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REVIEW

Glycine receptor mouse mutants: model systems for human hyperekplexia

Natascha Schaefer[1], Georg Langlhofer[1], Christoph J Kluck[2] and Carmen Villmann[1]

[1]*Institute for Clinical Neurobiology, Julius-Maximilians-University of Würzburg, Würzburg, Germany, and* [2]*Institute of Biochemistry, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany*

Correspondence
Carmen Villmann, Institute for Clinical Neurobiology, University of Würzburg, Versbacherstr. 5, Würzburg D-97078, Germany. E-mail: villmann_c@ukw.de

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Human hyperekplexia is a neuromotor disorder caused by disturbances in inhibitory glycine-mediated neurotransmission. Mutations in genes encoding for glycine receptor subunits or associated proteins, such as *GLRA1*, *GLRB*, *GPHN* and *ARHGEF9*, have been detected in patients suffering from hyperekplexia. Classical symptoms are exaggerated startle attacks upon unexpected acoustic or tactile stimuli, massive tremor, loss of postural control during startle and apnoea. Usually patients are treated with clonazepam, this helps to dampen the severe symptoms most probably by up-regulating GABAergic responses. However, the mechanism is not completely understood. Similar neuromotor phenotypes have been observed in mouse models that carry glycine receptor mutations. These mouse models serve as excellent tools for analysing the underlying pathomechanisms. Yet, studies in mutant mice looking for postsynaptic compensation of glycinergic dysfunction via an up-regulation in $GABA_A$ receptor numbers have failed, as expression levels were similar to those in wild-type mice. However, presynaptic adaptation mechanisms with an unusual switch from mixed GABA/glycinergic to GABAergic presynaptic terminals have been observed. Whether this presynaptic adaptation explains the improvement in symptoms or other compensation mechanisms exist is still under investigation. With the help of spontaneous glycine receptor mouse mutants, knock-in and knock-out studies, it is possible to associate behavioural changes with pharmacological differences in glycinergic inhibition. This review focuses on the structural and functional characteristics of the various mouse models used to elucidate the underlying signal transduction pathways and adaptation processes and describes a novel route that uses gene-therapeutic modulation of mutated receptors to overcome loss of function mutations.

Abbreviations

CLR, cys-loop receptor; ECD, extracellular domain; GEFS+, generalized epilepsy with febrile seizures plus; Gly, glycine; M1–M4, transmembrane domains 1–4; PTX, picrotoxin

History of human hyperekplexia

The ability of strychnine to influence inhibitory reflexes and to convert these into excitatory reflexes was originally shown by Owen and Sherrington (1911). Hyperekplexia (*Startle disease, Stiff baby syndrome, STHE* – startle disease or hyperekplexia, OMIM 149400) was first described by Kirstein and Silfverskiold (1958) long before recombinant systems became available. In 1966 Suhren *et al*. reported a family with 25 members through five generations that suffered from abnormal severe startle reactions. This family was clinically described as having an autosomal dominant inheritance with an abnormal startle reaction in affected patients, which was elicited by different stimuli that did not provoking similar reactions in healthy controls. With the help of electroencephalographic (EEG) observations, a subcortical origin, for example, some midline structure like the brainstem, was proposed as being the source for this abnormal startle reaction. Furthermore, the patients were conscious during the attacks and the observed EEG waves did not correspond to those of epileptic patients (Suhren *et al*., 1966). These patients were treated with clonazepam, a benzodiazepine and potent 5-HT agonist. It was also reported that alcohol, phenobarbital and chlordiazepoxide were able to decrease the intensity of the symptoms (Andermann *et al*., 1980).

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Later on, two other case reports of families with hyperekplexia were reported by Morley *et al.* (1982) and Kurczynski (1983), and in 1991, two Japanese families with several affected individuals were described with an autosomal dominant inheritance and abnormal startle responses. Recessive forms of inheritance were also depicted from other three families (Hayashi *et al.*, 1991). Only one year later, the genetic locus of hyperekplexia was deduced, with the help of linkage analysis and radiation hybrid mapping, as being on the long arm of chromosome 5. Two possible candidate genes, *GABRA1* and *GABRG2*, encoding GABA receptor subunits and three other loci, *RPS14*, *SPARC* and *GLRA1*, were determined as the source of the abnormal startle responses (Ryan *et al.*, 1992a; 1992b). Finally, Shiang *et al.* identified the *GLRA1* gene as the candidate gene for sequence alterations in affected patients because of the following reasons: (i) *GLRA1* was mapped on the long arm of chromosome 5 near *SPARC* (osteonectin) and *RPS14* (ribosomal protein S14), both of which were marked in the hyperekplexia region via radiation hybrid mapping; (ii) a detailed analysis showed that *GLRA1* was directly in the middle of the candidate region; and (iii) *GLRA1* encodes for a subunit of the inhibitory glycine receptor and can be antagonized by strychnine, which in sub-lethal doses causes hypertonia and exaggerated startle responses in mice. This phenomenon was similar to symptoms described in human patients suffering from hyperekplexia. Shiang *et al.* (1993) identified two mutations in four affected individuals from four different families with a nucleotide exchange G1192T/A. No mutation was found in 50 unaffected individuals. These single nucleotide exchanges resulted in an amino acid exchange from arginine at position 271 to leucine or glutamine (Figure 1). Meanwhile, several additional mutations have been identified, for example, Y279C (Shiang *et al.*, 1995), Q266H (Milani *et al.*, 1996), K276E (Seri *et al.*, 1997), P250T (Saul *et al.*, 1999), V260M (del Giudice *et al.*, 2001), S267N (Becker *et al.*, 2008) and Y128C (Chung *et al.*, 2010) (Figure 1; Table 1).

Glycine receptor structure and determinants

Overall architecture

The anion-conductive glycine (Gly) receptor is a pentameric ligand-gated ion channel that mediates fast synaptic inhibition in mammalian spinal cord and brain stem. The Gly receptor was purified via an affinity chromatography column using amino strychnine agarose as a resin and later several subunits were cloned (Pfeiffer *et al.*, 1982; Grenningloh *et al.*, 1990). Gly receptors belong to the superfamily of Cys-loop (CL) receptors, which also comprise GABA, type A and C ($GABA_A$), nicotinic ACh (nACh) and 5-hydroxytryptamine (type 3, $5\text{-}HT_3$) receptors (Karlin and Akabas, 1995; for receptor nomenclature see Alexander *et al.*, 2011). All members of this family share a common topological organization of different subunits in a pentameric conformation. Each of them consists of a large amino-terminal extracellular domain (ECD) that harbours the ligand-binding sites followed by four α-helical transmembrane domains (M1–M4) connected by intracellular and extracellular loop structures. The C-terminus is short and extracellularly localized (Lynch, 2004; Hilf and Dutzler, 2008).

The ECD is determined by 10 β-strands that are organized in two sheets to form a β-sandwich and a short α-helix. The loop between the sixth and seventh β-strand is constrained by a disulphide bond between two highly conserved cysteine residues, responsible for the name of the superfamily. The order of β-strands is similar to a modified Ig topology (Bork *et al.*, 1994). The M2 is followed by a large intracellular loop between M3 and M4. Gly receptors assemble from five subunits with each contributing one amphipathic M2 domain to the central ion channel pore localized 50 Å below the ligand-binding site (Unwin, 1995; Brejc *et al.*, 2001; Miller and Smart, 2010). The subunits are arranged like staves of a barrel around a symmetry axis that defines the ion permeation pathway.

The configuration of the native Gly receptor is still debatable. Independent approaches, such as the initial Gly receptor characterization of purified receptor protein, studies on concatameric receptors and the determination of subunit arrangements using atomic force microscopy of tagged α and β subunits, have revealed a heteromeric stoichiometry of 2α (48 kDa) and 3β subunits (58 kDa) (Pfeiffer *et al.*, 1982; Grudzinska *et al.*, 2005; Yang *et al.*, 2012). Recently, a heteromeric configuration of 3α:2β was determined using single-molecule imaging and stepwise photobleaching of venus fluorescent protein-tagged Gly receptor α and β subunits expressed in *Xenopus* oocytes (Durisic *et al.*, 2012). With regard to the existence of an α-α or β-β interface within the pentameric receptor complexes, the correct assignment of the ion channel subunit composition is important for developing specific therapeutic substances.

The ECDs form the agonist binding sites at subunit interfaces, which are determined by loops A, B and C from the principal (+) interface and loops D, E and F from the complementary (–) subunit. The interface between the ECD and the transmembrane domains is termed the transition zone. Following ligand binding, this receptor domain enables conformational changes that are required to allow ion channel opening. This zone comprises domains of the ECD, for example, loop 2, the Cys-loop, the pre-M1 linker and the M2–M3 loop (Brejc *et al.*, 2001; Unwin, 2005; Hibbs and Gouaux, 2011). A re-arrangement of the transition zone leads, in turn, to a reconfiguration of the M2–M3 loop. Rotation or tilting of the M2 helix during conformational changes ultimately produces channel opening (Pless *et al.*, 2007; Lee *et al.*, 2009).

