Hyperekplexia Phenotype of Glycine Receptor α1 Subunit Mutant Mice Identifies Zn⁴⁺ as an Essential Endogenous Modulator of Glycinergic Neurotransmission

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Summary

Zn²⁺ is thought to modulate neurotransmission by affecting currents mediated by ligand-gated ion channels and transmitter reuptake by Na⁺-dependent transporter systems. Here, we examined the in vivo relevance of Zn²⁺ neuromodulation by producing knockin mice carrying the mutation D80A in the glycine receptor (GlyR) α₁ subunit gene (Gira₁). This substitution selectively eliminates the potentiating effect of Zn²⁺ on GlyR currents. Mice homozygous for Gira₁(D80A) develop a severe neuromotor phenotype postnatally that resembles forms of human hyperekplexia (startle disease) caused by mutations in GlyR genes. In spinal neurons and brainstem slices from Gira₁(D80A) mice, GlyR expression, synaptic localization, and basal glycinergic transmission were normal; however, potentiation of spontaneous glycinergic currents by Zn²⁺ was significantly impaired. Thus, the hyperekplexia phenotype of Gira₁(D80A) mice is due to the loss of Zn²⁺ potentiation of α₁ subunit containing GlyRs, indicating that synaptic Zn²⁺ is essential for proper in vivo functioning of glycinergic neurotransmission.

Introduction

The mammalian central nervous system (CNS) contains high concentrations of the divalent metal ion Zn²⁺. Although acute Zn²⁺ deficiency impairs brain development and function in experimental animals and humans (overview in Choi and Koh [1998]), the roles of Zn²⁺ in the CNS remain enigmatic. Ninety percent of the total Zn²⁺ in brain tissue is tightly bound to proteins, and only 10% exists in a pool of chelatable “free” Zn²⁺. This chelatable Zn²⁺ is not evenly distributed in the brain but highly enriched in distinct glutamatergic nerve termve terminals (Choi and Koh, 1998). Furthermore, enrichment of Zn²⁺ at selected inhibitory synapses in cerebellum and spinal cord has also been reported (Velazquez et al., 1999; Jo et al., 2000; Danscher et al., 2001; Wang et al., 2001, 2002; Danscher and Stoltenberg, 2005). Estimates of free Zn²⁺ concentrations in the CNS vary between 100 and 200 nM in the cerebrospinal fluid and >200 μM within specific synaptic regions (Choi and Koh, 1998). Elevated concentrations of Zn²⁺ (>100 μM) may contribute to neuronal cell death during brain ischemia, seizures, trauma, and neurodegeneration (Choi and Koh, 1998; Doraiswamy and Finefrock, 2004), whereas lower levels of Zn²⁺ (<10 μM) protect neurons against glutamate-induced cell death (Chen and Liao, 2003; Chandra et al., 2005). Hence, proper regulation of Zn²⁺ homeostasis is thought to be crucial for brain function and development.

At synapses, Zn²⁺ is highly enriched in synaptic vesicles, from which it can be coreleased with glutamate in an activity-dependent manner (Assaf and Chung, 1984). Microfluorescence imaging of Zn²⁺ secretion during presynaptic stimulation has provided evidence for a quantal mode of Zn²⁺ release from hippocampal Mossy fiber terminals (Li et al., 2001; Qian and Noebels, 2005); however, the interpretation of the signals obtained in these studies has been a matter of debate (see Kay [2006]). The loading of synaptic vesicles with releasable Zn²⁺ is thought to be mediated by ZnT3, a member of the SLC30 superfamily of Zn²⁺ transporters (Cole et al., 1999). In addition, synaptic Zn²⁺ concentrations are controlled by other Zn²⁺ transporters of the ZnT (SLC30) and Zip (SLC39) families as well as by Zn²⁺-sequestering proteins, like metallothioneins (Baranano et al., 2001; Kambe et al., 2004; Smart et al., 2004). Since Zn²⁺ modulates both current responses mediated by excitatory and inhibitory neurotransmitter receptors and the efficacy of transporter driven neurotransmitter reuptake (Smart et al., 2004), synthetically released Zn²⁺ has been proposed to function as an important regulator of synaptic transmission and plasticity (Lu et al., 2000; Vogt et al., 2000) and/or “atypical neurotransmitter” (Baranano et al., 2001). This concept has, however, been challenged because (1) mice deficient in ZnT3 were found to be behaviorally normal despite a loss of synaptic Zn²⁺ staining (Cole et al., 1999) and (2) the available Zn²⁺ imaging techniques have been argued to be particularly sensitive to artifacts (Kay, 2006).

