperexcitability. Inherited phenotypes mimicking strychnine intoxication are found in two strains of mutant mice, *spastic* and *spasmodic* [18,19]. The *spastic* (*spa*) mutation is inherited by a recessive gene on chromosome 3 and characterized by a dramatic deficit of the adult receptor isoform $GlyR_A$ [19–21], which results from aberrant splicing of the GlyR β subunit pre-mRNA (Mülhardt et al., submitted). A motor disorder very similar to the *spastic* phenotype is seen in the *spasmodic* (*spd*) mouse, an unknown mutation linked to chromosome 11. In contrast to the *spastic* mutant, however, GlyR ligand binding has been reported to be unaltered in the CNS of *spd/spd* mice [22]. Synteny homology exists between the mouse chromosome 11 and the human chromosomal region 5q21-q31 [23,24] which includes the human locus of the α1 subunit gene of the inhibitory GlyR ([25]; T. Siddique and H. Betz, personal communication). Based on the genetic homology of human and murine chromosomal loci, and the similarity of *spastic* and *spasmodic* phenotypes, the GlyR α1 subunit gene was considered a candidate for the *spasmodic* mutation of the mouse. Here, we report a point mutation in the α1 subunit gene of the *spasmodic* mouse which predicts an alanine-to-serine exchange at position 52 of the mature polypeptide sequence and significantly affects agonist responses.

2. Experimental

2.1. Animal Care

Homozygous *spd/spd* mice were produced by mating tested B6C3Fe-*a/a*, *spd*/+ breeders purchased from the Jackson Laboratory (Bar Harbor, Maine 04609, USA). In contrast to earlier observations [22], an intermediate phenotype was present in adult heterozygotic animals (*spd*/+). Age-matched control animals were wild-type mice either of the B6C3Fe-*a/a* or the C57BL/6J strain.

2.2. Protein methods

Glycine-displaceable binding of [^{3}H]strychnine (16 nM; 29 Ci/mmol;

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DuPont NEN, Bad Homburg, Germany) to crude membrane fractions from spinal cord [17,26] was determined in triplicate by a filtration assay employing 80 μg of protein [21]. For radioligand displacement assays, membranes were incubated with 16 nM [^{3}H]strychnine and increasing concentrations of unlabelled ligands. Ligand binding data were analyzed using the computer program GraphPad. Monoclonal antibody (MAb 4a) was employed for immunodetection of GlyR by Western blot (11% acrylamide gel, 80 μg protein per lane) or dot immunoassay (8 mg protein/well) [26]. Immunoquantitation was performed in triplicate and reactivities were corrected for background absorbances obtained in the absence of the first antibody. The MAb 4a antigen content of *spd/spd* tissue was expressed as the mean ± S.E.M. of immunoreactivities standardized to paired wild-type samples ($n = 3$) producing specific absorbances of 0.314 ± 0.029 ΔOD, 0.250 ± 0.017 ΔOD, and 0.286 ± 0.013 ΔOD. For sucrose gradient sedimentation analysis of GlyR, 5–20% gradients were centrifuged and subjected to immunoassay [26]. Immunohistology of GlyRs was performed as described [27].

2.3. Reverse transcriptase-polymerase chain reaction

Total RNA (2–5 μg) extracted from spinal cord [28] was reverse-transcribed into cDNA using the SuperScript-Preamplification System (Gibco-BRL); appropriate aliquots (≈ 100 ng of cDNA) were subjected to PCR amplification performed in 15 mM Tris, pH 8.3, 2.25 mM $MgCl_2$, 50 mM KCl, 100 mM of each dNTP, and 0.2 mM of each PCR-primer using Taq DNA Polymerase (Boehringer Mannheim). During 34 cycles, conditions were as follows: heating to 95°C for 45 s, annealing at 55°C for 60 s, synthesis at 72°C for 90 s.

