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Disruption of a Structurally Important Extracellular Element in the Glycine Receptor Leads to Decreased Synaptic Integration and Signaling Resulting in Severe Startle Disease

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Functional impairments or trafficking defects of inhibitory glycine receptors (GlyRs) have been linked to human hyperekplexia/startle disease and autism spectrum disorders. We found that a lack of synaptic integration of GlyRs, together with disrupted receptor function, is responsible for a lethal startle phenotype in a novel spontaneous mouse mutant shaky, caused by a missense mutation, Q177K, located in the extracellular β8–β9 loop of the GlyR α1 subunit. Recently, structural data provided evidence that the flexibility of the β8–β9 loop is crucial for conformational transitions during opening and closing of the ion channel and represents a novel allosteric binding site in Cys-loop receptors. We identified the underlying neuropathological mechanisms in male and female shaky mice through a combination of protein biochemistry, immunocytochemistry, and both in vivo and in vitro electrophysiology. Increased expression of the mutant GlyR α1 Q177K subunit in vivo was not sufficient to compensate for a decrease in synaptic integration of α1 Q177Kβ GlyRs. The remaining synaptic heteromeric α1 Q177Kβ GlyRs had decreased current amplitudes with significantly faster decay times. This functional disruption reveals an important role for the GlyR α1 subunit β8–β9 loop in initiating rearrangements within the extracellular–transmembrane GlyR interface and that this structural element is vital for inhibitory GlyR function, signaling, and synaptic clustering.

Key words: β8–β9 loop; fast decay; glycine receptor; hydrogen bond network; shaky; startle disease

Significance Statement

GlyR dysfunction underlies neuromotor deficits in startle disease and autism spectrum disorders. We describe an extracellular GlyR α1 subunit mutation (Q177K) in a novel mouse startle disease mutant shaky. Structural data suggest that during signal transduction, large transitions of the β8–β9 loop occur in response to neurotransmitter binding. Disruption of the β8–β9 loop by the Q177K mutation results in a disruption of hydrogen bonds between Q177 and the ligand-binding residue R65. Functionally, the Q177K change resulted in decreased current amplitudes, altered desensitization decay time constants, and reduced GlyR clustering and synaptic strength. The GlyR β8–β9 loop is therefore an essential regulator of conformational rearrangements during ion channel opening and closing.

Introduction

Glycine receptors (GlyRs) are members of the superfamily of Cys-loop receptors (CLRs), whose structures have recently been resolved by x-ray crystallography or cryo-electron microscopy (EM; Du et al., 2015; Huang et al., 2015). Adult inhibitory GlyRs form pentameric ion channels with a 2α:3β stoichiometry reagents/analytic tools; N.S., A.B., I.v.B., F.Z., Y.Z., S.I., L.L., S.I., S.M., M.T., F.W., D.D., R.J.H., and C.V. analyzed data; C.V. wrote the paper.

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(Gru¨dzniska et al., 2005). Disturbances in glycinergic inhibition are associated with rare disorders such as startle disease (OMIM 149400, hyperekplexia, stiff baby syndrome) and autism spectrum disorders (Harvey et al., 2008; Bode and Lynch, 2014; Pilorge et al., 2016). The genetic causes for startle disease have been defined, with the most common gene GLRA1 (encoding the GlyR a1 subunit) followed by SLC6A5 (glycine transporter 2) and GLRB (GlyR b subunit; Harvey et al., 2008; Chung et al., 2010; James et al., 2013). Affected patients show exaggerated startle responses following unexpected acoustic or tactile stimuli, stiffness in infancy, tremor, and loss of postural control during startle episodes (Schaefe r et al., 2013). GlyRs are important in the spinal cord for feedback control mechanisms in the nerve–muscle circuit to balance motoneuron firing and in the brainstem for the generation of the respiratory rhythm (Bongianni et al., 2010; Schaefe r et al., 2012). The current view of startle disease pathologies differentiates between functional impairments and biogenesis defects (Bode and Lynch, 2014). A recent study (Schaefe r et al., 2015) demonstrated that startle disease mutations also affect GlyR folding and ER processing, suggesting a higher molecular complexity of disease mechanisms than was previously assumed.

Due to a similar startle phenotype in mice carrying Giral (spasmodic, oscillator) or Glrb (spastic) mutations, mouse models serve as excellent tools to study the underlying pathological mechanisms. Oscillator is a functional GlyR a1 subunit null mutation, while spasmodic harbors an A52S missense mutation in the GlyR a1 subunit (b1–b2 loop), leading to decreased ligand affinities but normal life span (Schaefe r et al., 2012). The novel spontaneous mouse mutant shaky carries a missense mutation in exon 6 of Giral, resulting in a Q177K substitution in the b8–b9 loop of the GlyR a1 subunit extracellular domain (ECD). In contrast to spasmodic, homozygous shaky mice suffer from serious neuromotor deficits progressively increasing from postnatal day 14 (P14) until death, indicating that disruption of the b8–b9 loop substantially impairs glycinergic function and is incompatible with life.

To date, the b8–b9 loop has mainly been investigated by in vitro mutagenesis studies in other CLRs (Thompson et al., 2006; Hibbs et al., 2009). These studies revealed effects on ligand efficiencies or affinities, arguing for an involvement of the b8–b9 loop in the ligand-binding process. This view was recently expanded by the identification of the b8–b9 loop forming an allosteric binding site for the antipsychotic chlorpromazine in the closely related Erwinia ligand-gated ion channel ELIC (Nys et al., 2016). Structural data have demonstrated that the b8–b9 loop is part of the signal transduction unit, transferring the closed state upon ligand-binding into the open configuration and back to the closed ion channel state (Du et al., 2015; Morales-Perez et al., 2016). However, the role of the b8–b9 loop in disease mechanisms is unclear.