The ECD

Considerable progress in the definition of amino acid residues involved in ligand binding has been made following the determination of the X-ray structure of the ACh-binding protein (AChBP). AChBP is a molluscan homologue of the ECD of nACh receptors. Meanwhile, the structures of two prokaryotic homologues ELIC and GLIC and the invertebrate glutamate-gated chloride channel (GluCl) from *Caenorhabditis elegans* have been resolved (Brejc *et al.*, 2001; Hilf and Dutzler, 2008; 2009; Hibbs and Gouaux, 2011). GLIC and ELIC presumably represent the closed and open state of CL receptors respectively (Bocquet *et al.*, 2009). Most conserved ECD residues are hydrophobic, resulting in two hydrophobic

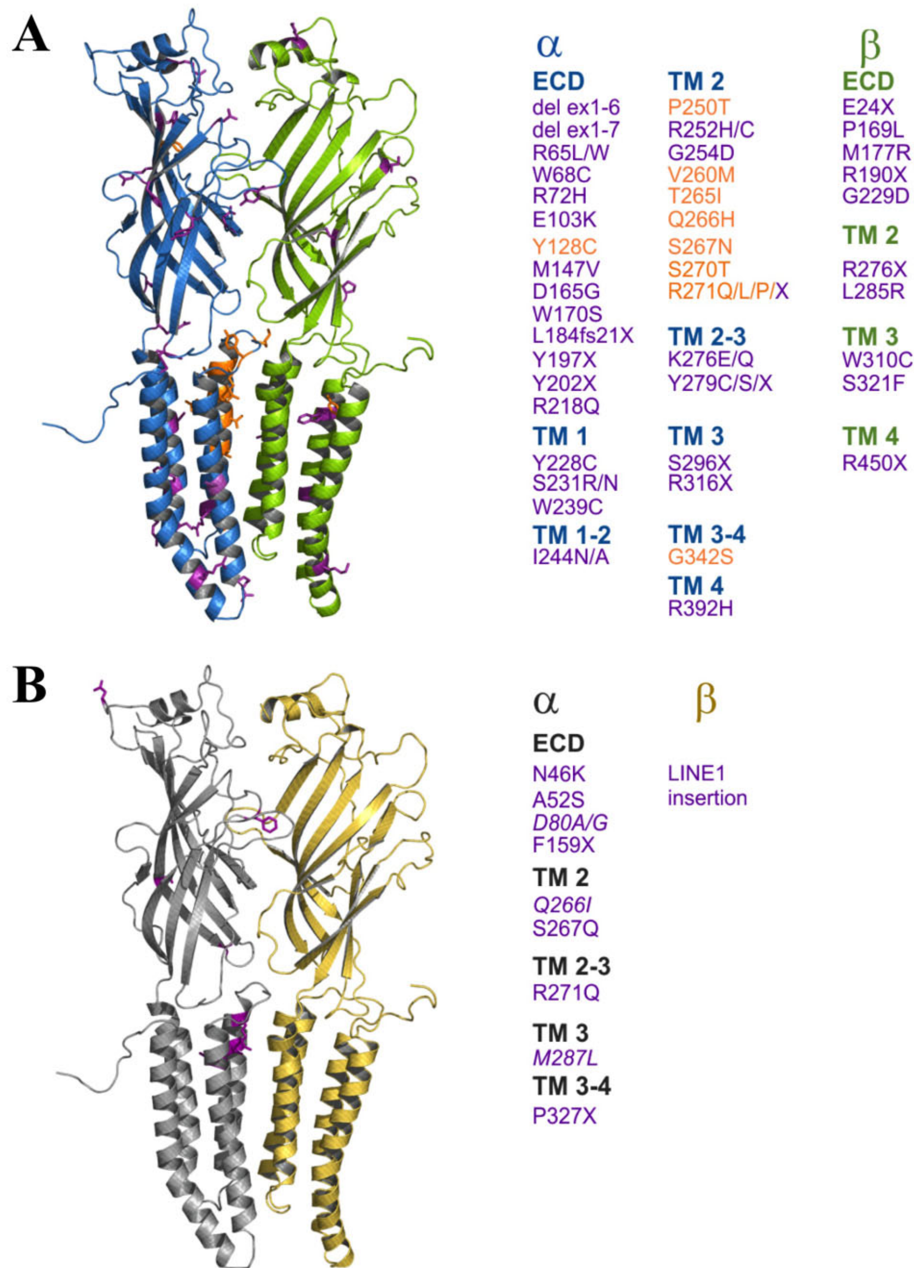

Figure 1

Homology models of the human (A, blue – α subunit, green – β subunit) and mouse (B, grey – α subunit, yellow – β subunit) Gly receptor subunits based on the crystal structure of the *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) solved by Hibbs and Gouaux (2011) showing the mutated amino acid positions affected in hyperekplexia. Two neighbouring subunits of a pentamer are shown. Amino acid exchanges due to recessive mutations, compound heterozygous mutations or deletions with frameshifts resulting in premature stop codons as well as those with no known mode of inheritance are shown as magenta sticks, while amino acid exchanges based on dominant mutations are depicted in orange. (B) Murine knock-in mutations are shown in italics. Note that in the homology models the large intracellular loop domain (ICD) between M helices 3 and 4 was left out as the crystallized GluCl variant also just had a minimal ICD of three amino acids. Therefore, Gly receptor variants having changes in the ICD region are listed, but not represented in the structures modelled.

pockets at the subunit interfaces (Brejc *et al.*, 2001). Several lines of evidence exist that these interfaces do indeed represent the ligand-binding sites (Corringer *et al.*, 2000). The binding pockets are rich in aromatic residues, which undergo cation–π interactions with the amine nitrogen of the agonist. These connections substantially contribute to agonist binding (Zhong *et al.*, 1998). The most common location for such an interaction is an aromatic residue on loop B (Trp[149] of the nACh receptor, Trp[183] of the 5-HT$_3$ receptor, Tyr[198] of the GABA$_C$ receptor and Phe[159] of the Gly receptor) (Pless *et al.*, 2008). The functional role of the Gly receptor ECD was further demonstrated by a pronounced effect on single

Table 1

Mutations in the human *GLRA1*gene

Mutation	Mode of inheritance	Effect	References
Deletion Exon 1–6 Deletion 1–7	Autosomal recessive	n.d.	Becker *et al.* (2006); Brune *et al.* (1996)
R65L/ R65W	Autosomal recessive	Non-functional	Chung *et al.* (2010)
W68C	Autosomal recessive	n.d.	Tsai *et al.* (2004)
R72H	Autosomal recessive	n.d.	Coto *et al.* (2005)
E103K	Autosomal recessive	Reduced maximal currents	Chung *et al.* (2010)
Y128C	Autosomal dominant	Reduced maximal currents	Chung *et al.* (2010)
M147V	Autosomal recessive	n.d.	Rees *et al.* (2001)
D165G	Autosomal recessive	Reduced maximal currents	Chung *et al.* (2010)
W170S	Autosomal recessive	n.d.	Al-Futaisi *et al.* (2012)
Y197X	Autosomal recessive	n.d.	Chung *et al.* (2010)
Y202X	Autosomal recessive	Reduced cell surface expression	Rees *et al.* (2001)
R218Q	Autosomal recessive	Reduced cell surface expression	Castaldo *et al.* (2004); Miraglia *et al.* (2003)
Y228C	Autosomal recessive	n.d.	Forsyth *et al.* (2007)
S231R/ S231N	Autosomal recessive	Reduced cell surface expression	Humeny *et al.* (2002); Villmann *et al.* (2009b); Chung *et al.* (2010)
W239C	Autosomal recessive	n.d.	Gilbert *et al.* (2004)
I244N A^{-1}	Autosomal recessive	Reduced glycine affinity, reduced maximal currents, increased desensitization	Rees *et al.* (1994); Lynch *et al.* (1997); Villmann *et al.* (2009b)
P250T	Autosomal dominant	Reduced single channel conductance, reduced glycine sensitivity	Saul *et al.* (1999)
R252H/R252C/R392H	Compound heterozygous, autosomal recessive	Reduced cell surface expression	Vergouwe *et al.* (1999); Rea *et al.* (2002); Villmann *et al.* (2009b); Chung *et al.* (2010)
G254D	Autosomal recessive	Non-functional	Chung *et al.* (2010)
V260M	Autosomal dominant	Changes in taurine binding	del Giudice *et al.* (2001); Castaldo *et al.* (2004)
T265I	Autosomal dominant	Co-transfection with β subunit required for functionality	Chung *et al.* (2010)
Q266H	Autosomal dominant	Reduced open probability, reduced glycine sensitivity	Milani *et al.* (1996); Moorhouse *et al.* (1999)
S267N	Autosomal dominant	Reduced glycine sensitivity	Becker *et al.* (2008); Findlay *et al.* (2005)
S270T	Autosomal dominant	n.d.	Lapunzina *et al.* (2003)
R271Q/L/P/X	Autosomal dominant	Reduced glycine sensitivity, reduced single channel conductance	Ryan *et al.* (1992b); Shiang *et al.* (1993); Langosch *et al.* (1994); Rajendra *et al.* (1994)
K276E/Q	Autosomal dominant	Reduced glycine sensitivity, reduced open probability	Elmslie *et al.* (1996); Lewis *et al.* (1998); Lynch *et al.* (1997)
Y279C/S/X	Autosomal dominant	Reduced glycine sensitivity, reduced maximal current responses	Filatov and White (1995); Lape *et al.* (2012); Lynch *et al.* (1997)
S296X	Autosomal recessive	n.d.	Bellini *et al.* (2007)
R316X	Autosomal recessive	n.d.	Tsai *et al.* (2004)
G342S	Compound heterozygous	n.d.	Rees *et al.* (2001)
R392H/R252H/R252C	Compound heterozygous, autosomal recessive	Reduced cell surface expression	Vergouwe *et al.* (1999); Rea *et al.* (2002); Villmann *et al.* (2009b); Chung *et al.* (2010)

n.d., not determined.

channel conductances when adjacent charged residues localized in β-strands β2 (D57 and R59) and β4 (K104, G105) were mutated into homologous residues of the nAChα1 receptors. These data revealed that the ECD determines the ion selectivity and permeation of Cys-loop receptors (Brams *et al.*, 2011a). Moreover, the highly conserved amino acid D97 on loop A was shown to construct an inter-subunit electrostatic bond, the disruption of which resembles a critical step in channel activation (Todorovic *et al.*, 2010). Previous studies have identified three residues within the ECD of Gly receptors including D97 that cause a spontaneous opening of the channel: D97R, F99A and Y128C (Beckstead *et al.*, 2002; Miller *et al.*, 2008; Chung *et al.*, 2010). Structural modelling of the Glyα1 receptor mutant Y128C predicted an induction of a short α-helix in the ECD directly overlying the M regions, suggesting a common mechanism of tonic channel opening (Chung *et al.*, 2010).