2.4. DNA blots and sequencing

cDNA fragments generated by PCR amplification were electrophoresed on a 1% agarose gel, transferred to a nylon membrane (Zeta-Probe GT; Bio-Rad), and hybridized to a random labeled 807 bp cDNA fragment comprising nt 253–1060 of the coding sequence of the murine α1 subunit [16]. Hybridization was in buffer containing 5 × SSC, 5 × Denhardt's, 25 mM NaP_i, pH 7.4, 1 mM $NaPP_i$, 100 μg/ml ssDNA, and 1% SDS at 65°C. Washings were performed in 2 × SSC, 0.1% SDS at 65°C. For analysis of genomic DNA samples isolated from the livers of two *spd/spd* and two wild-type mice, PCR amplification was performed as above, except that the elongation time was 30 s. After purification of genomic or cDNA and subcloning into pBluescript, DNA sequences were determined by the chain termination method according to standard procedures [29]. The murine α1 and $\alpha1^{A52S}$ subunit cDNA sequences obtained here differed from those published ([16]; GenBank/EMBL Data Bank X75832–40) in two non-coding nucleotide positions of exon 9 (A→G at nt position 1275, T→C at nt position 1287).

2.5. Oligonucleotide primers

Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems). For amplification of the murine α1 subunit cDNA [16] the following primers were designed to generate adequate restriction-sites flanking the open reading frame: sense, 5'-GCGGATCCATGTACAGCTTCAATACTCTTCGA (S1b/B; *Bam*HI); antisense, 5'-CGATGAATTCTCACTTGTTGTGGACATCCTC (AS9/E; *Eco*RI). DNA fragments encompassing exon 3 and flanking intronic sequences of the α1 subunit gene [16] were amplified from genomic DNAs using the primer combination Si2-1/B (intron 2, sense, 5'-CGGATCCAGGCACTTAGGGGTGG; *Bam*HI) and ASi3-1/E (intron 3, antisense, 5'-CGAATTCACAGCAGTTGCTGTAACGTACTC; *Eco*RI).

2.6. Heterologous expression of glycine receptors

GlyR α1 subunit cDNAs were cloned into a pSP64T-derived vector [30] and recombinant full-length plasmids were used to generate synthetic capped and polyadenylated cRNA employing SP6 polymerase (Promega) [29]. Injection of cRNAs into *Xenopus laevis* oocytes, and voltage clamp-recordings, were done as described [31]. We found that the use of polyadenylated cRNAs resulted in EC_{50} values of agonist responses exceeding those obtained by untailed α1 and $\alpha1^{A52S}$ subunit cRNAs by about an order of magitude (data not shown; compare [5,6,31]). Similar increases in affinities have also been observed after high-density expression in *Xenopus* oocytes of α1 subunit cDNA constructs [32].

3. Results

Glycine displaceable [^{3}H]strychnine binding to spinal cord membranes constitutes a reliable marker of $GlyR_A$ [2,17,21]. For mutant and wild-type mice, both [^{3}H]strychnine binding activity (*spd/spd* 1379 ± 272 fmol/mg; wild-type 1325 ± 98 fmol/mg) and affinity (Fig. 1, Table 1) were indistinguishable, confirming an earlier report [22]. For *spd/spd* mice, however, we consistently obtained ≈ 2-fold lower affinities for displacement of [^{3}H]strychnine binding by glycine, β-alanine and taurine (Fig. 1, Table 1). Monoclonal antibody (MAb) 4a defines an epitope common to all GlyR α subunits [3,17]. When spinal cord membranes from *spd/spd* and wild-type mice were subjected to Western blot analysis, MAb 4a detected a polypeptide of 48 kDa (Fig. 2A) corresponding to the previously described α1 subunit of murine $GlyR_A$ [19,21]. Upon immunoquantitation by MAb 4a [26], membrane samples from *spd/spd* mice produced 90.2 ± 20.0% of the wild-type signal, indicating similar spinal cord contents of GlyR. Moreover, when deter-