Here, we found that the b8–b9 loop is involved in GlyR synaptic clustering as well as neurotransmitter sensitivity and that a defect in these mechanisms causes severe startle disease. In neuronal cultures and spinal cord tissue from shaky mice, we observed an increased expression level of GlyR a1 Q177K, which was an unsuccessful attempt at compensation for an observed lack of GlyR integration into synapses. Decreased agonist efficacy and faster decay times of a1 Q177K GlyRs were recorded in artificial synapses and ex vivo brainstem slices. Recordings after the onset of neuromotor symptoms revealed significant reductions in current amplitudes, frequencies, and decay times but no changes in rise times in shaky homoygotes. Thus, shaky represents the first in vivo model highlighting that b8–b9 loop is a key regulator of GlyR signaling as it is essential for conformational rearrangements governing both receptor clustering and ion channel gating.
transcription (RT)-PCR. We used RevertAid M-MuLV (Mooney murine leukemia virus) RT (200 U/µl) provided with 5X reaction buffer and deoxy (d) ATP, dCTP, dGTP, dTTP (10 mM each), and random hexamers (50–200 ng; Thermo Fisher Scientific). Two microliters of cDNA were used for amplification of the housekeeping gene β-actin; GlyR α1, α2, and βi; gephyrin, and GlyT1 (95°F for 5 min; 25 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 10 min).

Cell lines and primary neurons. Human embryonic kidney 293 (HEK293) cells were grown in minimum essential medium (Thermo Fisher Scientific), supplemented with 10% fetal calf serum, 1-glutamine (200 mM), and 50 U/ml penicillin and streptomycin at 37°C and 5% CO2. Cells were transiently transfected using a modified calcium phosphate precipitation method. All experiments concerning protein biochemistry with HEK293 cells were performed 48 h after transfection. Primary spinal cord neuronal cultures were prepared at embryonic day 13 (E13). A piece of tail tissue of each embryo was used for genotyping. Briefly, every spinal cord was trypsinized using 1 ml of trypsin/EDTA (1 mg/ml) and 10 µl of DNase I (final concentration, 0.1 mg/ml), incubating the suspension at 37°C for 30 min. Trypsinization was stopped with 100 µl of fetal calf serum (final concentration, 10%). After a three-step trituration, the cells were centrifuged at 300 rpm for 15 min. Trituration was repeated. Cells were plated on a 3 cm dish with four poly-lysine-covered coverslips and incubated at 37°C with 5% CO2 at 95% humidity. Spinal cord neurons were grown in neurobasal medium plus 5 µl of 1-glutamine (200 mM) plus B27 supplement (Thermo Fisher Scientific) with an exchange of 50% medium after 6 d in culture. Neurons older than day in vitro 21 were used for experiments.

Membrane preparations and biotinylation of cell surface proteins. For membrane protein analysis, crude cell membranes were prepared from transfected cells or mouse tissue (Sontheimer et al., 1989). Biotinylation experiments were performed as described previously (Atak et al., 2015).

Radioligand-binding assays. [3H]strychnine displacement assays were performed using filtration assays with triplicates of 80 µg of membrane protein. Samples were incubated for 30 min either with 30 mM glycine or buffer B (25 mM potassium phosphate buffer, 200 mM KCl). Then either glycine or buffer B was replaced by a range of [3H]strychnine concentrations (1, 10, 20, 50, 100, and 200 nM; specific activity, 30 Ci/mmol; DuPont NEN; Kling et al., 1997). Binding data were analyzed by a nonlinear algorithm provided by the program Origin version 6.0 (Microcal Software).

Western blot and immunostaining. For SDS-PAGE, 11% polyacrylamide gels were freshly prepared, followed by Western blot on nitrocellulose membranes (GE Healthcare). Membranes were blocked for 1 h at room temperature in a 10% normal horse serum in PBS, incubated with the primary antibody (mAb2b; 1:1500; catalog #146 003, Synaptic Systems), Vyrl α1 (1:250; mAb2b; catalog #146 111, Synaptic Systems), VGAT (1:300; catalog #131 003, Synaptic Systems), gephyrin (1:200; catalog #147 003, Synaptic Systems), and synapsin (1:300; catalog #574778, Calbiochem) in 10% normal horse serum in PBS overnight at 4°C. After three washing steps with PBS for 10 min each, tissue was incubated with secondary antibodies coupled to Cy3 and Alexa Fluor 488 (1:1000, Dianova) diluted in 10% normal horse serum in PBS for 45 min at 22°C. For staining of the cell nuclei, slides were incubated in Molecular Probes DAPI solution (Thermo Fisher Scientific) diluted 1:1500 in PBS for 10 min at room temperature in a dark chamber. Finally, the slides were mounted with aqua polymount (Polysciences).

Confocal microscopy, and image acquisition and analysis. Images were acquired using an inverted IX81 microscope equipped with an Olympus FV1000 confocal laser scanning system, an EFD10 SPD spectral detector, and diode lasers of 405 nm (DAPI), 495 nm (Alexa Fluor 488), and 550 nm (Cy3). All images shown were acquired with an Olympus UPLSAPO 60X (oil objective; numerical aperture, 1.35) objective. The images were further developed and organized by Adobe Photoshop CS5 and Illustrator software or Corel Photopaint, Corel Graphic Suite X6.