In addition to the eponymous Cys-loop, the ECD of the Gly receptor carries two additional cysteine residues in loop C that are conserved in the recently solved GluCl receptor structure. These cysteines have been found to form another disulphide bond, which affects both the formation of the eponymous Cys-loop and the correct membrane integration needed for channel function (Vogel *et al.*, 2009; Hibbs and Gouaux, 2011). Several lines of evidence indicate a rigid body capping motion of loop C upon agonist binding, thereby trapping bound agonist molecules (Hansen *et al.*, 2005). At the complementary subunit interfaces, voltage-clamp fluorometry and site-directed mutagenesis analysis identified several residues on Gly receptor loops D, E to be crucial for ligand recognition and discrimination (Grudzinska *et al.*, 2005; Pless and Lynch, 2009). Apart from distinct loop structures, the pre-M1 linker at the interface between ECD and M1 of Gly receptors was demonstrated to be part of the transition zone translating ligand binding into channel opening (Lee *et al.*, 2009). It has been reported that electrostatic repulsion between a pair of residues that are in close proximity to each other in the tertiary structure, but are discrete from the pre-M1 and Cys-loop subdomains, respectively, prevents surface expression of Gly receptors (Shan and Lynch, 2012). These results emphasize the importance of an intact transition zone for both proper receptor assembly and transmission of agonist-induced conformational changes to the channel gate.

The ion channel pore

Most missense mutations located either in or near the M2 pore-lining domain affect ion channel properties (Harvey *et al.*, 2008). Structural analysis showed the M2 to be kinked radially inwards, attaining a minimum pore diameter at approximately its midpoint (Miyazawa *et al.*, 2003). Ion selectivity is controlled by sets of charged residues near the mouth of the channel (Hucho and Weise, 2001). The following Glyα1 receptor subunit residues are localized in the ion pore: G2′, T6′, R19′ and A20′ (Saitoh *et al.*, 1994; Shan *et al.*, 2001b). Other residues that most likely face the pore have been predicted by homology with other CL receptors: T7′, L9′, T10′, T13′, S16′ and G17′ (Akabas *et al.*, 1994; Xu and Akabas, 1996; Lynch, 2004). The M2 numbering system assigns position 1′ to the putative cytoplasmic end of M2 and 19′ to the outermost residue. When viewed from a α-helical net, these residues form a hydrophilic 'strip' along one site of an otherwise hydrophobic α-helix. The 9′ leucine ring is conserved throughout the CL receptor family, and substitution with hydrophilic residues in nACh, $GABA_A$ and Gly receptors has been shown to increase the tendency of the channel to open in response to agonist. Therefore, hydrophobicity of this ring stabilizes the closed state of the channel (Kwok *et al.*, 2001). Although other regions contribute to the modulation of conductance and selectivity of CL receptors, the minimal determinants of selectivity are the −1′Ala and −2′Pro positions for anions and the −1′Glu for cations, with no requirement for positively charged amino acids in the pore of anion-selective channels (Keramidas *et al.*, 2004; Sunesen *et al.*, 2006; Thompson *et al.*, 2010). For the Gly receptor, the mutations P250Δ (−2P′), A251E and T265V have been performed, which resulted in cation selectivity (Keramidas *et al.*, 2004). The critical effect of P250 was further demonstrated in a mutagenesis study, where isotropic forces, charge and hydrophobicity were identified as critical determinants of receptor function (Breitinger *et al.*, 2001) (Figure 2). Thus, P250 may serve a dual role (i) positioning neighbouring residues and (ii) representing a critical residue at the protein surface near the inner mouth of the ion channel. Apart from M2's function as an ion channel pore, it has been suggested that the main binding sites for allosteric modulation are located within the M-domain (Mihic *et al.*, 1997; Nury *et al.*, 2010; James *et al.*, 2013). These studies highlight the role of two cavities in the allosteric modulation processes, both are located in the upper part of the M-domains – one at the centre of the four helices bundle in each subunit and one at the interface between subunits (Duret *et al.*, 2011).

Pharmacology of glycine receptors

Low-efficacy agonists

Apart from the major agonist glycine, the β-amino acids taurine and β-alanine have been proposed as endogenous lower efficacy Gly recptor agonists (Laube *et al.*, 2002). At low concentrations, they competitively inhibit glycine responses, whereas high concentrations elicit significant membrane currents, albeit their affinities and efficacies are lower than that of glycine (Schmieden and Betz, 1995; Breitinger and Becker, 1998).

Zinc

Opposite concentration-dependent effects apply to Zn^{2+}-mediated modulation of Gly receptors. Low concentrations of Zn^{2+} (<10 μM) enhance glycine-mediated currents by increasing the agonist affinity without changing the maximal inducible current. Higher concentrations lead to an inhibitory effect (Laube *et al.*, 1995a; Lynch *et al.*, 1998). Both the potentiation and inhibition of Gly receptor currents by Zn^{2+} are reversible. It has been suggested that Zn^{2+} affects agonist association as well as the efficacy of channel opening (Laube *et al.*, 2000).

Antagonists

Strychnine and d-tubocurarine (d-TC) are alkaloids from poisonous plants that act as competitive antagonists with very

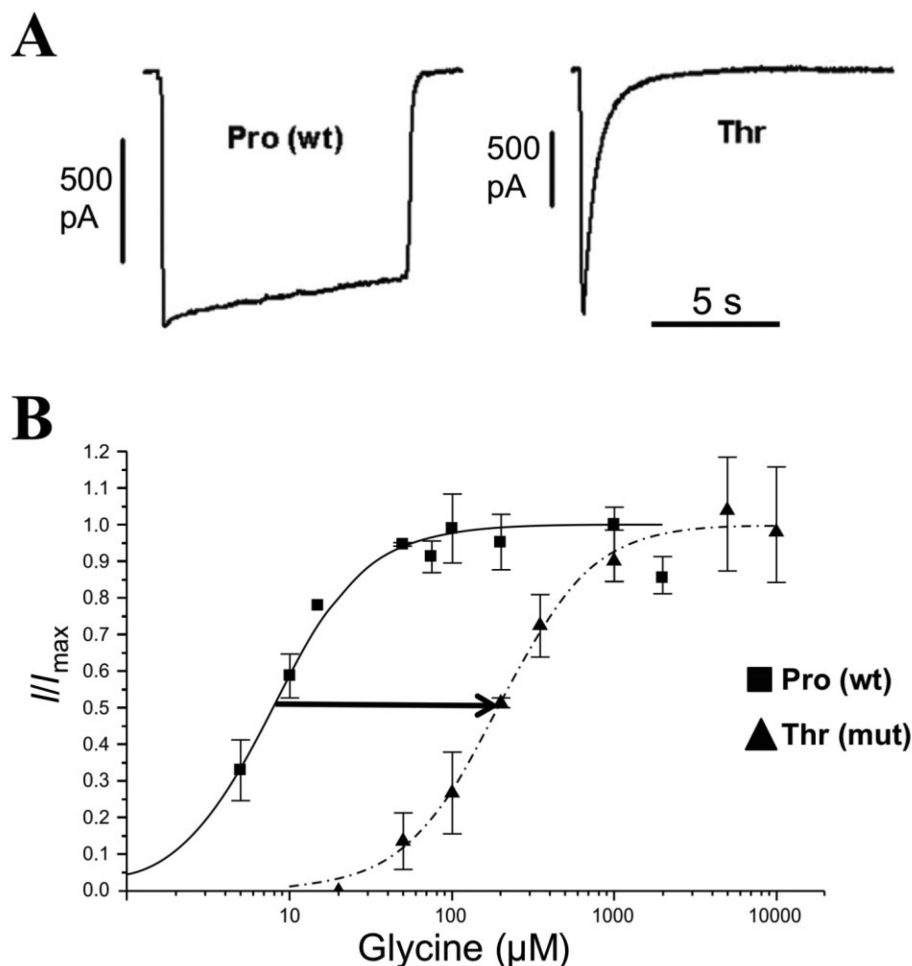

Figure 2

Dominant hyperekplexia mutants result in changes in pharmacological properties of the Gly receptor α1 channel (modified from Breitinger *et al.*, 2001). (A) The human mutation P250T (Thr) was introduced into the Gly receptor α1 cDNA and transfected into HEK293 cells. Following application of glycine (1 mM), mutant channels desensitize much faster that those of wild-type (wt Pro) controls (non-desensitizing wt compared to 121 ± 6 ms for P250T). (B) EC_{50} curves for glycine were measured using various glycine concentrations from 5 μM to 10 mM. With P250T, the glycine dose–response curve shows a rightward shift to lower glycine affinities (arrow, reduced by 24-fold compared with wild-type control).

high affinity for Gly and nACh receptors. In contrast to high affinity, both have low specificity as d-TC also antagonizes the effect of 5-HT on 5-HT_3 receptors and strychnine antagonizes some $GABA_A$ receptors. The low selectivity of both alkaloids has been attributed to a limited number of contacts in the ligand-binding pocket (Brams *et al.*, 2011b). Another antagonist displaying no subunit selectivity on Gly receptors is 3-[2′-phosphonomethyl[1,1′-biphenyl]-3-yl]alanine (PMBA). Although structurally distinct, PMBA and strychnine exhibit a number of similarities in their antagonism of recombinant Gly receptors. Therefore, they are assumed to bind to similar and possibly overlapping sites at the receptor (Saitoh *et al.*, 1994).