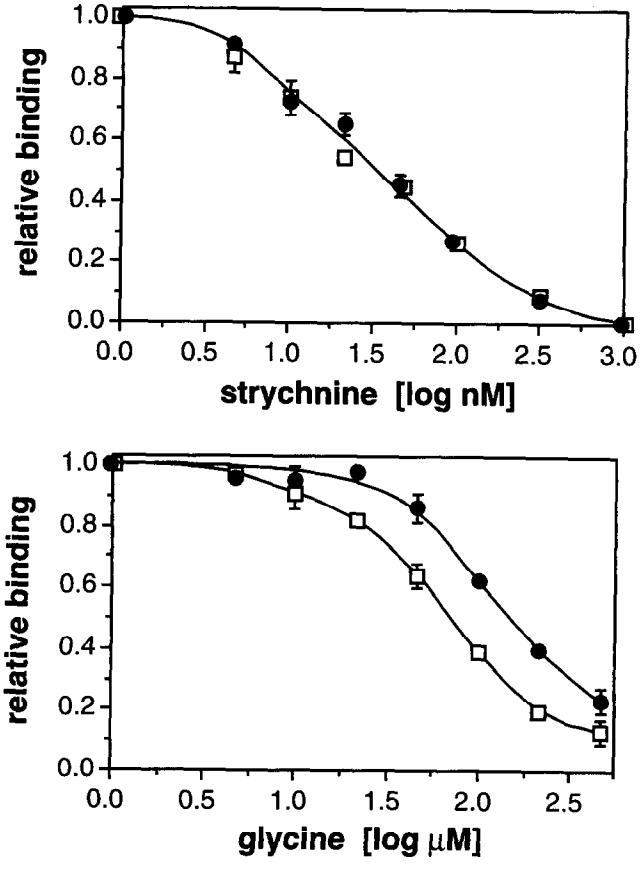


Fig. 1. Binding of GlyR ligands to membranes from spinal cord of *spd/spd* (●) and wild-type mice (□). Binding of [^{3}H]strychnine (16 nM) was displaced by increasing concentrations of either unlabelled strychnine (upper panel) or glycine (lower panel). Data are means ± S.E.M. of triplicate determinations of the fractional binding to crude membranes from either genotype. The 95% confidence intervals of glycine displacement were distinct for *spd/spd* and wild-type samples.

gent-solubilized GlyR preparations were subjected to sucrose gradient centrifugation, single peaks migrating with apparent sedimentation constants of ≈ 7.9 S were observed for both genotypes (Fig. 2B). Furthermore, immunohistology employing MAb 4a revealed that the neuronal localization of GlyRs was unaltered in spinal cord of *spd/spd* mice (not shown). We therefore conclude that the *spasmodic* mutation affects the agonist binding properties of $GlyR_A$ rather than its native protein structure, content, or cellular localization in spinal cord.

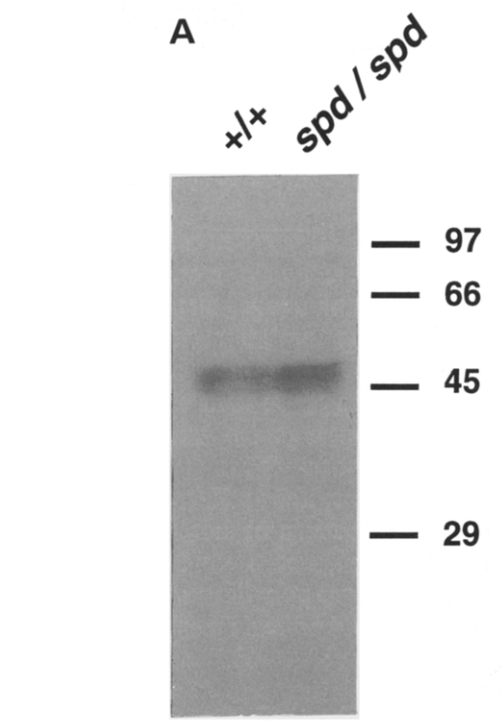
GlyR α1 subunit transcripts were investigated by PCR amplification of cDNA synthesized on spinal cord total RNA. A primer combination which covers the entire coding region of the α1 subunit [16] yielded amplification products indistinguishable in size (≈ 1.4 kb) for *spd/spd* and wild-type cDNAs (Fig. 3A). Cloning into pBluescript and sequencing of several recombinants identified a G→T substitution in nucleotide position 238 of the cDNA of *spd/spd* mice (Fig. 3B). This single nucleotide exchange resulted in an amino acid substitution at position 52 in the mature $\alpha1^{A52S}$ subunit of the *spasmodic* mouse where the alanine residue of the wild-type was replaced by a serine residue in the mutant. The $\alpha1^{A52S}$ subunit transcripts from *spd/spd* mice contained a further, however silent, T→C nucleotide substitution at position 198. Both point mutations were located in exon 3 (Table 2) of the GlyR α1 subunit [16]. Rodent α1 subunit transcripts comprise two splice variants resulting from alternative selection of the splice acceptor site of exon 9 [16,33]. Here, cDNAs were obtained from *spasmodic* mouse spinal cord which represented both splice variants of the $\alpha1^{A52S}$ subunit (not shown). To confirm the α1 subunit mutation of the *spasmodic* mouse at the genomic level, genomic DNA fragments encompassing exon 3 and neighbouring intronic sequences were amplified [16]. Sequence analysis of the 216 bp DNA fragments generated confirmed both nucleotide substitutions, indicating that the *spasmodic* mutation is an allele of exon 3 of the murine α1 subunit gene (Table 2).