In this study, we have chosen a genetic approach to unravel the importance of Zn²⁺ for synaptic...
transmission. Previously, we and others have shown that Zn$^{2+}$ affects the function of the strychnine-sensitive glycinergic receptor (GlyR; Bloomenthal et al., 1994; Laube et al., 1995), which mediates postsynaptic inhibition in motor and sensory pathways (overview in Betz and Laube [2003]). In spinal neurons and cells expressing recombinant GlyRs, low micromolar (<10 μM) concentrations of Zn$^{2+}$ enhance glycinergic currents, whereas higher concentrations (>10 μM) have an inhibitory effect. The effects of low concentrations of Zn$^{2+}$ have been attributed to an increase in agonist affinity resulting from decreased agonist dissociation; higher Zn$^{2+}$ concentrations produce a voltage-independent block by reducing the efficacy of channel opening (Laube et al., 2000; Lynch, 2004). Also, GlyR-mediated miniature inhibitory postsynaptic currents (mIPSCs) have been shown to be modulated by Zn$^{2+}$ (Suwa et al., 2001; Laube, 2002), indicating that Zn$^{2+}$ regulation is effective at synaptic sites. Mutational studies have localized the Zn$^{2+}$ binding sites mediating potentiation and inhibition of GlyRs in distinct regions of the N-terminal extracellular domain of GlyR $\alpha$ subunits (Laube et al., 2002; Lynch, 2004). Specifically, residues D80, E192, D194, and H107, H109, T112 of the adult-type GlyR $\alpha$1 subunit have been found to constitute important determinants of Zn$^{2+}$ potentiation and inhibition, respectively (Lynch et al., 1998; Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005). Neutralization of the $\gamma$-carboxylate of aspartate 80 by glycine or alanine substitution selectively eliminates Zn$^{2+}$ potentiation without affecting channel conductance and receptor kinetics (Lynch et al., 1998; Laube et al., 2000). Here, we introduced the D80A substitution into the murine GlyR $\alpha$1 gene locus (Girá1) via homologous recombination. Knockin mice homozygous for Girá1(D80A) show a phenotype, which mimicks that of human hyperekplexia (hereditary startle disease) patients and spastic or spastic GlyR mutant mice and is indicative of decreased glycinergic inhibition (Schofield, 2002). Our results indicate that potentiation of the GlyR by synaptic Zn$^{2+}$ is essential for proper glycinergic inhibition in vivo.

**Results**

**Generation of Girá1(D80A) Mice and GlyR Protein Expression**

The targeting strategy used for introducing the D80A substitution into exon 4 of Girá1 is depicted in Figure 1A. Using a replacement type targeting vector, the mutation was introduced into ES cells by homologous recombination together with a diagnostic XbaI restriction site and a floxed neomycin resistance cassette (neo), positioned in the 3’ adjacent intron. Homologous recombination events were identified by PCR, and confirmed by Southern blot analysis (Figures 1A and 1B). In a second step, the neo selection marker was deleted by transient transfection with a Cre recombinase expression vector (Figure 1C). The resulting mutant ES cells were injected into blastocysts and gave rise to two germine-transmitting chimeras. These animals sired heterozygous offspring without obvious abnormalities in morphology and behavior. Intercrosses of heterozygous animals generated Girá1(D80A) homozygous, heterozygous, and WT animals at the expected Mendelian ratios.

Correct introduction of the mutation Gira1(D80A) was confirmed by Southern blotting (Figure 1D) of XbaI-digested DNA and corroborated by sequencing the exon 4 region of the mutant locus (data not shown).

To examine whether GlyR $\alpha$1 subunit expression is unaltered in homozygous Gira1(D80A) mice, we analyzed GlyR expression in spinal cord tissue from postnatal day 21 (P21) mice. Immunoblot analysis of spinal membrane preparations with an antibody which recognizes all GlyR $\alpha$ subunits (mAb4a) or an antibody specific for the $\alpha$1 subunit (mAb2b) showed similar expression levels in all genotypes examined (Figures 2A and 2B).

Neither total GlyR $\alpha$ nor $\alpha$1 levels were significantly altered by the D80A substitution as revealed by normalizing densitometrically determined band intensities to syntaxin expression (mAb4a immunoreactivity in homozygous Gira1(D80A)—115% ± 10% of WT; n = 6; p > 0.05; mAb2b immunoreactivity in homozygous Gira1(D80A)—127% ± 34% of WT; n = 5; p > 0.05). Since the GlyR $\alpha$1 subunit is the predominant subunit in adult spinal cord and accounts for high-affinity $[^3]$Hstrychnine binding, we also performed binding assays on spinal cord membrane preparations at ligand concentrations ranging from 1 to 200 nM. $[^3]$Hstrychnine bound to WT and homozygous Gira1(D80A) membranes with similar dissociation constants (K_{D} = 13.2 ± 2.2 nM and 24.8 ± 5.9 nM; n = 3; p > 0.05) and B_{max} values (874 ± 76 and 1112 ± 165 fmol/mg protein, n = 3; p > 0.05; Figure 2C). Thus, the D80A mutation does not significantly affect receptor expression or ligand binding properties in vivo. We also examined the synaptic localization of the $\alpha$1(D80A) subunit by performing immunocytochemistry on spinal cord sections with the $\alpha$1-specific antibody mAb2b (Figure 2D). The punctate distribution of mAb2b staining, indicative of postsynaptically clustered GlyRs, was indistinguishable between WT and homozygous Gira1(D80A) sections, as revealed by respective cluster counts (43 ± 17 and 51 ± 13 puncta per 100 μm$^2$, respectively; n = 4 each; p > 0.05; Figure 2E). We therefore conclude that the D80A mutation has no significant effects on GlyR expression and synaptic localization.