Image analysis for quantification. Single coverslips were acquired using settings of a photomultiplier identically applied to all samples quantified in one experiment. Maximal projection images were created from confocal stacks using NIH Image 1.3e software (https://imagej.nih.gov/ij/). Nonspecific background was removed using threshold subtraction. In all experiments, clusters of GlyRs were defined semiautomatically by setting rectangular regions of interest (ROIs) with dimensions of approximately 8 × 8 pixels around local intensity maxima in the channel with GlyR α1-specific (mAb2b) staining using OpenView software (Tsuriel et al., 2006). Mean immunofluorescence (IF) intensities were measured in a 4 × 4 pixel box within every ROI in all corresponding channels. Obtained IF intensities were normalized to the mean intensity of control (shaky vs wild type). All results of IF analysis are shown as the mean ± SEM. All statistical analyses were performed with GraphPad Prism version 5.0 software (GraphPad Software).

Counting of motoneurons. Mice were deeply anesthetized and transcardially perfused as described previously (Jablonka et al., 2000). Slices were stained with cresyl violet, and motoneurons were counted in every 10th section of the lumbar spinal cord (L1–L6). The raw counts were corrected for double counting of split nuclei by as described previously (Masu et al., 1993). Differences between groups were evaluated with Student’s t test (unpaired; significance level, *p < 0.05), applying the Graphics Prism Program version 5.0 (GraphPad Software).

Molecular modeling of the GlyR α1 subunit Q177K mutation. The cryo-EM structure [Electron Microscopy Data Bank (EMDB), ID EMD-6345; Protein Data Bank (PDB), ID 3JA9] of the zebrafish GlyR α1 subunit was used to study the structural and functional effects of the Q177K substitution. The position of glycine in the binding site of GluCl (Pettersen et al., 2004) was modeled into the GlyR using the non-synonymous substitution Q177K using a superposition of the two receptors with MODELLER (Pettersen et al., 2004) using a superposition of the two receptors with MODELLER (Pettersen et al., 2004). The non-synonymous substitution Q177K was modeled into the GlyR using the swapaa command in Chimera (Pettersen et al., 2004) based on the Dunbrack backbone-dependent rotamer library (Dunbrack, 2002) and taking into account the lowest clash score, highest number of H-bonds, and highest rotamer probability. Flexible fitting resulted in moving R65 into the EM density.
sponses were measured at a holding potential of ~60 mV. All experiments were performed at 22°C. For desensitization analysis, whole-cell current traces were transferred to Microlab Origin version 6.0 (Microlab Software), and the decaying current phase was analyzed using a single exponential function plus a constant, as shown in Equation 1:

$$I_{\text{dec}} = I_1 \cdot e^{-t/\tau_1} + I_{\text{const}}$$  \hspace{1cm} (1)$$

where $I_{\text{dec}}$ is the observed total current amplitude, $I_1$ is the fraction of current desensitizing with time constant $\tau_1$, and $I_{\text{const}}$ is the amplitude of the nondenensitizing current fraction. A single exponential decay plus a constant term were sufficient to describe desensitization behavior. Functional constants of the coexpressed subunits were compared using a t test. A probability of error of $p < 0.05$ was considered significant ($^{*} p < 0.01$, $^{**} p < 0.001$).

Electrophysiological recordings from artificial synapses. Experiments were performed as described by Zhang et al. (2015).

Brainstem slice preparation and whole-cell recordings. Electrophysiological experiments were performed on brainstem slices from 18- to 24-d-old mice. After anesthesia and decapitation, brainstem tissue was rapidly removed and immersed in ice-cold “high-sucrose” artificial CSF (aCSF) containing the following (in mM): 75 sucrose, 125 NaCl, 3 KCl, 0.3 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 2 5 NaHCO3, and 30 t-glucose and bubbled with carbogen (95% O2/5% CO2) at pH 7.4. Transverse slices, 250–350 μm thick, containing the PreBötzing complex (PreBotC) were cut and transferred into warm (35°C) aCSF for 15 min and kept at room temperature thereafter in normal aCSF for at least 1 h before using. Recordings were performed in normal aCSF bathing, pH 7.4, that contained the following (in mM): 125 NaCl, 3 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, and 10 t-glucose at 30°C. The brainstem region of interest was identified based on their location relative to nearby landmarks such as the inferior olive, hypoglossal nerve and nucleus ambiguous (for PreBotC). Whole-cell recordings were performed with patch pipettes filled with an internal solution composed of (in mM): 130 CsCl, 3 MgCl2, 5 EGTA, 5 HEPES, 2 Na2-ATP, 0.3 Na2-GTP, and 5 QX-314, pH 7.3. The electrode resistance ranged from 3 to 5 MΩ when filled with internal solution. Whole-cell currents were recorded at a holding potential of −70 mV (corrected for liquid junction potential), filtered (2–6 kHz), and sampled at 20 kHz using a Multiclamp 700B Amplifier in conjunction with a Digidata 1440A interface and pClamp10 software (Molecular Devices).