Recombinant expression of the α1 subunit in eukaryotic cells generates GlyR channels resembling the ligand pharmacology of native receptors [5,31,34]. Upon injec-

Table 1
Affinity constants of GlyR ligand binding to spinal cord membranes from +/+ and *spd/spd* mice

Ligand	Wild-type		*spd/spd*	
	K_I	n_H	K_I	n_H
[^{3}H]Strychnine	22.6 ± 3.6 nM	1.0*	21.7 ± 3.5 nM	1.0*
Glycine	37.3 ± 2.2 μM	1.3	68.1 ± 10.9 μM	1.9
β-Alanine	55.3 ± 10.0 μM	1.1	135.7 ± 39.7 μM	1.4
Taurine	152.7 ± 7.6 μM	1.7	318.8 ± 44.1 μM	1.4

K_I, inhibition constant for displacement of [^{3}H]strychnine binding by the respective unlabelled ligand [2,21]; n_H, Hill coefficient.
*Assuming a single-site competition, n_H for displacement by [^{3}H]strychnine was defined as 1.0.



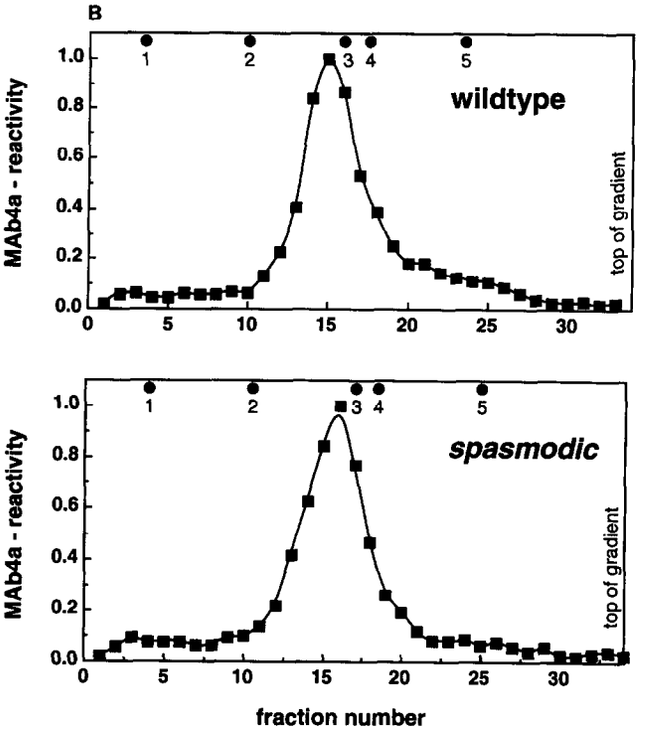


Fig. 2. Structural properties of the GlyR protein from wild-type and *spd/spd* mouse spinal cord. (A) Western blot analysis. In crude membranes from wild-type (+/+) and *spd/spd* mice, MAb 4a recognizes an α1 polypeptide of ≈ 48 kDa molecular weight. (B) Sedimentation profile of detergent-solubilized GlyRs as analyzed by MAb 4a. On 5–20% sucrose gradients, GlyRs from either genotype migrated at positions corresponding to an apparent S value of 7.9. Positions of marker enzyme reactivities (●) were as indicated: (1) β-galactosidase, 15.95 S; (2) catalase, 11.3 S; (3) aldolase, 7.4 S; (4) LDH, 7.0 S; (5) MDH, 4.3 S.