**Homozygous Gira1(D80A) Mice Develop a Severe Neuromotor Phenotype**

During the second postnatal week, at around P12, homozygous Gira1(D80A) mice developed a severe neuromotor phenotype characterized by an increased muscular tone and inducible tremor, which persisted throughout adulthood. Nevertheless, the homozygous animals were fertile, survived normally, and showed only a marginal reduction in body weight (<10%) as compared to their WT and heterozygous littermates (data not shown). To characterize the phenotypic properties of adult homozygous Gira1(D80A) mutant mice more closely, simple handling assays were performed. When picked up by the tail, homozygous mutants displayed a characteristic hind feet clapping behavior (Figure 3A). In contrast, WT and heterozygous mice balanced their position by spreading out the legs (Figure 3A). As a second criterion to evaluate neuromotor performance, littermates were screened for the development of tremor after suspending them by their tails onto an electromechanical transducer, as described previously.
In contrast to WT littermates, homozygous Glra1(D80A) mice developed inducible tremor, as demonstrated by high amplitudes of the electromechanical tracings which reflect tremor-induced movements (Figure 3B). Also, the righting response of the mutants was strongly impaired. WT animals immediately turned themselves over to get back on their feet (Figure 3C). In contrast, homozygous Glra1(D80A) mice displayed a mean righting time of >10 s (Figure 3C). Furthermore, footprint analysis disclosed clear differences in motor performance. WT animals displayed alternating positioning of hind- and forepaws, resulting in overlapping footprints and a uniform gait width (Figure 3D). In contrast, homozygous Glra1(D80A) mice produced traces that never showed overlapping footprints (Figure 3D). Quantification of the gait analysis revealed that homozygous Glra1(D80A) show a significantly shorter stride (WT, 61 ± 11 mm versus Glra1(D80A), 38 ± 9 mm; n = 7; p < 0.05), as well as a narrower base of the hind limbs (WT, 35 ± 7 mm versus Glra1(D80A), 27 ± 6 mm; n = 7; p < 0.05).

Altered Sensory Processing in Homozygous Glra1(D80A) Mice

To address whether adult homozygous Glra1(D80A) mice also show alterations in sensory functions, we analyzed parameters of visual and acoustic signal processing. First, we recorded dark- (DA) and light-adapted (LA) electroretinograms (ERGs) from homozygous Glra1(D80A) and WT mice (n = 6, each). Figure 4A shows representative DA and LA ERGs recorded from a WT and a homozygous Glra1(D80A) mouse in response to full-field flash stimuli at ten different stimulus intensities. We found a moderate increase in scotopic (DA) b-wave amplitude in homozygous Glra1(D80A) mice, which was even more prominent under LA conditions when cones are stimulated (Figures 4A and 4B, indicated by filled arrows). However, no difference was seen in the
case of the a-wave (Figures 4A and 4B, indicated by open arrows). Plotting the intensity response functions for the b-wave peak amplitudes revealed significant differences (Figure 4C; n = 6). Whereas in the DA condition the median of the b-wave amplitude in homozygous Glra1(D80A) mice was only slightly above the 95% quantile of that of the WT controls (the upper limit of the normal range), the median of the b-wave amplitude in the LA condition was significantly increased in homozygous Glra1(D80A) mice (Figure 4C). Furthermore, the mice carrying the D80A substitution had increased photopic oscillatory potentials (OPs), seen as rhythmic oscillations superimposed on the b-wave of the ERG (compare OPs of WT and homozygous Glra1(D80A) mice in Figures 4A and 4B). Thus the Glra1(D80A) substitution alters transmission of the visual signal by bipolar cells (b-wave) and AII amacrine cells (OPs), consistent with an impaired glycinergic suppression of cone signal transmission in Glra1(D80A) mice.

An increased acoustic startle response (ASR) is a hallmark of mutations affecting GlyR genes and seen in both mice and humans (Schofield, 2002). We also found an enhanced response of homozygous Glra1(D80A) mice to sudden loud acoustic stimuli (ASR), as detected by
coordinated muscle contraction. When measuring ASRs to three different stimulus intensities, mutant mice showed already at relatively low intensity levels (70 dB) a pronounced startle reaction, whereas WT mice were largely insensitive to this stimulus intensity (Figure 4D). Only at high intensities (>80 dB), ASR was also seen in most WT animals. Together, the sensorimotor phenotype of Glra1(D80A) mice resembles those of previously described GlyR mutants in mouse (spasmodic, spastic, or oscillator) and man (hyperekplexia) as well as the symptoms produced upon subconvulsive strychnine poisoning (Schofield, 2002).

Analysis of Glycine-Mediated Currents in Glra1(D80A) Spinal Neurons
To examine the functional consequences of the D80A substitution, we recorded GlyR currents in cultured spinal neurons prepared from E13 WT and homozygous Glra1(D80A) mice after 21 days of in vitro differentiation, i.e., a time period corresponding to the end of the second postnatal week. The analysis of glycine dose-response curves in these cultures revealed indistinguishable glycine potencies in cells from WT (EC50 = 27 ± 3.8 μM) and homozygous Glra1(D80A) mice (EC50 = 34 ± 5.3 μM; n = 5; p > 0.05; Figure 5A). Analysis of Zn2+ dose-response curves revealed a significant reduction in the potentiation of glycine currents by low concentrations of Zn2+ in cells from homozygous Glra1(D80A) mice as compared to WT neurons (53% ± 28% versus 117% ± 33% at 5 μM Zn2+, respectively; n = 7; p < 0.05; Figure 5B). In contrast, Zn2+-mediated inhibition at higher concentrations of the metal ion was not affected (Figure 5B).