Picrotoxin (PTX)

In contrast to strychnine and PMBA, picrotoxin (PTX) has been shown to discriminate between homo- and heteromeric Gly receptors (Pribilla *et al.*, 1992). While homomeric α1 and α2 Gly receptors are blocked at low micromolar concentrations, α1βGly receptors are relatively resistant to PTX (Lynch *et al.*, 1995). Nevertheless, PTX is not specific for Gly receptors as it also modulates $GABA_A$ receptors. Inhibition by PTX is sensitive to mutations of residues in the ion channel pore (Zhang *et al.*, 1995). However, the possibility that it induces classical pore block seems unlikely. Further studies have revealed that PTX acts in an allosteric mode inducing conformational changes in the M2–3 loop and thereby transmitting glycine-induced conformational changes in the ECD to the channel gate (Hawthorne and Lynch, 2005). In this respect, Wang *et al.* (2006) showed that PTX binds to Gly receptors in the open state and is likely to be trapped while glycine dissociates from the receptor.

Cyanotriphenylborate (CTB)

CTB represents a subunit-selective open-channel blocker of Gly receptors (Rundstrom *et al.*, 1994). The antagonistic

effect of CTB is non-competitive and depends on a single glycine residue at position 254 of the α1Gly receptor subunit, which is exchanged by an alanine residue in the CTB-resistant α2 subunit. Although positively and negatively modulating agents of the inhibitory Gly receptors exist, with the exception of CTB, there is no known specific blocker of Gly receptors or even a subunit-specific blocker.

Ivermectin

Endogenous neurotransmitter agonists activate rapidly reversible currents at Gly receptors. In contrast, the anthelmintic ivermectin activates slowly developing currents that are usually irreversible (Shan *et al*., 2001a). There is evidence that binding sites for glycine and strychnine are distinct from that for ivermectin and that different conformational changes are involved in activation by the respective agonists. Ivermectin inserts deeply into the subunit interface, wedged between the M3 α-helix on the principal and the M1 helix on the complementary subunit (Hibbs and Gouaux, 2011). The reversible agonist activity of ivermectin is slow and is most likely due to its accumulation in the membrane associated with weak binding, which induces a global receptor conformational change. The weak interaction might either facilitate agonist binding or agonist-induced conformational changes associated with channel activation.

Alcohols and anaesthetics

Gly receptors are modulated by ethanol and anaesthetics. It has been hypothesized that amino acids in all four transmembrane domains contribute to a drug-binding cavity (Cheng *et al*., 2008). Using chimeric Glyα1/GABAρ1 receptor constructs, a region of 45 amino acids within the M2 and M3 domains of Gly receptors was identified, which appeared to be essential and sufficient for the enhancement of receptor function by ethanol. Within this region, two specific residues, S267 in M2 and A288 in M3, have been found to play an unique role in the allosteric modulation of both $GABA_A$ and Gly receptors by alcohols and anaesthetics (Mihic *et al*., 1997). Furthermore, hyperekplexia-associated mutations of S267 and neighbouring residues have been found to reduce glycine sensitivity and to convert β-alanine and taurine into competitive antagonists (Rajendra *et al*., 1995; Becker *et al*., 2008) (Figure 1; Table 1). The potency of *n*-alcohols on recombinant Gly receptor responses increased with alkyl chain length up to 12 carbon atoms (Mascia *et al*., 1996). Moreover, anaesthetic concentrations of propofol, an aromatic alcohol, as well as volatile hydrocarbons, such as halothane, enflurane, isoflurane, methoxyflurane and sevoflurane, enhance the effects of low glycine concentrations (Machado *et al*., 2011; Lee *et al*., 2013). Molecular models suggest that residues of M2 and M3 surround a water-filled pocket, in which alcohol binding could preferentially stabilize the open state (Cheng *et al*., 2008; Schaefer *et al*., 2012). Recently, the X-ray structure of a GLIC mutant was solved in the presence and absence of ethanol and other modulators representing the structural basis for ethanol potentiation of CL receptors (Sauguet *et al*., 2013). GLIC is a prokaryotic member of the CL receptor family. A mutation in the pore-lining M2 helix F238A (corresponding residues in other CL receptors: F in nACh receptor subunits, Q in Gly receptors, L in $GABA_A$α subunits) of GLIC resulted in a marked potentiation of function by pharmacologically relevant concentrations of ethanol (Howard *et al*., 2011). The structural data in the presence of ethanol or anaesthetics suggest a stabilization of the open form of the ion channel while binding to a transmembrane cavity between receptor subunits.

Endocannabinoids and other allosteric modulators

Gly receptor activity can be allosterically modulated by certain endocannabinoids (ECs) in a G-protein independent manner (Hejazi *et al*., 2006; Yevenes and Zeilhofer, 2011a). Accordingly, chemically neutral ECs (e.g. anandamide) are positive modulators of α1, α2 and α3 Gly receptors, whereas acidic ECs (e.g. *N*-arachidonoyl-glycine, NA-Gly) potentiate only α1 Gly receptors but inhibit α2 and α3. The sites for the EC modulation of Gly receptors are different from the M sites responsible for ethanol modulation and involve a basic residue within the large intracellular loop between M3 and M4 that is also required for Gβγ modulation (Yevenes *et al*., 2006; Yevenes and Zeilhofer, 2011b). It has recently been proposed that the positive allosteric modulation of Gly receptors exerted by the derivatives of Δ9-tetrahydrocannabinol and cannabidiol are highly relevant for cannabinoid-induced analgesia in animal models of pain. Therefore, these ligands could represent a promising strategy for the development of new pain therapeutics (Xiong *et al*., 2011; 2012).

Curcumol, another allosteric modulator of the Gly receptor, is one of the two sesquiterpenes present in the volatile essential oil of *Rhizoma curcumae*. This compound inhibits glycine-mediated currents reversibly and in a concentration-dependent manner. The most prominent effect was observed on α2 homomers and α2β heteromers. Site-directed mutagenesis identified T59 in the ECD of α2 that confers curcumol sensitivity. When T59 was exchanged by an A, as present in the α1 or α3 subunit (A52), the curcumol effect was almost absent. However, the inhibitory effect of curcumol can be transferred to α1 or α3 carrying a threonine instead of an alanine 52 (Wang *et al*., 2012). The exact mechanism of curcumol's action is still unclear and needs further investigation.

Sesterterpenes isolated from Australian sponges of the genus *Psammocinia* represent a new class of Gly receptor modulators. Electrophysiological recordings on stable transfected HEK293 cell lines expressing either the α1 or the α3 Gly receptor subunits revealed that (–)-ircinianin, (–)-ircinianin lactam A sulphate and (–)-oxoircinianin lactam A react preferentially with the α1 subunit, whereas (–)-ircinianin lactam A was specific for the α3 subunit (Balansa *et al*., 2013a; b).

Glycine receptor mutants and human hyperekplexia

Following the identification of *GLRA1* as a candidate gene associated with hyperekplexia and the cloning of Gly receptor subunits from human samples, the consequences of known *GLRA1* mutations have been analysed *in vitro*. Most of the dominant mutations accumulate in the ion channel domain M2 as well as adjacent loop structures (Figure 1).