Constant current pulses (width, 0.01 ms) of 100–400 μA were delivered every 10 s to a bipolar tungsten electrode located in close vicinity to the preBotC to evoke synaptic responses. Glycinergic IPSCs were pharmacologically isolated by ionotropic glutamate receptor antagonist kynurenic acid (KA; 2 mM) and GABA_A receptor antagonist bicuculline methiodide (BMI; 10 μM). Glycine (50 μM) was used to induce postsynaptic response in the presence of tetrodotoxin (TTX; 1 μM) and kynurenic acid/bicuculline. Strychnine (10 μM) was applied in some experiments to verify the glycineric origin of IPSC or to block glycine-induced currents. The identification of a GlyR missense mutation provided a plausible explanation for the observed severe motor phenotype similar to other startle disease mouse models. The synonymous change in exon 3 was not causative and is a common variant that comes from the hybrid C57BL/6129Sv background (rs26948271) of shaky. However, breeding of heterozygotes for the missense mutation Q177K resulted in an autosomal-recessive inheritance of the mutation following backcrosses from the original mixed background of C57BL/6129Sv over >10 generations (614 wild-type animals, 303 heterozygous animals, 138 shaky animals). Sequencing of other candidate genes affected in startle disease excluded further pathogenic sequence variations, as only common single nucleotide variants were found (Glrb: exon 5, c.A555G, p.L163L, rs13477223; SLC6A5: exon 2, c.A109G, p.T37A, rs31048165).

To confirm that shaky results from defects in glycineric transmission, heterozygous shaky mice were bred with heterozygous spasmodic or oscillator mice. Spasmodic carries a different missense mutation A52S in the GlyR α1 subunit that affects glycine efficacy but results in a mild phenotype with tremor episodes but normal life span for the animals (Buckwalter et al., 1994; Saul et al., 1994). Eight matings of shaky and oscillator heterozygotes generated 10 mice with severe motor deficits with onset of symptoms at P14 and a life span of 4 weeks (Fig. 1D). Genotyping of the pups exhibited heterozygosity of affected mice for the oscillator and shaky mutations (Glr1++/−), confirming the validity of this test for allelism. By contrast, heterozygous Glr1++/− mice survive until P14, and a life span of 4 weeks (Fig. 1D). Together, these data provided strong evidence that the pathophysiologically mechanism in the spontaneous mouse model shaky is a missense mutation (Q177K) in Glr1.

Motor deficits in homozygous shaky mice are improved by benzodiazepine treatment. Due to their impaired motor control, shaky mice performed very poorly on the rotarod test (Glr1++/− time on rod, 288 ± 5 s; Glr1++/− time on rod, 15 ± 3 s; ***p = 0.935E-27, t test; Fig. 1E). Humans suffering from startle disease respond to treatment with benzodiazepines such as clonazepam, which improves symptoms.
by potentiating GABAergic transmission. Therefore, shaky mice were tested on the rotarod prior to (baseline) and after intraperitoneal injection of 0.5 mg/kg diazepam. After this treatment, shaky mice showed a significant improvement in their performance (after diazepam: $t = 34 \pm 7$, $n = 10$; **$p = 0.00102$, t test) on the rod (Fig. 1E). Again, improvement of the symptoms following diazepam treatment provides further evidence for a glycinergetic transmission defect in shaky mice.

Shaky GlyRs have disrupted ligand binding, but the neuromotor phenotype does not result from disturbed receptor biogenesis

A first analysis of key proteins at the glycinergetic synapse (GlyR $\alpha_2$, GlyR $\beta$, gephyrin, GlyT1) in wild-type (+/+) and heterozygous (+/sh) mice revealed no obvious differences at the mRNA level (Fig. 2A). At the protein level (Fig. 2B–E), the distinct expression of GlyR $\alpha$ subunits was detected in brainstem and spinal cord, but there was only faint expression in cortex (presumably, $\alpha_2$ or $\alpha_3$ subunits). Furthermore, specific $\alpha$ expression was enhanced in spinal cord of Glyra1<sup>sh/sh</sup> mice ($p = 0.09$, nonsignificant, t test) and significantly increased in brainstem ($p = 0.019$, t test) at P21 when shaky mice exhibit a severe neuromotor phenotype (Fig. 2B, C). No GlyR $\alpha$ expression was observed in cortex. Gephyrin was also increased concomitantly with GlyR $\alpha$ in the brainstem ($p = 0.045$, t test) of affected animals (Glyra1<sup>sh/sh</sup>; Fig. 2B, D). The broad expression of gephyrin in the cortex is consistent with a major role in GABAA receptor clustering within this brain area (Tyagarajan and Fritschy, 2014). A developmental analysis of spinal cord, brainstem, and cortex from P0 to P28 indicated that GlyR $\alpha$ subunit expression in Glyra1<sup>sh/sh</sup> was indistinguishable from that observed in Glyra1<sup>+/+</sup> mice and began at P6 (Fig. 3A). Furthermore, the subunit switch
to increased GlyR α1 levels after birth was completed before the onset of symptoms at P14. Backcross into the oscillator line revealed a slight but nonsignificant decrease in GlyR α1 protein in heterozygous Glra1±/± (sh/h) and homozygous animals (sh/sh). GlyR α1 expression in shakymice was increased, binding of the antagonist strychnine is diminished. To analyze synaptic localization, we performed immunostaining of spinal cord tissue and samples from spinal cord cultures from Glra1sh/sh mice. In contrast to wild-type GlyRα1, a fraction of GlyRα1Q177K was not colocalized with presynaptic markers synapsin and VGAT (Fig. 4A, B). Quantification of synaptic GlyRα1 in neuronal cultures demonstrated a significant decrease in Glra1sh/sh compared with Glra1+/+, p = 0.25.
GlyR α1 comparison between Glra1<sup>+/+</sup> and Glra1<sup>sh/sh</sup>; *p = 0.018; gephyrin compared with GlyR α1 in Glra1<sup>+/+</sup>, *p = 0.5; gephyrin compared with GlyR α1 in Glra1<sup>sh/sh</sup>, *p = 0.017, t test). An apparent increase in α<i>Q177K</i> expression observed in <i>shaky</i> neurons may result from an attempt to compensate for the lack of synaptic α1-containing GlyRs (Fig. 4D, E). Hence, GlyR α1<sup>Q177K</sup> expression is enhanced in brainstem and spinal cord, but synaptic localization is decreased. At the functional level, GlyRs in <i>shaky</i> mice display a lower strychnine-binding affinity, suggesting an additive effect of sorting deficiencies and functional disruption.