In recombinant α1 GlyRts, the D80A substitution abolishes Zn2+ potentiation of glycine-mediated currents (Lynch et al., 1998). The residual Zn2+ potentiation seen in whole-cell currents recorded from homozygous Glra1(D80A) mice may therefore reflect the expression of GlyR α subunits other than α1, which also are potentiated by low concentrations of Zn2+ (Laube et al., 1995). Immunostainings of the cultured neurons with an antibody specific for the perinatally expressed α2 subunit of the GlyR revealed that virtually all cells exhibiting α1 GlyR staining by mAb2b also showed α2

Figure 4. Sensory Perception in Homozygous Glra1(D80A) Mice
(A–C) Electrophysiological evaluation of retinal function in 3-month-old homozygous Glra1(D80A) mice.
(A) Individual traces of dark- and light-adapted ERGs recorded from WT (black) and homozygous Glra1(D80A) (red) mice in response to stimuli of increasing intensities. Note differences between WT and Glra1(D80A) mice in photopic b-wave amplitude at higher flash intensities (b-wave and a-wave are indicated by filled and open arrows, respectively).
(B) Superposition of responses shown in (A). By increasing stimulus luminance, the difference in b-wave but not a-wave amplitudes becomes obvious (indicated by solid arrow).
(C) Qualitative comparison of homozygous Glra1(D80A) and WT mice. b-Wave amplitudes plotted as a function of the logarithm of flash intensity. Data are from six homozygous Glra1(D80A) and WT littermates each. Crosses indicate the median, boxes the 25%–75% quantile range and bars the 5% and 95% quantiles, respectively, of the Glra1(D80A) data (red). The Glra1(D80A) mice show significantly larger responses in the cone-driven light-adapted ERG than WT controls (5% and 95% quantiles of WT are indicated in black). DA: dark-adapted; LA: light-adapted.
(D) ASR incidence in WT and homozygous Glra1(D80A) mice at three different stimulus intensities (70–90 dB). Note significantly increased ASRs to 70 and 80 dB stimuli in homozygous Glra1(D80A) as compared to WT mice (n = 7; p < 0.05). Error bars indicate ± SEM.
GlyR immunoreactivity in double-staining experiments (Figure 5C). As demonstrated in Figure 5C, the α2 immunoreactivity was distributed over the entire cell and appeared to be less punctate as compared to the focal pattern found with antibodies against the α1 subunit or the postsynaptic GlyR clustering protein gephyrin (Figure 5D). Quantification of the immunoreactive structures revealed that only 28% ± 14% and 17% ± 13% (n = 5) of the α2 puncta colocalized with α1 and gephyrin, respectively (Figure 5E). Thus, most α2 GlyRs are not synaptically localized but represent extrasynaptic receptors. Consequently, the residual Zn²⁺ potentiation seen in homozygous Glra1(D80A) neurons mainly results from the modulation of nonsynaptic GlyRs.

Loss of Zn²⁺-Mediated Potentiation of Glycinergic mIPSCs in Homozygous Glra1(D80A) Spinal Neurons

To investigate the effect of the D80A substitution on GlyR-mediated synaptic currents, we first analyzed the pharmacological properties of recombinant hetero-oligomeric α1β GlyRs, which are thought to be synthetically localized (Laube et al., 2002). Electrophysiological analysis of glycine dose responses revealed indistinguishable glycine EC₅₀ values at WT α1β (EC₅₀ = 74 ± 18 μM) and α1(D80A)β (EC₅₀ = 68 ± 13 μM; n = 4, each; p > 0.05) coexpressing human embryonic kidney (HEK)293 cells (Figure 6A). To reveal how the mutation affects Zn²⁺ modulation of hetero-oligomeric α1β GlyRs, Zn²⁺ dose-response curves were analyzed in the presence of submaximal (50 μM) and saturating (500 μM) glycine concentrations. Figure 6B shows that potentiation of glycine currents by low concentrations of Zn²⁺ was lost in HEK cells expressing α1(D80A)β heterooligomers. Thus, the α1(D80A) substitution suppresses Zn²⁺ potentiation in both homo- and hetero-oligomeric GlyRs. In contrast, at saturating glycine concentrations, where Zn²⁺ exerts only an inhibitory effect on glycine currents (Laube et al., 1995), no difference in the IC₅₀ values of Zn²⁺ was found between WT α1β- and α1(D80A)β-expressing HEK cells (208 ± 41 μM and 167 ± 34 μM, respectively; n = 5; p > 0.05). This confirmed that the α1(D80A) mutant selectively suppresses Zn²⁺-mediated potentiation rather than inhibition of the heteromeric receptor. To exclude that the bath solution might be contaminated by low concentrations of Zn²⁺, we added the chelator tricine (10 mM). This had no significant effect on both glycine currents (Figure 6B) and Zn²⁺ dose-response curves when recorded in defined Zn²⁺ buffers (data not shown; see Paoletti et al. [1997]). Similar results were obtained with 1 mM Ca²⁺-EDTA, another effective Zn²⁺ chelator (data not shown). Thus, Zn²⁺ contamination was not relevant under our recording conditions.