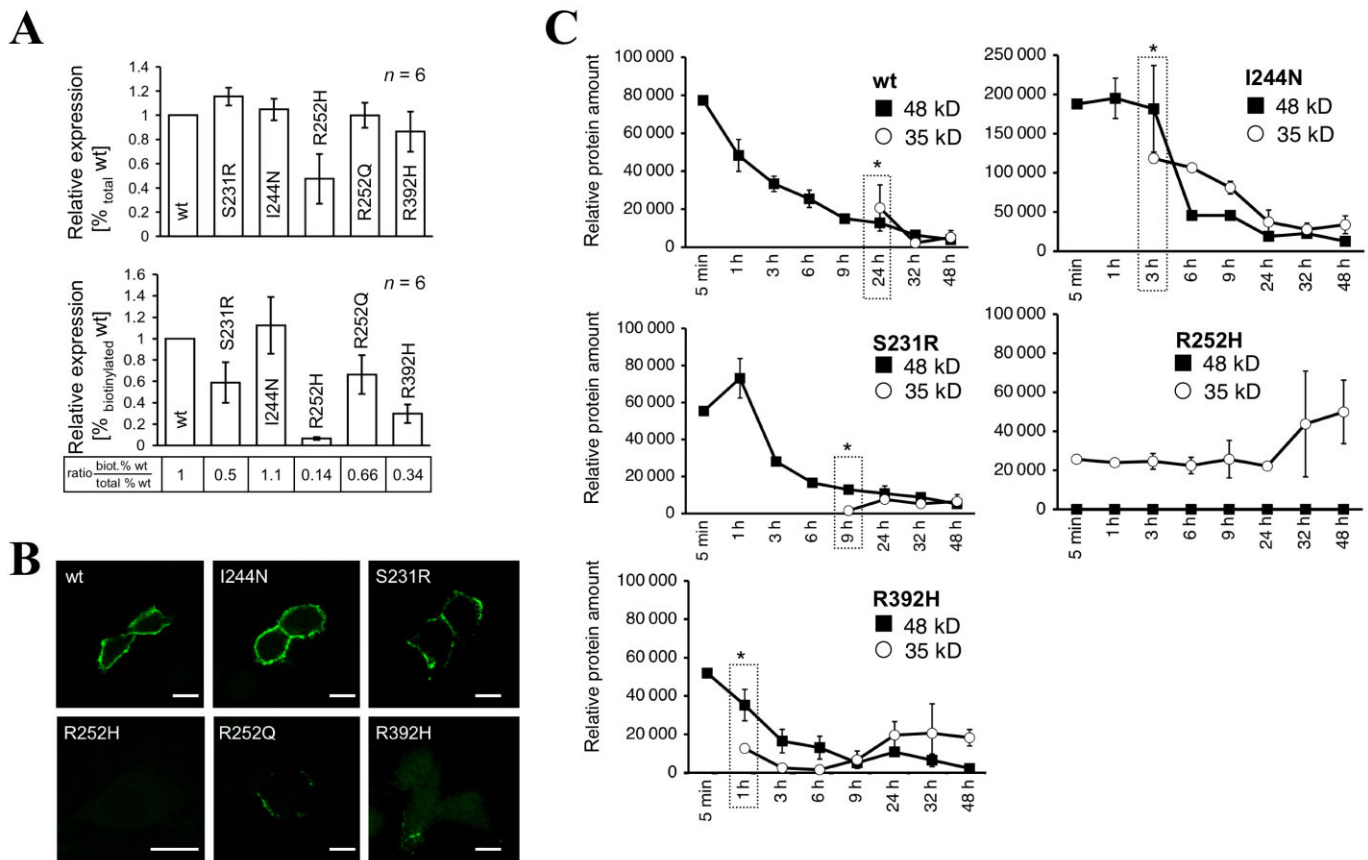

Figure 3

Recessive hyperekplexia mutants have disordered trafficking and protein stability (modified from Villmann *et al.*, 2009b). Comparison of cell surface and intracellular protein of recessive Gly receptors α1 subunit variants from transfected COS7 cells. (A) Quantified reduced surface expression of Gly receptors α1 mutants (14–50% of wt expression) is evident from labelled surface proteins by NHS-SS-biotin. (B) Cell surface expression was less in non-permeabilized transfected HEK293 cells using the monoclonal anti-Gly receptor α1 antibody mAb2b. While M1-mutants (I244N, S231R) are still expressed at the cell surface, only some antigenic clusters were observed for the arginine mutants R252Q and R392H (lower pictures). (C) Protein stability of Gly receptor α1 subunit mutants analysed by pulse-chase radiolabelling. The radiolabelled mutants from transfected HEK293 cells were immunoprecipitated with the monoclonal antibody mAb2b. The intensities of radiolabelled Gly receptor α1 receptor bands from independent experiments ($n = 3$) were quantified using the Image Quant software (Molecular Dynamics). Wild-type α1 protein and mutants displayed obvious differences in membrane accumulation and half-life of radiolabelled protein. Protein band of 48 kD and intermediate bands (35 kD) are shown. The dashed lines marked by an asterisk (*) indicate the first appearance of the lower MW Gly receptor α1 protein band at 35 kD.

Their close location to the ion channel pore suggests that these mutations might change ion channel properties. Human mutations such as P250T, Q266H, S267N, R271Q/L, K276E and Y279C showed reduced maximal glycine-induced currents, decreased open probability and reduced glycine sensitivity (Lynch *et al.*, 1997; Saul *et al.*, 1999; Chung *et al.*, 2010) (Table 1). For some dominant mutants, reduced surface expression was also observed. No changes in glycine binding, but less taurine binding, were observed for V260M, which is localized in the inner ion channel (del Giudice *et al.*, 2001). Furthermore, mutations I244N and P250T have been described, which lead to an enhanced desensitization of the Gly receptor channel. Residues I244 and P250 are localized at the end of M1 or within the loop between M1–M2 at the cytoplasmic side of the membrane (Lynch *et al.*, 1997; Breitinger *et al.*, 2001) (Figure 2). These data support the proposal that the intracellular structural domains are involved in the modulation of ion channel properties such as desensitization.

Recessive hyperekplexia due to mutations in the *GLRA1* or *GLRB* gene are more dispersed over all the Gly receptor subunits (Figure 1). Among the ECD mutants, most variants show disturbances in assembly and trafficking to the cell surface (Chung *et al.*, 2010; 2013; James *et al.*, 2013). As a result, their surface expression is reduced or absent. However, there are exceptions such as M1 mutants; S231R and I244N are transported but exhibit highly reduced maximal glycine-gated currents (Lynch *et al.*, 1997; Humeny *et al.*, 2002; Villmann *et al.*, 2009b) (Figure 3). Intracellular arginine mutations are also associated with recessive hyperekplexia (Vergouwe *et al.*, 1999; Rea *et al.*, 2002). Residues R252 and R392 are located next to M2 or M4 and seem to play an

important role as topological start or stop signals for the membrane integration of the appropriate M domains. Mutations of these arginines almost completely inhibit their expression at the cell surface and they accumulate in the ER, where they undergo proteasomal degradation. Their co-expression, together with the Glyβ receptor subunit, was not able to circumvent this process. Furthermore, recessive hyperekplexia mutants display a higher turnover rate and are unstable compared to wild-type α1 Gly receptor protein (Figure 3). Degradation of mutated α1 proteins was faster by around a factor of 5 for M1 mutants (S231R and I244N; 9 and 3 h compared to 24 h for the wild type) but 20-fold faster for intracellular arginine mutations R252H and R392H (Villmann *et al.*, 2009b) (Table 1).

From studies on human patients, *GLRB* has only rarely been found to be a target gene for hyperekplexia (Filatov and White, 1995; Shan *et al.*, 2001b; Rees *et al.*, 2002). Three novel amino acid substitutions M177R, L285R and W310C have recently been reported in patients with hyperekplexia, demonstrating that *GLRB* is the third most common gene associated with hyperekplexia in humans (Chung *et al.*, 2013; James *et al.*, 2013). Similar to the effects observed with α1 mutations, these substitutions affect the physiology of the ion channel. The α1/βM177R Gly receptors were demonstrated to have increased EC_{50}s and decreased maximal responses. The Gly receptors composed of α1/βL285R display a leak conductance associated with spontaneous Gly receptor activity. These data again show that the Gly receptor β subunit is not only a structural component of the receptor complex via its binding to the scaffolding protein gephyrin, but these β residues are important for the characteristic properties of the Gly receptor channel.

Hyperekplexia-like phenotypes in mice with glycine receptor mutations

The genetic causes underlying hyperekplexia-like phenotypes in different mouse models were determined in the early 1990s after successful cloning of the Gly receptor subunits from mice. *Spasmodic* (*spd*), *oscillator* (spd^{ot}) and *spastic* (*spa*) are spontaneous mouse mutants with mutations either in the *Glra1* or *Glrb* gene (Keramidas *et al.*, 2004). A normal life expectancy was only observed for homozygous *spasmodic* animals. These mice carry a point mutation A52S in the ECD of the α1 Gly receptor protein, leading to a reduced affinity for glycine (Ryan *et al.*, 1994; Saul *et al.*, 1994) (Table 2). Physiological characterizations of these mutant receptors on hypoglossal motoneurons from *spd/spd* animals showed reduced mIPSC amplitudes but significantly faster decay times compared with control mIPSCs. However, the single channel open probability was unchanged (Graham *et al.*, 2006). These slight changes in ion channel properties in *spasmodic* mice are in line with the observed mild hyperekplexia-like phenotype; these mice display exaggerated startle responses after touching but have a normal life expectancy. In contrast, *spastic* and *oscillator* mice show a much more severe phenotype. *Oscillator* affects the *Glra1* gene (Figure 4; Table 2). Homozygous *oscillator* mice do not survive the third post-natal week. At the molecular level, a microdeletion together with a microinsertion generated a 7 bp frameshift deletion, resulting in a functional null mutation. Consistent with the non-sense mutation, two different transcripts are formed dependent on the use of an alternative splice acceptor site 3′ to the

Table 2

Mutations in mice

Name gene mutation	Mode of mutation	Mode of inheritance	Effect	References
Spasmodic Glra1 A52S	Spontaneous	Autosomal recessive	Decrease in glycine affinity	Ryan *et al.* (1994); Saul *et al.* (1994)
Nmf11 Glra1 N46K	ENU-induced	Autosomal recessive	No physiological data	Traka *et al.* (2006)
Glra1 D80A/G	Knock-in	Autosomal recessive	Selective loss of synaptic Zn^{2+} potentiation	Hirzel *et al.* (2006)
Cincinnati Glra1 F159X	Spontaneous	Autosomal recessive	Loss of function	Holland *et al.* (2006)
Glra1 Q266I	Knock-in	Autosomal recessive	Reduced functionality of Gly receptors	Blednov *et al.* (2012); Borghese *et al.* (2012)
Glra1 S267Q	Transgene and knock-in	Autosomal recessive	No enhancing effects with ethanol	Findlay *et al.* (2002; 2005)
Glra1 R271Q	Transgene	Autosomal recessive	Decrease in glycine affinity	O'Shea *et al.* (2004)
Glra1 M287L	Knock-in	Autosomal recessive	Reduced functionality of Gly receptors	Blednov *et al.* (2012); Borghese *et al.* (2012)
Oscillator Glra1 ins/del P327X	Spontaneous	Autosomal recessive	Loss of function	Buckwalter *et al.* (1994); Kling *et al.* (1997); Villmann *et al.* (2009a)
Spastic Glrb LINE1 insertion	Spontaneous	Autosomal recessive	Less full-length Gly receptor β	Kingsmore *et al.* (1994); Mulhardt *et al.* (1994); Becker *et al.* (2012)

Figure 4

Phenotype of a homozygous *oscillator (spd^{ot}/spd^{ot})* mouse compared with a wild-type control littermate (+/+). *Oscillator* mice are usually smaller than wild-type controls. Shown are animals at post-natal day (P)19. The *oscillator* mutatant usually dies by P21. Symptoms start around P15 and develop progressively during P17–P21. Rigidity of the whole body and massive tremor in forelimbs, hindlimbs and even the tail are seen compared with a healthy control mouse.

mutation. If the splice acceptor site is used, a 24 bp shorter transcript is generated. However, at the protein level, a non-sense protein is formed, which is longer than the normal wild-type protein. If the splice acceptor site is not used, a short truncated protein is generated with an early stop codon within the 8 amino acids encoded by the alternative splicing cassette in the M3–M4 loop of α1 Gly receptor (Malosio *et al*., 1991). In *in vitro* recordings from transfected cells, it was found that both splice variants had similar physiological properties. α1 Gly receptor proteins have never been detected in homozygous *oscillator* animals, most probably due to non-sense-mediated decay mechanisms (Kling *et al*., 1997; Villmann *et al*., 2009a).