The α<i>Q177K</i> mutation impairs the functionality of GlyR channels <i>in vitro</i>

Using overexpression of GlyRs in transfected HEK293 cells, quantification of whole-cell and plasma membrane protein of α1<sup>Q177K</sup>β GlyRs was compared with wild-type α1β receptors. This revealed a significant decrease of α1<sup>Q177K</sup>β surface expression (heteromeric α1<sup>Q177K</sup>β 33 ± 5% of wild-type α1β, **p = 0.003, t test; Fig. 5A). In whole-cell recordings from transfected cells, it became obvious that α1<sup>Q177K</sup>β cell surface GlyRs could form functional ion channels with no changes in maximal current amplitudes upon 1 mM glycine application (Fig. 5B) but with significantly lower current values at 100 μM glycine (α1β, 4.7 ± 0.3 nA; α1<sup>Q177K</sup>β, 1.13 ± 0.3 nA; ***p = 6.616E-06, t test; Fig. 5B). In contrast, on application of 1 mM β-alanine or taurine, α1<sup>Q177K</sup>β GlyRs exhibited significantly reduced current amplitudes (β-alanine, 3.2 ± 0.3 nA, n = 11; compared with α1β, 4.5 ± 0.6 nA, n = 9; **p = 0.033, t test; taurine, 2.8 ± 0.4 nA, n = 6; compared with α1β, 5.1 ± 0.7 nA, n = 5, *p = 0.014, t test). A concentration of 100 μM for both partial agonists generated again significantly decreased agonist-induced currents compared with wild-type receptors (β-alanine α1<sup>Q177K</sup>β, 0.23 ± 0.02 nA, n = 6; compared with α1β, 1.1 ± 0.2 nA, n = 6, **p = 0.0014, t test; taurine, α1<sup>Q177K</sup>β 0.1 ± 0.02 nA, n = 6; compared with α1β, 0.9 ± 0.09 nA, n = 5, ***p = 6.72E-06, t test; Fig. 5B).

Hence, the EC<sub>50</sub> value for mutated α1<sup>Q177K</sup>β channels activated by glycine increased by a factor of 6 (α1β EC<sub>50</sub> = 40 ± 6 μM; α1<sup>Q177K</sup>β EC<sub>50</sub> = 241 ± 29 μM; Fig. 5C). The potencies of the partial agonists β-alanine (α1<sup>Q177K</sup>β EC<sub>50</sub> = 318 ± 22 μM; α1β EC<sub>50</sub> = 260 ± 50 μM) and taurine (α1<sup>Q177K</sup>β EC<sub>50</sub> = 389 ± 51 μM; α1β EC<sub>50</sub> = 212 ± 34 μM) were almost unaffected with a slight decrease of 1.2- and 1.8-fold, respectively (Fig. 5C).

Desensitization decay time constants were significantly decreased for mutant receptors, arguing for faster ion channel closure of α1<sup>Q177K</sup>β heteromers with wild-type channels (α1β τ = 1.9 ± 0.14 s; α1<sup>Q177K</sup>β τ = 0.88 ± 0.19 s; *p = 0.049, t test; Fig. 5D). Last, artificial synapses were used to analyze the α1<sup>Q177K</sup> mutation in the context of spontaneous glycine release from neighboring spinal cord interneurons. Spontaneous IPSPs (IPSCs) of α1<sup>Q177K</sup>β compared with α1β exhibited again a significant decrease in the decay time constant (***p = 5.2066E-5, t test; Fig. 5E).
The mutation Q177K disrupts GlyR function by faster ion channel closure

To investigate glycinergic synaptic signaling in intact synapses in situ, we prepared brainstem slices from wild-type and shaky mice and performed whole-cell recordings from neurons of the Pre-Bötz, a nucleus rich in GlyR/1 expression and important for respiration. When strychnine-sensitive glycinergic IPSCs (Fig. 6A) were evoked at different stimulus intensities in Pre-Bötz neurons at P18 to P24, a dramatically flattened input–output relationship in the mutant preparation was obtained (**p < 0.001 for all stimulus intensities; for values see the legend of Fig. 6B). Bath-applied glycine (50 μM) caused a significant smaller baseline shift in Glra1sh/sh neurons compared with control, again suggesting a deficit in postsynaptic GlyR function (**p < 0.0004, t test; Fig. 6C). mIPSC recordings (in 1 μM TTX) from Pre-Bötz neurons to study individual glycinergic synapses revealed significantly lower frequency, smaller amplitudes, and accelerated decay in Glra1sh/sh neurons (mIPSCs: Glra1+/+ compared with Glra1sh/sh, amplitude *p = 0.032; frequency, *p = 0.031; decay, **p = 0.0017; rise time, p = 0.423, nonsignificant). These effects were independent from the block of spontaneous spiking by TTX (spIPSCs; Glra1+/+ compared with Glra1sh/sh: amplitude, ***p = 4.27E-06; frequency, ***p = 2.55E-07; decay, ***p = 2.58E-06; rise time, p = 0.211, nonsignificant; Fig. 6D,E).