We next examined the effect of the D80A substitution on synaptic currents by analyzing glycineric mIPSCs in spinal cord cultures prepared from E13 WT and homozygous Glra1(D80A) mice after 21 days of in vitro differentiation (Figure 6C). Neurons from WT and homozygous Glra1(D80A) animals showed mIPSCs with variable, but similar frequencies (WT, 9.4 ± 1.4 Hz; n = 6; p > 0.05) and mean amplitudes (WT, 44 ± 14.7 pA versus Glra1(D80A), 49 ± 21.4 pA; n = 6; p > 0.05). Application of 500 nM strychnine abolished mIPSCs in both WT and homozygous Glra1(D80A)
Figure 6. Electrophysiological Characterization of Synaptic α1(D80A) GlyRs

(A and B) Whole-cell patch-clamp analysis of glycine responses elicited in HEK cells expressing α1β and α1(D80A)β hetero-oligomeric GlyRs.

(a) Glycine dose-response curves for α1β and α1(D80A)β cells. Current traces elicited by 50 μM glycine in the absence and presence of 5 μM Zn2+ (left) and respective Zn2+ dose-response curves (right) (n = 5, each). Note that addition of tricine (10 mM) had no effect on the glycine current.

(b) Consecutive traces showing mIPSCs prior to (upper trace) and during (middle trace) the application of 500 nM strychnine (+Stry). Note that addition of tricine (10 mM) had no effect on the glycine current.

(c) Superimposed averaged glycinergic mIPSCs (n = 50) recorded from WT and homozygous Glra1(D80A) mice at DIV21. Altered Zn2+-Dependence of Glycinergic IPSCs in Homozygous Glra1(D80A) Hypoglossal Motoneurons

To examine the effects of Zn2+ on glycineergic neurotransmission in situ, spontaneous IPSCs were recorded in hypoglossal motoneurons of acute brainstem slices.

Glrα1(D80A) neurons. Mean changes are expressed as percentages of control values obtained for mIPSCs recorded in the absence of Zn2+ for both WT (black bars) and homozygous Glrα1(D80A) (white bars) neurons (n = 6; p < 0.01).

(F) Summary graph showing the relative changes in mIPSC mean amplitudes and frequencies found in WT and homozygous Glrα1(D80A) neurons. Note similar decay kinetics in both genotypes. (G) Immunohistochemical localization of α1 subunit containing GlyR clusters in homozygous Glrα1(D80A) spinal neurons at DIV21. Scale bar, 10 μm. Error bars indicate ± SEM.
from P8 WT and homozygous Glra1(D80A) mice. At this age, α1 subunit containing GlyRs represent already >60% of the total GlyR protein in rodent spinal cord (Becker et al., 1992). Hypoglossal motoneurons from WT animals generated glycineric IPSCs with a mean amplitude of 67.0 ± 27.0 pA and a decay time of τ = 13.5 ± 3.8 ms (n = 6; Figures 7A and 7B). In homozygous Glra1(D80A) neurons, mean IPSC amplitudes and decay kinetics were similar (67.3 ± 21.5 pA and τ = 12.3 ± 4.2 ms, respectively; n = 8; p > 0.05; Figures 7A and 7B). In addition, mean IPSC frequencies in WT and homozygous Glra1(D80A) cells were not statistically different (WT, 11.1 ± 5.1 Hz versus Glra1(D80A), 13.9 ± 3.4 Hz; n = 6; p > 0.05). Upon addition of 5 μM Zn2+, a concentration which efficiently potentiates both recombinant α1(i) GlyRs and mIPSCs in dissociated spinal cultures, IPSCs were not potentiated, consistent with endogenous free Zn2+ being present in slice preparations at concentrations sufficient to preclude potentiation by the exogenously applied metal ion (Lu et al., 2000; Suwa et al., 2001). We therefore complexed endogenous Zn2+ by tricine (10 mM). This reduced the mean amplitude of IPSCs in WT slices by 44.1% ± 18.5% (from 67.3 ± 27.0 pA to 38.4 ± 24.2 pA; n = 6; p < 0.01; paired Student’s t test; Figures 7A and 7C), while no significant effect on IPSC frequency was seen (11.1 ± 5.1 Hz versus 9.5 ± 5.2 Hz; n = 6; p > 0.05). Readjustment to a calculated free Zn2+ concentration of ca. 5 μM by adding 500 μM Zn2+ to the tricine buffer (Paolelli et al., 1997) resulted in a significant rescue of glycineric IPSC mean amplitude (Figure 7A, increase by 35.4% ± 12.9%; n = 8; p < 0.05; paired Student’s t test). This indicates that endogenous Zn2+ indeed contributes to the regulation of glycineric IPSC amplitudes in our WT slice preparations. In contrast, slices made from homozygous Glra1(D80A) mice showed no significant effect of tricine on IPSC amplitude, with the mean amplitude being reduced by only 14.0% ± 8.6% (from 67.3 ± 21.5 pA to 57.7 ± 17.5 pA; n = 8; p > 0.05; paired Student’s t test); again, no effect on IPSC frequencies was detected (13.9 ± 3.4 Hz versus 11.2 ± 3.8 Hz; n = 8; p > 0.05). Although mean IPSC amplitudes did not differ significantly between WT and homozygous Glra1(D80A) cells after the tricine treatment (38.4 ± 24.2 pA versus 57.7 ± 17.5 pA; n = 8; p = 0.054), the differential effects of tricine on IPSC mean amplitudes in WT and Glra1(D80A) preparations were highly significant (decrease in WT, 44.1% ± 18.4% versus Glra1, 12.8% ± 7.3%; n = 8; p < 0.001; Figure 7C). Moreover, the coefficient of variation calculated for the mean amplitude values was reduced upon tricine addition in the case of WT but not homozygous Glra1(D80A) slices (from 1.0 ± 0.37 to 0.7 ± 0.2 for WT; n = 8; p < 0.05, paired Student’s t test, and from 0.78 ± 0.20 to 0.78 ± 0.27 for Glra1(D80A), respectively; p > 0.05, paired Student’s t test). Thus, endogenous Zn2+ accounts for the higher variability of glycineric IPSC amplitudes seen in WT as compared to homozygous Glra1(D80A) neurons.