The α3 splice variants are also generated by alternative splicing in the M3–M4 loop, resulting in fast desensitizing currents for the short variant Gly receptor α3K and non-desensitizing currents for the long splice variant Gly receptor α3L. Both variants differ in the absence or presence of an intracellular, 15 amino acids-long, alternative splice cassette in the M3–M4 loop. Mutations within this splice cassette of the Gly receptor α3, its deletion or duplication further confirmed these findings (Nikolic *et al*., 1998; Breitinger *et al*., 2009). In contrast to α3K, the α3L variant has been shown to be more mobile and is able to cluster in hippocampal neurons. In addition, *in vivo* the α3L variant is usually predominant in the brain. A redistribution of α3L towards α3K due to post-transcriptional processing seems to be associated with some forms of mesial temporal lobe epilepsy (Eichler *et al*., 2009; Notelaers *et al*., 2012).

The Gly receptor α1 is the most important α subunit at post-natal stages. During embryogenesis, the α2 subunit is prominent, most probably generating homomeric Gly receptor pentamers. After birth between P5 and P14, the α2 subunit is down-regulated and the appearance of α1 increases continuously. Similar time frames have been described for the transformation of mixed GABA/glycine synapses into glycinergic presynaptic terminals in many spinal and brainstem synapses in wild-type mice (Jonas *et al*., 1998; Kotak *et al*., 1998; Muller *et al*., 2006). Postsynaptic Gly receptor complexes present after this stage of development are mainly composed of α1/β subunits. *In vivo* electrophysiological recordings on superficial dorsal horn neurons, which concentrated on compensatory mechanisms by other Gly receptor subunits, showed that there are remaining functional α3/β complexes (Graham *et al*., 2006; 2011). Nevertheless, in the *oscillator* mice these α3/β complexes are not sufficient to sustain life.

The *spastic* mouse mutant is, as yet, the only known Gly receptor β mutation in rodents (Becker *et al*., 1986; Kingsmore *et al*., 1994; Mulhardt *et al*., 1994). At the molecular level, two independent groups found that the underlying cause was an insertion of a transposable LINE1 element into intron 6 of the *Glrb* gene (Kingsmore *et al*., 1994; Mulhardt *et al*., 1994). The consequences of this LINE1 insertion are skipping of exons 5 and 6 or both. Therefore, much less full-length Gly receptor β protein is available for the formation of postsynaptic α1/βGly receptor complexes in adult homozygous *Glrb$^{spa/spa}$* mice (Keramidas *et al*., 2004; Becker *et al*., 2012). These highly reduced functional α1/βGly receptor complexes are the underlying reason for the hyperekplexia-like phenotype in *spastic* mice.

Recently, it has been shown that the *spastic* allele of the murine *Glrb* is a two hit mutation. The original *spastic* mutation was demonstrated in a hybrid mouse background B6C3Fe. A minigene approach using DNA from both parental backgrounds C57BL/6J and C3H/HeJ of *Glrb* wild-type and *spastic* animals identified a single nucleotide polymorphism in exon 6 between both mouse backgrounds. Thus, the disturbed splicing pattern of *Glrb* is not only a result of the LINE1 insertion but is also due to a SNP that disrupts an exonic splicing enhancer (ESE) sequence within exon 6 leading to missplicing. The ESE is important for the binding of an essential splicing regulatory protein SFRS1 (Becker *et al*., 2012).

These data indicate that in addition to a mutation in a disease-associated gene the mouse background might also have an effect on the observed neuromotor phenotype. Similar findings have been obtained in other mouse models of movement disorders, for example, ataxia, dystonia, tremor and progressive paralysis, are associated with mutations in the *Scn8a* gene encoding a widely distributed voltage-gated sodium channel (Buchner *et al*., 2003).

Two other Gly receptor mutations in mice have been described. The *cincinnati* mouse possesses a premature stop codon (F159X) representing another example of a functional null mutation (Holland *et al*., 2006). An ENU-induced mutation N45K localized in the ECD of the Gly receptor α1 protein has been identified and although it has not been characterized at the physiological level (Traka *et al*., 2006), it was hypothesized that due to the close localization of N45K to A52 (*spasmodic*), this mutation might also affect glycine affinity.

Studies on the spontaneous mouse mutants have provided lots of details on the effects of alterations in the mechanisms of inhibitory neuronal circuits in rodents. In turn, that allowed us to draw conclusions for the human situation in various types of hyperekplexia. The top-down approaches using knock-out and knock-in mouse models carrying human mutations have helped to relate and understand the consequences of the human variants on channel physiology *in vivo*.

R271 mutations: the first knock-in mouse models

The R271Q/L mutations were discovered in human hyperekplexia patients by Shiang *et al.*; R271 is located in the M2–M3 loop close to the ion channel pore (Shiang *et al.*, 1993). *In vitro* experiments by Langosch and Rajendra demonstrated that the mutations at position 271 do not alter ligand binding to glycine, but do significantly affect binding of β-alanine and taurine (Langosch *et al.*, 1994; Rajendra *et al.*, 1994; 1995). Laube *et al.* (1995b) demonstrated that an iso-functional substitution of R271K led to reduced affinities and maximal current responses (I_{max}) following application of the low-efficacy agonists β-alanine and taurine. All data on R271 substitutions suggest that it is associated with a highly diminished agonist sensitivity without differences in agonist affinity. From the *in vitro* data, it was concluded that ligand binding in R271 is uncoupled from the channel activation mechanisms.

An introduction of R271Q by a transgenic approach into mice generated tg271Q animals with a hyperekplexia-like phenotype even in heterozygous animals. Like *spastic* mice, homozygous tg271Q animals did not reach adulthood. It should be noted that the symptoms were gene dose-dependent and strains with the lowest expression rate did not develop any phenotype (Becker *et al.*, 2002). Notably, an inter-cross of tg271R into spd^{ot} mice could prevent the lethal phenotype of the *oscillator* loss-of-function mutation. This suggested that the tg271R enabled the formation of functional interspecies Gly receptor complexes in spd^{ot} mice (Becker *et al.*, 2002).

O'Shea *et al.* (2004) successfully restored the function of R271Q/L with the help of propofol in *in vitro* experiments and also in a mouse model *in vivo*. The maximal glycine-induced currents of oocytes transfected with R271Q and R271L were markedly potentiated in the presence of propofol, whereas the wild-type maximal currents were not affected. In addition, tg271Q mice were tested before and after propofol treatment for startle reaction and righting reflexes. The startle responses of propofol-treated tg271Q mice were temporarily highly reduced and resembled those of wild-type mice. However, within 30 min after injection of propofol, the hyperekplexia-like phenotype reappeared. The same was observed for the righting time of propofol-treated tg271Q mice (O'Shea *et al.*, 2004). Therefore, propofol might be a treatment for human hyperekplexia as it also enhances $GABA_A$ receptor activity in addition to glycine-gated current enhancement, but propofol also possesses anaesthetic and sedative properties.

Other knock-in mice to study the modulation by *n*-alcohols

Humans produced and consumed alcohol even a millennia ago. Nowadays, alcohol is the most widely used drug worldwide (Vallee, 1998). However, the mechanism by which alcohol acts on ion channels is still unclear.

A huge step towards discovering the effect of ethanol on the Gly receptor was carried out by Mihic *et al.* (1997). With the creation of various chimeric receptor constructs, a 45 amino-acid region was found to be necessary and sufficient for the enhancement of receptor function in the presence of ethanol. S267 and A288 turned out to be critical for the modulatory action of ethanol. The mutated receptors harbouring S267I did not enhance receptor function (Mihic *et al.*, 1997). It was found that A288 has a critical role in the pharmacology of Gly receptors and this residue contributes to the binding cavity for *n*-alcohols and volatile anaesthetics (Lobo *et al.*, 2008). Ethanol was also demonstrated to inhibit strychnine-induced seizures in mice (McSwigan *et al.*, 1984). But it is not known how or whether the behavioural effects of ethanol are linked to the potentiation of Gly receptor function.

To determine this, a mouse line carrying a S267Q transgene under the control of the rat synapsin 1 promoter was created. S267Q showed normal sensitivity to glycine and no enhancing effects in the presence of ethanol. Moreover, high concentrations of ethanol inhibited the function of this mutant receptor *in vitro*. The ability of ethanol to eliminate strychnine-induced seizures was reduced in transgenic mice, which is in line with earlier reports (Findlay *et al.*, 2002). In three behavioural tests, a decreased sensitivity to ethanol was observed: (i) ethanol inhibition of strychnine-induced seizures; (ii) motor incoordination (rotarod); and (iii) loss of righting reflex (LORR). One disadvantage of mice carrying the mutation S267Q on a transgene was that they still had endogenously expressed, alcohol-sensitive wild-type Gly receptors. Therefore, the generation of S267Q knock-in mice was required.