Table 2
Nucleotide and deduced amino acid sequences encoded by exon 3 of rodent GlyR α subunit variants

α1	*wt*	GGTCCTCCTGTGAATGTAAGTTGCAACATCTTCATCAACAGTTTCGGTTCCATCGCTGAGACAACCATG	(252)
	spd	.....C.....T.....	
	wt	G P P V N V S C N I F I N S F G S I A E T T M	(56)
	spd	. . . . . S	
α2A		. . . . . T . . . . . V T	(62)
α2B		. . . . . T	(62)
α3		. . . . . T	(56)
α4		. . . . . T . . . . . V T	(62)

The α1 subunit nucleotide and deduced polypeptide sequences from wild-type mice (*wt*) and *spd/spd* mutants (*spd*) are aligned with rodent α subunit variants (murine α2A, α2B, α4; rat α3) [13–16]. The first codon is split into exons 2 and 3. Numbering starts with the first nucleotide of the respective cDNA coding sequence, and with the N-terminal first amino acid of the mature polypeptide. Identical nucleotides and amino acids are indicated by dots.

tion of synthetic $\alpha1^{A52S}$ subunit cRNA, *Xenopus* oocytes produced glycine-induced inward currents as analyzed by voltage-clamp recording (Fig. 4). Using $\alpha1^{A52S}$ cRNA, half-maximal currents were observed at concentrations (EC_{50}) of glycine, β-alanine and taurine which were significantly lower than EC_{50} values obtained with α1 cRNA (Fig. 4, Table 3). The currents induced by $\alpha1^{A52S}$ and α1 cRNAs were highly sensitive to strychnine, with half-maximal inhibition at antagonist concentrations (Table 3) matching affinities derived from radioligand binding (Table 1). In oocytes injected with α1 cRNA, the maximum currents elicited by application of either β-alanine ($I_{\beta ala}$) or taurine (I_{tau}) equaled those evoked by glycine (I_{gly}): $I_{\beta ala}/I_{gly} = 0.95 \pm 0.04$; $I_{tau}/I_{gly} = 0.90 \pm 0.08$ (Fig. 4). When $\alpha1^{A52S}$ cRNA was injected, however, saturating concentrations of β-alanine or taurine produced responses of smaller amplitude ($I_{\beta ala}/I_{gly} = 0.80 \pm 0.06$; $I_{tau}/I_{gly} = 0.57 \pm 0.15$). This agonist profile is reminiscent of recombinant α2 and α3 GlyRs which also exhibit lower maximum current responses to these β-amino acids [4,5,13,31]. Thus, the *spasmodic* mutation appears to impair functional properties of $GlyR_A$, resulting in a dysfunction of glycinergic inhibition.

4. Discussion

As an important mediator of neuronal post-synaptic inhibition, $GlyR_A$ contributes to mammalian motor regulation [1,35]. Here we report that the α1 subunit gene, which encodes the ligand binding subunit of $GlyR_A$ [2,3], is mutated in the motor-deficient *spasmodic* mouse. The