To investigate whether tonic glycineric inhibition mediated by extrasynaptic GlyRs may also be affected by Zn2+ chelation, we analyzed the holding currents (Ih_hold) of WT and Glra1(D80A) neurons before and after adding 10 mM tricine. Mean holding currents in WT and homozygous Glra1(D80A) cells were neither before (WT, −363 ± 191 pA versus. Glra1(D80A), −394 ± 139 pA) nor after the addition of tricine statistically different (WT, −425 ± 199 pA versus Glra1(D80A), −594 ± 157 pA; n = 6; p > 0.05, ANOVA). Apparently, ambient glycine concentrations do not contribute to tonic inhibition of hypoglossal motoneurons. Consistent with this interpretation, 1 μM strychnine did not alter Ih_hold values in both WT and Glra1(D80A) cells (WT, −201 ± 115 pA versus Glra1(D80A), −216 ± 117 pA; n = 8; p > 0.05, paired Student’s t test). We conclude that tricine affects IPSCs primarily due to a loss of synaptic rather than extrasynaptic GlyR potentiation.

We also recorded sIPSCs from hypoglossal motoneurons of WT and homozygous Glra1(D80A) mice at P14–P16, i.e., at a stage at which the phenotype of the Glra1(D80A) mouse is fully manifest. Hypoglossal motoneurons from WT animals generated glycineric IPSCs with a mean amplitude of 99.4 ± 8.0 pA and a decay time of τ = 6.97 ± 0.26 ms (n = 17, Figures 7D and 7E). In homozygous Glra1(D80A) neurons, mean IPSC amplitudes and decay times were significantly reduced (58.3 ± 7.7 pA and 2.99 ± 0.27 ms, respectively; n = 7; p < 0.01; Figure 7F). Thus, differences in IPSC characteristics apparently become detectable when the transition from α2- to α1-containing receptors is complete (Becker et al., 1992). Together, these data show that glycineric IPSCs are altered in hypoglossal motoneurons of Glra1(D80A) mice due to a loss of Zn2+ potentiation.

**Discussion**

In this study, we report the first in vivo evidence for Zn2+ modulation of a ligand-gated ion channel in the mammalian CNS. By introducing a point mutation into the murine Glra1 gene, we generated mice which display a severe hyperekplexia phenotype characterized by the development of inducible tremor, a delayed righting reflex, an abnormal gait, an increase in the b-wave amplitude of the ERG, and an enhanced ASR. These symptoms are hallmarks of impaired glycineric neurotransmission (Schofield, 2002). As demonstrated by electrophysiological recordings in spinal cultures and brainstem slices, the Glra1(D80A) mutation caused a significant reduction in the Zn2+-mediated potentiation of glycineric IPSC amplitudes without altering basic GlyR function. Our results establish an essential role for Zn2+ in CNS inhibition and indicate that Zn2+ binding to synaptic GlyRs is crucial for proper sensorimotor coordination and function.

In murine spinal cord, the subunit composition of GlyRs changes during postnatal development (Becker et al., 1988). During the first and second postnatal week, the neonatal α2 subunit is replaced by adult α1 subunit containing receptors. At P8, ≥60% of the total GlyRs in WT spinal cord contain α1, and at P14 >90% (Becker et al., 1992). This GlyR isoform switch is the cause for the late onset of neuromotor symptoms at the end of the second postnatal week in the mutant mice spasmodic and oscillator which carry mutations in Glra1 (Schofield, 2002). The appearance of the sensorimotor phenotype in Glra1(D80A) mice at around P12 thus coincides with the loss of GlyR α2 isoform expression and the onset of hyperekplexia symptoms in the natural Glra1 mouse mutants. This suggests that the
developmental pattern of α1 subunit expression is unchanged in homozygous Glra1(D80A) mice.