Many Pre-Bötz neurons receive mixed GABAergic/glycinergic inhibitory synaptic input with a corelease of both neurotransmitters from the same synaptic terminal (Rosenmund and Stevens, 1996). GABAergic spIPSCs, however, did not differ in frequency between the genotypes (p = 0.067, nonsignificant; Fig. 7), arguing against a generalized reduction in inhibitory synaptic terminal number or release probability as the main factor behind defects in glycinergic inhibition. To further exclude an overall decrease in motoneuron number as a modifier of the underlying pathology in shaky mice, the number of motoneurons was counted in lumbar spinal cord. No differences between Glra1+/+ and homozygous Glra1sh/sh animals were observed in six to seven

The mutation Q177K results in a lack of synaptic integration. A–C, Immunohistological stainings of spinal cord tissue from Glra1+/+ compared with homozygous Glra1sh/sh animals. Spinal cord slides (9 μm) were stained for the GlyR α1 subunit with the monoclonal antibody mAb2b together with presynaptic markers synapsin (syn; A) vesicular transporter (VGAT; B) and the postsynaptic marker gephyrin (geph; C). Ventral or dorsal horns are marked by a white dotted line. DAPI was used to stain nuclei. Right panels represent enlarged images of each staining. Note that there is less colocalization of GlyR α1 and gephyrin in homosynous shaky mice (white arrows). D, Spinal cord neuronal cultures from E13 embryos with genotypes Glra1+/+ or Glra1sh/sh were differentiated for 3 weeks in culture and stained for α1 (mAb2a) and gephyrin. An upregulation of GlyR α1 in shaky neurons was observed, but less colocalization in synaptic clusters with gephyrin (white arrows). Right panels represent enlarged dendrites of spinal cord neurons, which are marked by white boxes. E, Quantification of synaptic clusters in neurons from Glra1+/+ and Glra1sh/sh, n = 11 from two independent experiments. The relative expression of gephyrin and GlyR α1 is shown (p < 0.05, n.s.), comparison of expression levels between Glra1+/+ and Glra1sh/sh and within each group.

Figure 4.
Figure 5. GlyR α1<sup>Q177K</sup> leads to reduced agonist potency and faster decay times. A, Expression of wild-type and mutant heteromeric receptor complexes following cotransfection with the GlyR β subunit in HEK293 cells. Biotinylation assays were used to discriminate between surface and whole-cell protein. Left, Quantification of wild-type and mutant GlyR α1 protein with or without the GlyR β-subunit in whole-cell and surface pools normalized to pan-cadherin; data were obtained from at least three independent sets of experiments (n = 3–6). Right, Western blot analysis of biotinylation assays. The monoclonal pan-α antibody was used for recognition of the GlyR α1 subunit (48 kDa), the membrane protein pan-cadherin served as a loading control (LC, 135 kDa). UT, Untransfected cells; MOCK, GFP-transfected cells. B, Functional parameters from whole-cell recordings of transfected HEK293 cells for α1β and α1<sup>Q177K</sup>β heteromeric receptor configurations, with current amplitudes at 1 mM (α1β, n = 9; α1<sup>Q177K</sup>β, n = 16) and 100 μM glycine (α1β, n = 5; α1<sup>Q177K</sup>β, n = 5); 1 mM (α1β, n = 11; α1<sup>Q177K</sup>β, n = 9) and 100 μM β-alanine (α1β, n = 6; α1<sup>Q177K</sup>β, n = 6); and 1 mM (α1β, n = 6; α1<sup>Q177K</sup>β, n = 5) and 100 μM taurine (α1β, n = 5; α1<sup>Q177K</sup>β, n = 6). C, Ligand-binding potencies (EC<sub>50</sub>) determined by current measurements of transfected cells expressing the heteromeric receptor configurations α1β and α1<sup>Q177K</sup>β according to the adult in vivo receptor configuration at seven different glycine, β-alanine, and taurine concentrations (0.3–3.000 μM, n = 5) for each receptor configuration and for the agonist used. The 1 mM concentration of the agonist glycine or the partial agonists β-alanine and taurine were used to determine I<sub>max</sub> values. D, Decay times for desensitization. The decay currents were calculated in the presence of agonist (5 s). Right, Representative scaled current traces with α1β (n = 5, black) and α1<sup>Q177K</sup>β (n = 6, red). E, Faster decay of Shaky channels expressed in artificial synapses. Spontaneous IPSCs of R65 and S129 from one subunit (B) and F159, Y202, T204, and F207 from the adjacent subunit (A; Fig. 9C). R65 is crucial for ligand binding as it interacts with the α-carboxyl and α-amino groups of glycine (Grudzinska et al., 2005). The guanidinium group of R65 has an electrostatic interaction with the glycine carboxylate group (in the pocket), but also forms an additional H-bond with the Q177K substitution is predicted to not only abolish the formation of the H-bond between Q177 and R65 but is also likely to alter the position of R65 side-chain due to the additional positive charge contributed by the lysine side-chain. This in turn could lead to destabilization of the glycine-binding pocket (Fig. 9E). In animals analyzed for each genotype (p = 0.44, nonsignificant, t test; Fig. 8A, B). To conclude, the dramatically decreased amplitudes in glycine-evoked currents, mIPSCs, and spIPSCs explain the severity of the observed shaky phenotype. Similar to recordings from overexpressed cells as well as artificial synapses, decay times were accelerated in brainstem slice recordings, suggesting a function of the GlyR β8–β9 loop in the glycineergic signal transduction pathway.