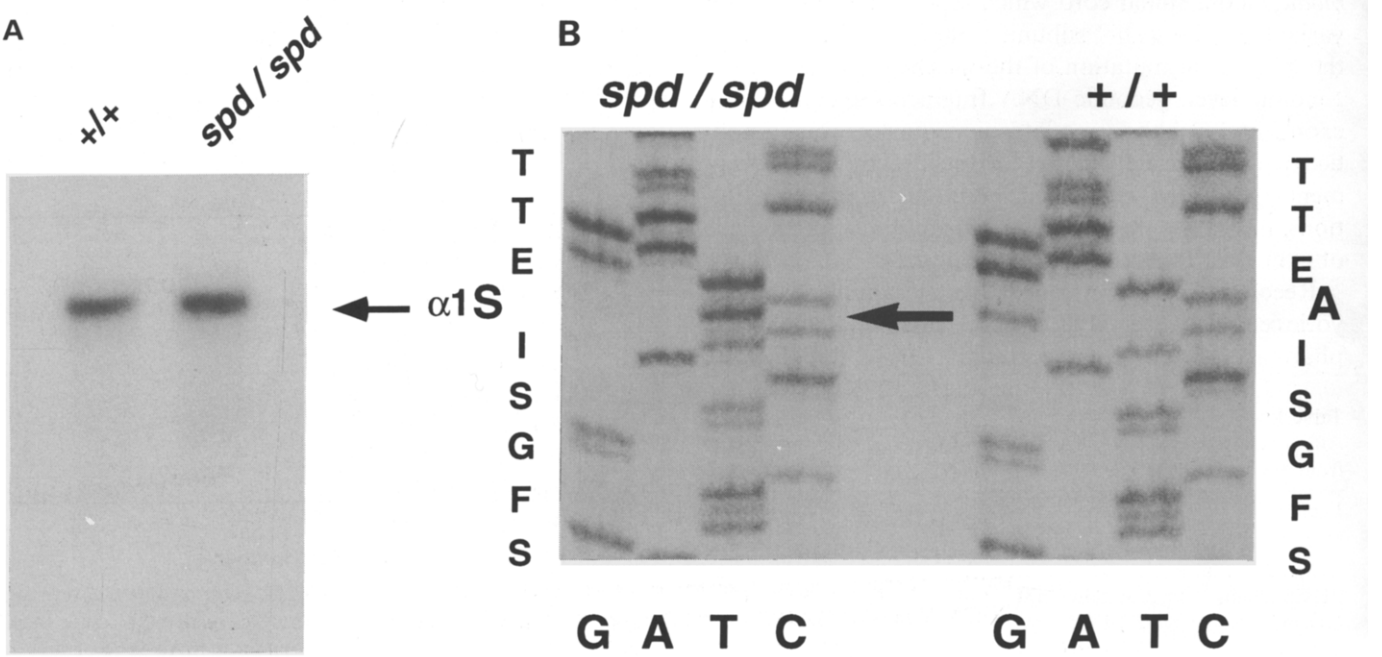


Fig. 3. Analysis of GlyR α1 subunit transcripts in mouse spinal cord. (A) PCR amplification of α1 subunit cDNAs from +/+ and *spd/spd* mice, using a primer combination covering the entire coding sequence (exons 1 through 9). PCR products were identified by Southern blots hybridized with an α1 subunit-specific cDNA probe comprizing exons 4–8 [16]. (B) Nucleotide substitution (G → T) at position 238 of the GlyR α1 subunit cDNA resulting in an amino acid exchange in *spd/spd* mice. The nucleotide difference between +/+ and *spd/spd* (arrow) leading to the substitution of alanine by serine is revealed by the cDNA sequence (coding strand, gel lanes: G, A, T, C). The amino acid sequences (single letter code) encoded by the two DNA ladders and reading from bottom to top are listed next to the gel patterns.

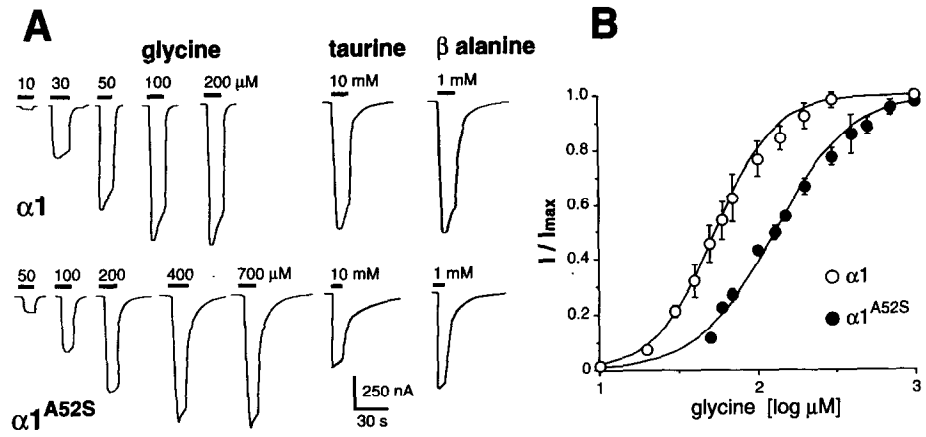


Fig. 4. Glycine-elicited membrane currents recorded from *Xenopus* oocytes injected with α1 and $\alpha1^{A52S}$ cRNAs. (A) Current responses of oocytes injected with the corresponding cRNA and superfused with agonists. Downward deflections denote inward currents recorded at a holding potential of −70 mV, horizontal bars = duration of agonist application. (B) Dose–response relationship of glycine-induced inward currents in oocytes injected with α1 (○) and $\alpha1^{A52S}$ cRNA (●). For both cRNAs, data are normalized to peak amplitudes (I_{max}) obtained at saturating concentrations of glycine; α1 subunit cRNA I_{max} = 1304 ± 757 nA; $\alpha1^{A52S}$ subunit cRNA I_{max} = 720 ± 173 nM.