Different lines of evidence indicate that the phenotype of homozygous Glra1(D80A) mice can be confidently attributed to a selective loss of synaptic Zn²⁺ potentiation. First, in heterologous expression experiments substitution of aspartate 80 by either glycine or alanine has been shown to selectively suppress Zn²⁺ potentiation of α1 GlyRs without altering the glycine dose-response relationship, open probability and single-channel conductance (Lynch et al., 1998; Laube et al., 2000). Similarly, here no changes in basic GlyR physiology could be detected with recombinant α1D80Aβ GlyRs and homozygous Glra1(D80A) neurons. Second, GlyR expression and synaptic localization as monitored by Western analysis, [3H]strychnine binding, and immunocytochemistry were not significantly altered in the homozygous mutant mice. Third, glycinergic IPSC amplitudes, decay kinetics, and frequencies were similar in cultured spinal neurons and P8 brainstemhypoglossal motoneurons prepared from WT and homozygous Glra1(D80A) animals. Only when recording glycinergic IPSCs from hypoglossal motoneurons at P14, i.e., a time point where the α2 to α1 subunit switch is complete and the hyperekplexia phenotype manifest, significantly reduced amplitudes and shorter decay times were found in Glra1(D80A) cells. This is consistent with ambient Zn²⁺ increasing the IPSC amplitudes and decay time of WT α1 but not Glra1(D80A) GlyRs (Suwa et al., 2001; Laube, 2002). Concurrently, chelation of Zn²⁺ by tricine disclosed comparable differences in glycinergic IPSCs between P8 WT and Glra1(D80A) hypoglossal neurons, confirming that the majority of GlyRs present at this stage contains the α1 subunit. Notably, the hhold of the hypoglossal neurons was neither affected by strychnine nor tricine, indicating that the hyperekplexia phenotype of homozygous Glra1(D80A) mice is not due to altered tonic inhibition mediated by extracellular GlyRs. In contrast to our findings with homozygous Glra1(D80A) mice, the decrease in glycinergic IPSC amplitudes observed in spasmotic animals is caused by a reduced glycine affinity resulting from the A52S mutation in Glra1 (Graham et al., 2006). In conclusion, the phenotype of Glra1(D80A) mice is due to impaired Zn²⁺ potentiation rather than a reduction or malfunctioning of the synaptically localized mutant GlyRs.

Earlier reports showing that histochemically detectable Zn²⁺ is highly enriched within glutamatergic mossy fiber terminals of the hippocampus had led to the proposal that synaptic Zn²⁺ modulates primarily excitatory neurotransmission (Li et al., 2003). Indeed, low micromolar concentrations of exogenous Zn²⁺ potently inhibit NMDA receptor currents (Paoletti et al., 1997). More recent studies revealed an enrichment of Zn²⁺ also in inhibitory nerve terminals (Velázquez et al., 1999; Danscher et al., 2001; Wang et al., 2001, 2002;
At central synapses, the Zn$^{2+}$ transporter ZnT3 is thought to accumulate Zn$^{2+}$ within synaptic vesicles (Cole et al., 1998). The question therefore arises why homozygous Glira1(D80A) mice develop hyperekplexia, whereas no behavioral impairments have been found in ZnT3-deficient mice (Cole et al., 1999). One possible explanation could be that Zn$^{2+}$ transporters other than ZnT3 mediate synaptic vesicle loading at inhibitory synapses where the consequences of ZnT3 deficiency have not been studied. Alternatively, nonvesicular mechanisms may allow for Zn$^{2+}$ loading of and release from synaptic structures. ZnT3 independent accumulation of Zn$^{2+}$ has been demonstrated in hippocampal neurons (Lee et al., 2000) and in synaptic regions of the developing barrel cortex (Liguiz-Lecznar et al., 2005). Apparently, the large repertoire of 10 Slc30s (ZnT) and 14 Slc39s (Zn) genes allows to functionally compensate for ZnT3 deficiency which likely accounts for the lack of hyperekplexia symptoms in the ZnT3 knockout mice.

The basal concentrations of free Zn$^{2+}$ at inhibitory synapses are unknown. However, in our brainstem slices, tricine reduced IPSC amplitudes and the corresponding coefficient of variation, whereas application of 5 μM Zn$^{2+}$ had no effect. This again indicates that at least low micromolar concentrations of endogenous Zn$^{2+}$ must have been present in our preparation. Zn$^{2+}$ has been shown to not only increase the amplitude of GlyR responses at nonsaturating agonist concentrations (Laube et al., 2000) but to also inhibit glycine uptake mediated by the glial glycine transporter GlyT1 (Laube, 2002). Hence, Zn$^{2+}$ might act synergistically by both potentiating postsynaptic GlyR currents and reducing glycine reuptake from the synaptic cleft, thereby producing a higher occupancy of the synaptic receptors. In conclusion, our in vivo data support the view that nonsaturating amounts of glycine are released during physiological inhibitory transmission.

Recent data indicate an enrichment of Zn$^{2+}$ also in terminals of dorsal spinal and retinal neurons (Velázquez et al., 1999; Danscher et al., 2001; Ugarte and Osborne, 2001), suggesting that Zn$^{2+}$ participates in the processing of sensory signals. Here, we found that Glira1(D80A) mice display profound changes in ERG and ASR. Our ERG recordings from Glira1(D80A) mice indicate an impaired glycinerigic suppression of cone-mediated transmission. Endogenous Zn$^{2+}$ levels are high in the vertebrate retina, and Zn$^{2+}$ has been shown to modulate light responses of color-opponent bipolar and amacrine cells (Luo et al., 2002). Since the GlyR α1 subunit has been shown to localize at synapses between all amacrines and bipolar cells (Haverkamp et al., 2004), we conclude from these morphological and functional data that the loss of Zn$^{2+}$ potentiation at the α1(D80A) subunit reduces inhibition in the retina. This is consistent with the enhancement of the b-wave amplitude and the oscillatory potentials found in our experiments. Likewise, the enhanced ASR seen in the Glira1(D80A) mice suggests disinhibition of sensory input processing. In conclusion, our data reveal an essential role of endogenous Zn$^{2+}$ in shaping sensory transmission within visual and auditory circuits.