Q177K disrupts hydrogen bonding with residues in the ligand-binding pocket

The recently uncovered cryo-EM structure of zebrafish GlyR α1 (PDB ID, 3JAE) provided evidence that the β8–β9 loop harboring the Q177K mutation is involved in ion channel opening/closing processes (Du et al., 2015). Glycine was modeled into the GlyR structure based on the position of glutamate in the binding pocket of GluCl (Hibbs and Gouaux, 2011) by superposition of GluCl (PDB ID, 3RHW) onto the GlyR structure. The Q177K substitution was inserted into the glycine-bound open-channel state as part of β-strand 9 (Fig. 9A–C). The glycine-binding pocket comprises residues R65 and S129 from one subunit (B) and F159, Y202, T204, and F207 from the adjacent subunit (A; Fig. 9C). R65 is crucial for ligand binding as it interacts with the α-carboxyl and α-amino groups of glycine (Grudzinska et al., 2005). The guanidinium group of R65 has an electrostatic interaction with the glycine carboxylate group (in the pocket), but also forms an additional H-bond with the Q177K substitution is predicted to not only abolish the formation of the H-bond between Q177 and R65 but is also likely to alter the position of R65 side-chain due to the additional positive charge contributed by the lysine side-chain. This in turn could lead to destabilization of the glycine-binding pocket (Fig. 9E). In
summary, disrupted hydrogen bonding affecting key ligand-binding residues is likely to underlie the functional impairment shown by biochemical and physiological approaches and explains the severity of the shaky phenotype.

Discussion

Our results comprehensively illustrate the importance of the β8–β9 loop structure within the extracellular domain for GlyR ligand binding and the subsequent conformational changes enabling signal transduction and synaptic clustering. The current understanding of GlyR ion channel function suggests the coupling of movements within the ECD (loop C, β1–β2, β6–β7) upon ligand binding proceeding to elements of the ECD–transmembrane domain interface (β10–pre-M1, the M2–M3 loop). These conformational rearrangements result in tilting of transmembrane domains, enabling ion channel opening and closing.

(Hassaine et al., 2014; Du et al., 2015; Gielen et al., 2015; Huang et al., 2015). The role of the β8–β9 loop, a flexible region localized at the complementary side of adjacent subunits underneath the ligand-binding pocket, has remained elusive due to the overall low electron density observed for this unstructured region of the ligand-binding site in earlier studies (Brejc et al., 2001; Hansen et al., 2005). Here, using the shaky mutant mouse that harbors a β8–β9 loop alteration, we found in vivo evidence that this extracellular loop is involved in stabilizing the GlyR ligand-binding site and ion channel gating. The pathogenic mechanism in this mouse mutant delineates a combined pattern of biogenesis defects resulting in defective synaptic integration, together with lower opening frequency, smaller amplitudes, and accelerated decay rates for remaining GlyRs, functional disturbances that are incompatible with survival.

Figure 6. Shaky mice have impaired glycinergic synaptic transmission in PreBötC neurons. All recordings were made in whole-cell voltage-clamp mode in the presence of the ionotropic glutamate receptor antagonist KA (2 mM) and the GABA receptor antagonist bicuculline (10 μM). A, Traces from a wild-type PreBötC neuron illustrate the evoked IPSC (at stimulation intensity of 200 μA for 0.1 ms) and sensitivity to strychnine (10 μM). B, Input–output curves of evoked IPSCs in Gira1+/– (n = 24 from 12 mice) and Gira1sh/sh (n = 25 from 12 mice) mice; comparison of Gira1+/– and Gira1sh/sh at 100 μA: ***p = 3.198E-09; at 200 μA, ***p = 1.539E-12; at 300 μA, ***p = 3.20E-12; at 400 μA, ***p = 3.32E-12, t-test). C, Traces from wild-type and Gira1sh/sh neurons show postsynaptic current responses to glycine application (50 μM), recorded in TTX (1 μM). Note the increase in baseline noise with glycine in Gira1sh/sh neuron, despite no shift in the holding current. Diagram on the right summarizes glycine-induced currents (Gira1+/–, n = 7 from 3 mice; Gira1sh/sh, n = 9 from 4 mice). D, Traces of spontaneously occurring mIPSCs, recorded in TTX (1 μM) from both genotypes, illustrate the reduced action potential-independent synaptic activity in Gira1sh/sh mice. E, Traces of spontaneously occurring events (spIPSCs: Gira1+/–, n = 17 from 10 mice; Gira1sh/sh, n = 21 from 12 mice; mIPSCs: Gira1+/–, n = 6 from 3 mice; Gira1sh/sh, n = 6 from 4 mice). *p < 0.05; **p < 0.01; ***p < 0.001.
In humans and rodents, mutations in GlyR subunit genes are associated with startle disease, characterized by exaggerated responses to acoustic stimuli and uncontrolled falling (Bode and Lynch, 2014) with an increased risk of cognitive deficits, such as learning difficulties or delay in speech acquisition (Thomas et al., 2013). The phenotype of the shaky mouse is in line with that of human patients, as well as strychnine poisoning (Buckwalter et al., 1994) and with other glycinergic mouse models (e.g., spastic, oscillator, and Nmf11; Traka et al., 2006; Schaefer et al., 2012). Our in vitro analysis revealed differences in surface expression between heteromeric wild-type α1β and mutant α1Q177Kβ GlyRs. Mutant α1Q177Kβ GlyRs were able to form functional ion channels with increased EC50 values. However, the desensitization time constant decreased for α1Q177Kβ GlyRs, indicating faster ion channel closure. The observed changes in vitro provided some explanations for the glycinergic defect present in vivo; however, the lethality of homozygous shaky mice presented a conundrum. A fivefold to sixfold reduction of GlyR agonist potency has also been documented in spastic mice carrying an A52S substitution in the β1–β2 loop of the GlyR α1 subunit ECD. In contrast to shaky mice, homozygous spastic mice display a normal life span and a mild neuromotor phenotype, arguing that the shift in agonist potency alone cannot explain lethality (Graham et al., 2011). Moreover, in vivo expression levels of α1Q177K were increased in spinal cord and brainstem, although synaptic integration was significantly diminished. Synaptic integration of GlyRs is enabled by the scaffolding protein gephyrin via high-affinity binding to the intracellular M3–M4 domain of the GlyR β subunit (Triller and Choquet, 2005; Dumoulin et al., 2010). A consequence of lower synaptic integration is less synaptic strength, which is normally regulated by lateral diffusion of synaptic and extrasynaptic receptors in and out of synapses (Triller and Choquet, 2005). The lower number of synaptic complexes argues that the extracellular binding site mutation is able to transduce conformational changes to the M3–M4 loop domain, thereby disrupting gephyrin binding and, thus, synaptic anchoring of α1Q177Kβ GlyRs. In turn, these data suggest that glycine sensitivity or gating properties of the receptor can be altered by the clustering status of the GlyR at the synapse. Another option that may explain the reduced synaptic integration is an enhanced turnover of the synaptic receptor pool via endocytosis as a neuronal adaptation to the impaired functionality of α1Q177Kβ GlyRs in vivo. We therefore conclude that the enhanced expression of α1Q177K represents an unsuccessful attempt at neuronal compensation.