spasmodic mutation produces an alanine-to-serine exchange at position 52 of the N-terminal domain of the mature α1 polypeptide, resulting in altered GlyR agonist binding and current responses upon recombinant expression in *Xenopus* oocytes. A sequence comparison of GlyR α subunit variants reveals that alanine residues are conserved at homologous positions in α1 [11,12], α2B [14], and α3 [13] subunits. In contrast, threonine residues are encoded by the α2A splice variant which is highly responsive to glycine [12,14,15], and the α4 subunit gene [16]. The functional significance of this subunit heterogeneity is not understood. Within the N-terminal domain of the α1 subunit, non-continuous structural motifs are involved in ligand binding and discrimination [3]: (i) amino acid residues 159–161 comprise determinants of agonist and high affinity strychnine binding [4,6,7]; (ii) residue 111 is essential for GlyR channel activation by taurine [5]; (iii) a β-sheet–β-turn motif formed by residues 200–212 is thought to contribute to agonist–antagonist discrimination [7,8]. The *spasmodic* mutation lies outside of these regions and thus defines a new determinant of agonist-mediated channel activation. As components of a multisubunit protein, α and β polypeptides also carry characteristic 'cassettes' governing receptor assembly [9] which, in the $\alpha1^{A52S}$ subunit, indeed flank the site of the mutation. Although single amino acid exchanges may interfere with oligomerization of recombinant α1 polypeptides [10], no evidence was obtained here that the *spasmodic* mutation alters the native protein structure of $GlyR_A$.

Several lines of evidence indicate that the amino acid substitution identified in the $\alpha1^{A52S}$ subunit is causal for the *spasmodic* phenotype: (i) *spasmodic* symptoms [22] mimic states of GlyR dysfunction as encountered in the $GlyR_A$-deficient *spastic* mouse [19–21], or induced by sublethal strychnine poisoning [2]; (ii) the functional impairment of $\alpha1^{A52S}$ subunit GlyRs is consistent with a loss of glycine-mediated motor inhibition [1]; (iii) the *spasmodic* locus on chromosome 11 [22,24] is linked by synteny homology to the human chromosomal region 5q21-q31 [23] where the α1 subunit gene has been mapped [25]. Recently, Shiang et al. [25] identified point mutations in the human α1 subunit gene which cause the dominant neurological disorder, hyperekplexia, which resembles the *spasmodic* phenotype. These mutations predict amino acid substitutions delineating transmembrane segment M2 [25] which, upon heterologous expression, render homomeric α1 GlyRs functionally inactive [10]. While both, human hyperekplexia and the *spasmodic* mouse are associated with GlyR α1 subunit mutations, the predominant expression of this subunit in adult rodents [3,17] deviates from the postnatal alleviation of hyperekplexia symptoms [25]. Thus, GlyR regulation may differ between these species or the phenotype of hyperekplexia is effectively attenuated by epigenetic

Table 3
Current responses of α1 and α^{A52S} receptor channels generated by cRNA expression in *Xenopus* oocytes

Ligand	α1		$\alpha1^{A52S}$	
	EC_{50}	n_H	EC_{50}	n_H
Glycine	61 ± 7 μM	2.4 ± 0.2	176 ± 41 μM	1.8 ± 0.1
β-Alanine	106 ± 32 μM	1.8 ± 0.1	373 ± 163 μM	1.8 ± 0.1
Taurine	757 ± 234 μM	2.0 ± 0.1	1300 ± 284 μM	1.9 ± 0.1
Strychnine*	42 ± 5 nM	–	55 ± 10 nM	–

EC_{50} values for agonists were obtained by single sigmoid fits of normalized agonist-evoked peak currents. Data represent average ± S.E.M. of 3–5 determinations; n_H, Hill coefficient.
*For strychnine, IC_{50} values were determined at glycine concentrations producing half-maximal responses.

mechanisms. Our analysis of the *spasmodic* mouse corroborates the functional importance of distinct GlyR isoforms and shows that a point mutation affecting transmitter sensitivity may provide the molecular basis of a neurological disorder.

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