Homozygous S267Q knock-in mice exhibit a hyperekplexia-like phenotype and like spd^{ot} mice die by 3 weeks of age. Compared to the wild-type mice, no differences in Gly receptor expression were detectable in S267Q knock-in mice. However, the observed decrease in maximal responses to glycine and the decreased ion influx suggested a disruption of ion channel function (Findlay *et al.*, 2003). From the results of NMR studies, it was postulated that S267Y stabilizes the closed state of the inhibitory Gly receptor (Tang *et al.*, 2002), which could explain the reduced open channel time and the observed decrease in potency of glycine in S267Q mice.

In summary, S267 is involved in the effects of alcohols and volatile anaesthetics and seems to be part of a binding site for these modulators. These findings strongly suggest that S267 is also involved in channel gating. Further support came from a human mutation S267N, which was found in a patient suffering from hyperekplexia. In electrophysiological studies in transfected HEK293 cells, the S267N mutation resulted in an increased EC_{50} for glycine, but no changes in I_{max} values (Becker *et al.*, 2008). Although S267 lies in an

alcohol-sensitive domain, no alterations in alcohol sensitivity were apparent in transfected HEK293 cells, the effect of alcohol was visible only when this mutation was expressed in *Xenopus* oocytes (Valenzuela *et al.*, 1998; Findlay *et al.*, 2003). These findings provide further evidence that the Gly receptor M2 domain is sensitive to alcohol.

In order to generate a knock-in mouse to elucidate the effects of alcohol on Gly receptors, it is necessary to use a model where Gly receptor function is generally unaffected. Two other candidates from previous *in vitro* experiments were Q266I and M287L (Mihic *et al.*, 1997). Here, channel properties of mutated Gly receptors expressed in *X. laevis* oocytes and HEK293 cells were similar to those observed in recordings from isolated neurons and membrane preparations from corresponding knock-in mice. Heterozygous as well as homozygous animals revealed decreased glycine-induced maximal currents and a lack of sensitivity to ethanol suggesting a reduced functionality of Gly receptors in both knock-in models (Borghese *et al.*, 2012). In turn, strychnine sensitivity was increased. The single-point mutations Q266I and M287L resulted in the death of homozygous mice during the third post-natal week. Moreover, in behavioural studies, heterozygous mice displayed an increased startle response. In these mice, five behavioural experiments have been performed in which the effects of alcohol were reduced compared with the wild-type mice: (i) acute alcohol withdrawal; (ii) ethanol induced LORR; (iii) alcohol consumption; (iv) recovery from rotarod ataxia; and (v) alcohol-induced taste aversion. Taken together, these data show that the Q266I mutation produced a much higher suppression of alcohol action than the M287L mutation (Blednov *et al.*, 2012).

In conclusion, structural data and transgenic mouse models demonstrate that ethanol acts via an inter-subunit cavity close to the M2–M3 regions, thereby stabilizing the open channel conformation.

D80A knock-in mice: the first *in vivo* evidence for Zn^{2+} modulation of ligand-gated glycine receptor channels

Zn^{2+} modulation is important in many neurological signal transduction pathways. Data from *in vitro* studies on Zn^{2+} modulation have identified residues within the Gly receptor α1 subunit responsible for potentiating as well as inhibitory effects of Zn^{2+}. A knock-in mouse mutant D80A with a disruption of the high-affinity Zn^{2+} binding site was generated to study Zn^{2+} neuromodulation *in vivo*.

Interestingly, D80A knock-in mice develop a hyperekplexia-like phenotype, which persists into adulthood and have a normal lifespan and fertility. In electrophysiological studies of spinal cultures and brainstem slices, a reduction in the Zn^{2+}-mediated potentiation of Gly receptors with no changes in its basic functions was detected. These mice showed an increase in b-wave amplitudes of the ERG (electroretinogram), which is a parameter for visual signal processing and an enhanced ASR (acoustic startle response). This also indicates deficits in the development of sensory transmission and auditory circuits.

From the various results it was concluded that the phenotype of D80A mice is due to a selective loss of synaptic Zn^{2+} potentiation. In heterozygous expression experiments, D80A does not alter the glycine dose–response relationship, open probability and single channel conductance (Laube *et al.*, 1995a; 2000). This was also apparent in recombinant $\alpha1^{D80A}\beta$ Gly receptors and homozygous *Glra1* (D80A) neurons. There was no change in synaptic localization in spinal cord neurons from homozygous animals. Glycinergic IPSCs, decay kinetics and frequencies were similar in cultured spinal cord neurons and P8 brainstem hypoglossal motoneurons from wild-type and D80A animals. Changes occurred first in hypoglossal motoneurons at P14 with reduced amplitudes and shorter decay time. This onset fits with the developmental change of the embryonic Gly receptor isoform α2 towards the adult isoform composed of α1 and β subunits. Zn^{2+} seems to play an important role in glycinergic neurotransmission in the context of normal behaviour (Hirzel *et al.*, 2006). Further support for a critical role of zinc in the modulation of Gly receptor function came from an *in vivo* study on heterozygous D80A mice, in which zinc binding and ethanol modulation were investigated. Knock-in female mice have been found to consume less ethanol, demonstrate a decreased preference for alcohol and have an increased startle response, in agreement with previous findings for sex differences in alcohol consumption (Blednov *et al.*, 2012; McCracken *et al.*, 2013). These data emphasize the important role of residue D80 for both zinc binding and modulation of ethanol actions *in vitro* and *in vivo*.

Domain co-expression: a way to rescue protein function

Gly receptors are composed of independent folding domains. This has been shown in a series of rescue of function experiments with the non-functional truncated α1 variant present in the mouse model *oscillator*. The *oscillator* mutation is a frameshift mutation. Depending on which alternative splice acceptor site is used, two transcript variants are generated, leading to either a truncated or an elongated protein. *In vitro* experiments showed a non-functionality of both proteins lacking most of the M3-M4 loop, M4 and the C-terminus. If the portion of the α1 protein lacking was co-expressed as a second independent domain, Gly recptor function was restored *in vitro* (Villmann *et al.*, 2009a) (Figure 5A).

Reorganization of domains was a significant factor in the evolution of prokaryotic channels and has been described as being important for the distribution of prokaryotic members of the ACh receptor family (Li *et al.*, 2006). Such rearrangements of known protein domains from prokaryotes or other less developed species were most probably an evolutionary advantage for the generation of eukaryotic proteins with novel functions. The class of excitatory glutamate (Glu) receptors evolved from protein domains of other origin. Here, domain exchange between an ancestral Glu receptor channel pore Glu receptor 0 into another donor receptor GluK2 generated functional ion channels (Hoffmann *et al.*, 2006). Studies on a functional chimera with the ECDs from the prokaryotic CL receptor GLIC (*Gloeobacter violaceus*) and the