In addition to the postsynaptic effects of the shaky mutation, there may also be presynaptic consequences for functionally impaired homomeric α1Q177K GlyRs. A potential role for presynaptic homomeric GlyRs in hyperekplexia has recently been demonstrated (Xiong et al., 2014). Presynaptic GlyR α1 subunit homomers have been described in calyceal synapses in the medial nucleus of the trapezoid body, in spinal cord and the ventral tegmental area (Turecek and Trussell, 2001; Jeong et al., 2003). Activation of these presynaptic GlyRs by glycine spillover triggers weakly depolarizing Cl− currents. The generated depolarization leads to enhanced transmitter release by Ca2+ channel activation and increased Ca2+ concentrations in the nerve terminal (Turecek and Trussell, 2001). The increase in α1Q177K expression in vivo may result in an enhanced expression of presynaptic homomeric GlyRs generating impaired presynaptic GlyR activity and thus, diminished glycine release in the brainstem and spinal cord of shaky mice. Consequently, disrupted presynaptic GlyR func-
tion would be consistent with the significantly reduced IPSC frequencies we observed in brainstem slice recordings.

Functional analysis in brainstem nuclei that are rich in glycineric synapses revealed largely reduced current amplitudes and significantly lower frequencies of spontaneous and miniature IPSCs in *shaky* mice, which are likely to be consequences of the low numbers of postsynaptic functional receptors and enhanced expression of functionally impaired presynaptic homomeric GlyRs. As the reduced ligand potency of *shaky* GlyRs is still within the range of glycine concentrations that can be achieved during synaptic activation, the observed functional deficits must result from disturbed translation of ligand binding into ion channel opening (gating). Impaired gating was further confirmed by reduced efficacies of the partial agonists /H9252-alanine and taurine.

Moreover, residue Q177 normally undergoes H-bond formation with the ligand-binding residue R65, which is disrupted by the positively charged lysine. The positional change of the R65 side-chain due to the introduction of additional positive charge from K177 would in turn destabilize the glycine-binding pocket, which is in agreement with the observed decrease in glycine potency. The importance of the /H9252–/H9252 loop contribution to a hydrogen bond network in bound and unbound receptor states has been shown previously for /H9253 GABA<sub>A</sub> and 5HT<sub>3A</sub> as well as nAChR subunits (Nys et al., 2013).

The observed decrease in the decay time constant for the /α<sub>1Q177K</sub> mutation, resulting in faster ion channel closure is similar to *spasmodic* mice (Graham et al., 2006), arguing for a significant impact of ion channel decay mechanisms on the startle phenotype and in the case of *shaky* a contribution to the lethality in this mouse model. The functional analogy between *spasmodic* and *shaky* mice suggests a defect in the same signal transduction pathway determined by coupling of ECD movements following ligand binding to finally ion channel opening and closing (Du et al., 2015). Functional synaptic /α<sub>1Q177K</sub> GlyRs close much faster
than wild-type channels, implying a fast unbinding process of the agonist. Since binding of glycine was only marginally affected in spinal cord tissue, the functional defects of α1Q177K must result from downstream processes (e.g., fast transduction of ligand-bound receptor into the closed conformation). These data are in line with the recently proposed model of signal transduction for GlyRs (Du et al., 2015; Morales-Perez et al., 2016; Nys et al., 2016). Thus, the integrity of the β8–β9 loop is a prerequisite for conformational rearrangements and is crucial during gating processes of the GlyR channel, which opens a novel window for therapeutics, resulting in prolonged open times. Accordingly, both the reduced synaptic integration and faster ion channel closure observed for GlyRs in shaky mice may also represent novel pathogenic mechanisms for human startle disease mutations. Together, our data reveal that the β8–β9 loop in the GlyR α1 ECD is a key regulator of glycinergetic signaling. Furthermore, shaky represents the first in vivo mouse model demonstrating the incompatibility of a disrupted β8–β9 loop with life.

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