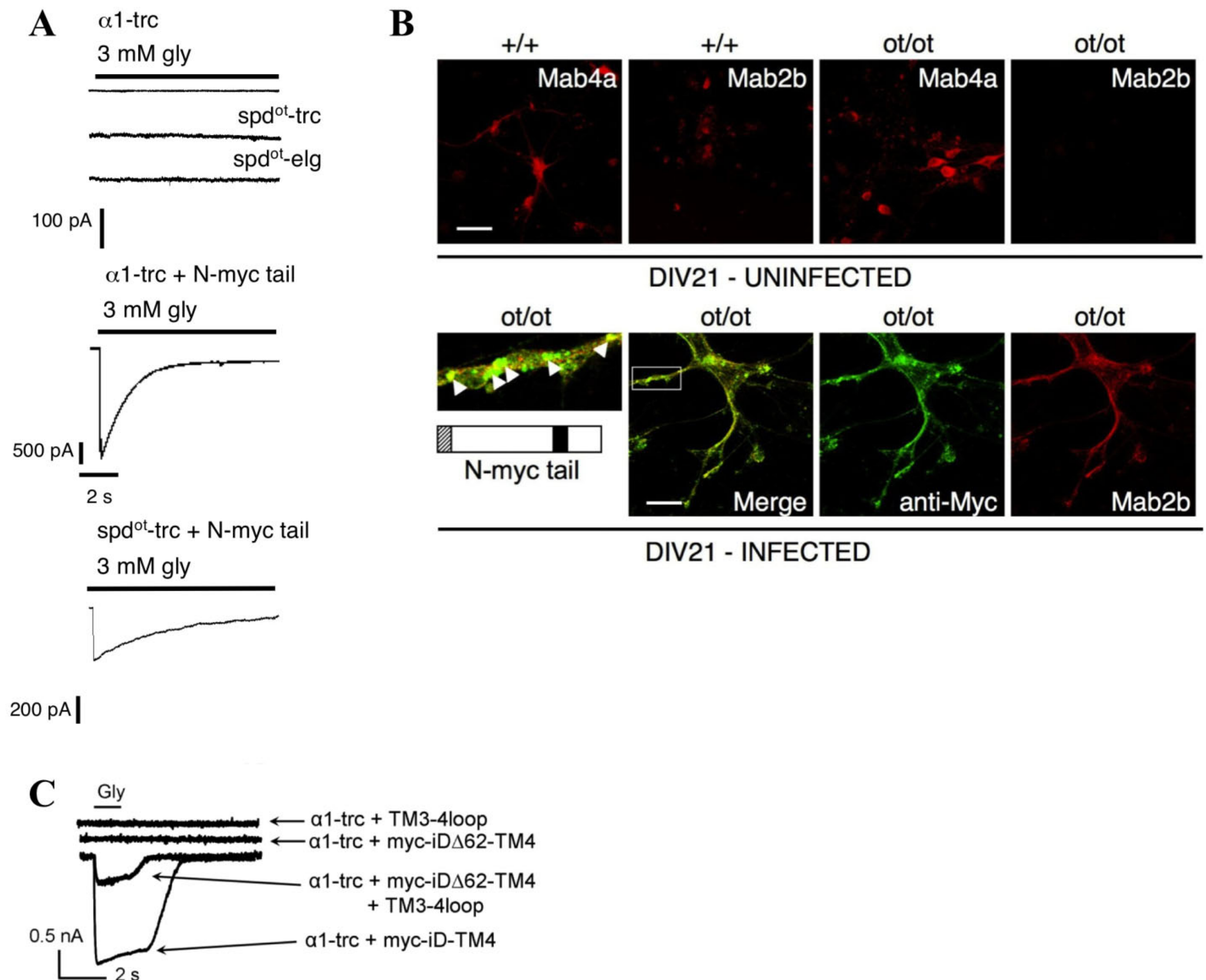

Figure 5

Domain complementation for rescue of ion channel function (modified from Unterer *et al.*, 2012 and Villmann *et al.*, 2009a). (A) Rescue of function experiments of the truncated *oscillator* Gly receptor α1 variant *spd^ot^* compared with truncated wild-type α1. When single domains of the Gly receptor α1 were expressed, no functional channels were observed following 3 mM glycine application (upper panel). If the complementation domain (N-myc tail) was co-expressed, functional rescue was observed for the truncated wild type and the *oscillator* Gly receptor α1 variants (middle and lower panel). (B) Comparison of infected (DIV21; bottom) and uninfected spinal cord neurons isolated from either homozygous wild type (+/+) or *oscillator* (*ot/ot*) mice (DIV21 uninfected) with the N-myc tail construct encoded on a pAAV vector. Infected neurons show a signal for the endogenous Gly receptor α1 protein (bottom 3 panels, right picture specific Gly receptor α1 signal in red) that co-localized (merged pictures) with the N-myc tail complementation domain. (C) Functionality of the Gly receptor α1 expressed from three domains. The functionality of the truncated N-terminal domain of the Gly receptor α1 (α1-trc) was not restored by either the M3–M4 loop or the shortened complementation domain (myc-iDΔ62-TM4). Co-expression of all three domains restored Gly receptor function (middle trace).

M-domains of Gly receptor α1 showed that both domains contributed to the physiological properties of the generated chimeric channels. The chimera could be activated by protons, a prerequisite of GLIC but also harbours properties of the human Gly receptor α1 such as the allosteric modulation by propofol (Duret *et al.*, 2011).

The special feature in the rescue of function experiments is that the domains are separately expressed and not connected like chimeric proteins. Similar to the complementation of *oscillator* domains, for 7-TM receptors it has been shown that domains of the GPCRs fold independently, for example, the vasopression receptor V_2 and the muscarinic ACh receptor (Schoneberg *et al.*, 1995; 1996).

A functional rescue of Gly receptor function was not only shown *in vitro* in transfected cell lines. Following an infection of the rescue domain via a viral construct into primary spinal cord neurons isolated from homozygous *oscillator* animals, the Gly receptor α1 protein, which has never been detected in

uninfected neurons, was up-regulated (Villmann *et al.*, 2009a) (Figure 5B). Usually *oscillator* mice die during the third week after birth and a lack of expression of the Gly receptor α1 protein is the underlying reason for this (Buckwalter *et al.*, 1994; Kling *et al.*, 1997). With the help of an independent rescue domain, it appears to be possible to overcome the non-functionality of truncated receptor variants. Nevertheless, *in vivo* data are still missing in this context. Using the *in vitro* rescue of function experiments, several domains have been identified that are important for trafficking of Gly receptor domains as well as rescue effectiveness (Unterer *et al.*, 2012). At the 3′ sequence to the *oscillator* mutation, the wild-type harbours a basic motif RRKRR in the M3–M4 loop. These residues have been demonstrated to ensure the cell surface expression of the α1 protein (Sadtler *et al.*, 2003). When RRKRR was introduced into the *oscillator* GDIT sequence, the rescue efficiency was increased from 10% towards wild-type responses (100%). Studies on intracellular basic residues, which are affected in hyperekplexia patients showed the importance of these positively charged residues for protein biogenesis and function (Rea *et al.*, 2002; Villmann *et al.*, 2009b).

To gain further insights into the mechanisms of functional rescue by independent domains, *in vitro* studies on truncated rescue domains have been performed. Truncation from the N-terminus corresponding to residues in the beginning of the M3–M4 loop generated functional channels when co-expressed with a truncated α1 wild-type up to a deletion of 49 amino acid residues of the M3–M4 loop (the last with a rescue efficiency of 58%). Further deletions generated almost no functional chloride channels (Unterer *et al.*, 2012). The M3–M4 loop is the most highly variable among all the CL receptor members. The Gly receptor M3–4 loop can be divided into variable (V) and conserved (C) regions (Melzer *et al.*, 2010; Unterer *et al.*, 2012). The conserved region C3 seems to act as a barrier between functional and non-functional complementation in a two domain Gly receptor configuration. C3 is localized before M4 and harbours a high number of charged residues. Large truncations of the M3–M4 loop deleted all negatively charged residues and generated positively charged N-terminal ends. Ionic interactions between positively and negatively charged residues in the M3–M4 loop might represent the mechanism that mediates the rescue of Gly receptor function from independent domains. It is striking that all charged residues are localized in conserved regions in human, rat and mouse Gly receptor α subunits (α1-α4). Moreover, it is known that the last aspartate in front of M4 is conserved in 45 known CL receptor subunits. This aspartate is required for $GABA_A$ receptor assembly as its mutation resulted in ER accumulation and thus reflects impaired forward trafficking (Rampon *et al.*, 1996). Hence, an interaction between charged residues in the M3–M4 loop could also be required for the transduction of conformational changes of the whole Gly receptor protein translating ligand binding into opening and closing of the ion channel. The Gly receptor domain architecture does also allow for the generation of a functional inhibitory neurotransmitter receptor from three independent domains. The co-expression of the lacking M3–M4 loop in addition to the truncated N- and C-terminal Gly receptor α1 protein fragments again resulted in the rescue of channel function (Figure 5C) (Unterer *et al.*, 2012).

The domain study demonstrated that the concomitant transport of Gly receptor subdomains covering the entire Gly receptor subunit sequence passes the ER control system; otherwise, most of the truncated Gly receptor domains accumulate in the ER compartment and get degraded similar to the assembly of the nACh receptor from different subunits (Villmann *et al.*, 2009a; Valkova *et al.*, 2011). These extensive studies that use domain complementation as a way of producing functional protein rescue suggest novel strategies for gene-therapeutic approaches.

Conclusions and perspectives

The inherited neuromotor disorder hyperekplexia is associated with mutations in glycine receptor genes, genes encoding for Gly receptor interacting proteins or even the presynaptic glycine transporter 2. Gly receptors are most important for inhibitory neurotransmission in the adult spinal cord and brainstem in humans and in rodents. Dysfunction of these receptors in humans results in startle attacks and excessive muscle tremor induced by tactile or acoustic stimuli. Interestingly, similar phenotypes have been detected in mice carrying mutations in the *Glra1* or *Glrb* genes. Characterization of the spontaneous mouse models *spasmodic*, *spastic* and *oscillator* has provided lots of detailed information on the single-channel properties from homozygous but also from heterozygous animals. With the use of protein biochemical techniques, it was possible to show that in mutant mice adaptation processes at the presynapse occur. Here, mixed GABA/glycine vesicles change to GABAergic vesicles, in contrast to the normally occurring developmental shift to presynaptic glycinergic vesicles in brainstem neurons of wild-type mice.

The continuous increase in solved X-ray structures from various members of the CL receptor superfamily has helped to explain the data obtained *in vitro* and *in vivo*. Modern knock-in techniques have enabled the study of human mutations in mice. All single-point mutations known from human mutations have led to hyperekplexia-like phenotypes when introduced into mice. Homozygous animals died 3 weeks after birth similar to the spontaneous mouse mutant *oscillator*. At this developmental stage, the change from the embryonic Gly receptor to the adult isoform should be completed. Knock-in mice indicated the phenotype but have also been used to demonstrate the distinct pharmacological and therapeutic profiles. Clonazepam is the drug of choice for hyperekplexia patients. Unfortunately, in mice clonazepam has only a small therapeutic window where the positive modulation induced by an enhanced GABAergic response helps to improve the hyperekplexia-like symptoms. All efforts to show an up-regulation in the number of GABA receptors as a response to the treatment have failed so far. The mechanism behind clonazepam's action is still not completely understood; an increase in the GABAergic response by a factor of 1.5 at the molecular level seems unlikely to be the only reason for the improvement in symptoms. Other *in vivo* data demonstrated that although α3β receptors are present and functional in α1 mutant mice, these receptors are not sufficient to prevent homozygous mutant mice from dying after 3 weeks of age nor do they rescue the disturbed ion channel

function in heterozygous animals. Furthermore, in human patients, symptoms become less severe when the patients become 3 years old. How the pathomechanism underlying hyperekplexia are able to change with age has to be elucidated. An appropriate mouse model to study this ageing phenomenon would help to further understand the pathology of the neuromotor disorder hyperekplexia.

Functional rescue experiments using domain co-expressions are helpful tools for testing the mechanisms behind truncated receptor variants. One of the first conclusions obtained from these experiments was that Gly receptors could have evolved from independent domains from other origins. Secondly, postsynaptic receptors get assembled in the ER and are concomitantly transported to the cell surface. Thirdly, glycine receptors are not the only truncated receptor variants known to be associated with neurological disease. Null mutations of the $GABA_A\gamma2$ subunit, another member of the CL receptor superfamily, have been reported in patients suffering from a special form of epilepsy, GEFS+. A functional rescue by domain co-expression might represent a gene-therapeutic strategy to overcome a neurological phenotype. Hence, it has been shown that similar to the Gly receptor, ion channel function can be restored by independent co-expression of receptor domains. However, there is still a lack of evidence for the successful rescue of Gly receptor function *in vivo*.

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Conflict of interest

The authors declared that they have no conflict of interest.

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