The data presented in our paper disclose a pivotal role of ambient synaptic Zn$^{2+}$ for glycinerigic neurotransmission in the context of normal animal behavior. Whether activity-dependent alterations in extracellular Zn$^{2+}$ concentration contribute to different forms of plasticity seen at inhibitory synapses, will have to be clarified in future studies. Our results imply that manipulation of synaptic Zn$^{2+}$ levels by systemic Zn$^{2+}$ intake or Zn$^{2+}$-specific chelators should affect glycinerigic inhibition.

**Experimental Procedures**

**Generation of Glira1(D80A) ES Cells**

A genomic fragment encompassing Glira1 exons 4 to 6 was isolated from a P1 phage library. A 1 kb BglII-HindIII fragment containing exon 4 was subcloned, and the substitution D80A and an additional silent diagnostic XbaI restriction site were introduced by site-directed mutagenesis. The mutant BglII-HindIII fragment was then used to generate a D80A 6.1 kb HindIII fragment which formed the long arm of homology. This fragment was blunt-end inserted into HindIII/XbaI-digested pEasyflox, 5’ of the loxP site flanking the neo-resistance cassette, thereby disrupting the XbaI site of the vector (Figure 1A). A 735 bp HindIII/XbaI short arm of homology was blunt-end inserted into the BamHI site flanking the 5’-loxP site of the vector, disrupting the XbaI site of the genomic fragment. After linearization with NotI at the 3’-end of the short arm, the targeting construct was transfected into E14 ES cells. Colonies resistant to G418 (Life Technologies) and 1-(2-deoxy-β-D-ribofuranosyl)-5-iodouracil (FIAU) were selected and screened for homologous integration of the construct by PCR using primers P1 (TGCTGGGGAAAACCTGCTGACTAGG) and P2 (TGCTGAAGTGCCTTGGGACATCAC) (Figure 1A). Homologous recombination events were confirmed by Southern blot analysis. Removal of the loxP-flanked neomycin resistance cassette was achieved by transfection with the Cre recombinase expression plasmid ptkCrebpa and verified by PCR using primers P3 (GCCCTAACATGATACCCGTGCGTAA) and P4 (GGACACTCTGAAAAGCTGATCCATAACTTC), as well as by Southern blot analysis.

**Generation of Glira1(D80A) Mice**

Glira1(D80A) homozygous mutant mice were produced by injecting ES cells with one engineered Glira1(D80A) allele into C57BL/6 blastocysts, which resulted in germline-transmitting chimeras. With C57BL/6 females, these animals sired offspring heterozygous for the mutation. For the analysis of sensorimotor behavior, chimeras were backcrossed over five generations to C57BL/6 females, and heterozygous offspring were then interbred to yield all genotypes.

**Southern Blot Analysis**

Genomic DNA of ES cells or mouse tissue was digested with XbaI, or BglII and XbaI, separated by agarose gel electrophoresis, and transferred to Hybond N+ membrane (Amersham). 32P-labeled internal probes were generated by random primer labeling (Prime It II, Stratagene) of a 735 bp HindIII/XbaI fragment and hybridized to the membrane.

**Miscellaneous Methods**

Western blot analysis of crude membrane fractions prepared from mouse spinal cord with antibodies specific for GlyR α subunits (mAb4a, 1:1000), the GlyR α1 subunit (mAb2b, 1:100), and syntaxin (1:5000) and glycine-displaceable binding of [3H]strychnine (DuPont NEN) were performed as described previously (Becker et al., 1988). Western blots were scanned, and digitalized images were analyzed for quantification using the software NIH-Image 1.63.

**Behavioral Characterization of Glira1(D80A) Mice**

The methods used for monitoring the neuromotor performance of Glira1(D80A) mice (hind feet clamping, tremor recordings, righting
response quantification) have been described previously (Becker et al., 2002). Footprint tracings were generated using P20–P30 mice from four different breeding pairs. Mouse footpads were painted with Tempera paint, and mice were placed onto a sheet of paper in a 50 cm long tube. The ASR was measured as described (Plappert and Pitz, 2001). The protocol was designed to determine strain sensitivity to startle stimuli of three intensities between 70 and 90 dBs presented randomly in 50 ms pulses, with an interval of 30 s. In this protocol each mouse experienced 10 trials at each noise level. ERGs were obtained as previously described (Seelig et al., 2001). At the age of 3 months, mice were dark adapted over-night and anesthetized with ketamine (66.7 mg/kg) and xylazine (11.7 mg/kg). The pupils were dilated, and single-flash ERG recordings were obtained under scotopic (DA) and photopic (LA) conditions. Stimuli were presented with increasing intensities, reaching from 10−4 cd °s/25 to 25 cd °s/°, divided into ten steps. Ten responses were averaged with an interstimulus interval (ISI) of 5 s (for 0.1, 1, 10, 30, 100, 300 mcd°s/°), 10 s (for 1 and 3 cd°s/°), or 20 s (for 10 and 25 cd°s/°). In all tests conducted, WT littersmates served as controls for the respective homozygous mutant animals. All procedures were approved by the animal care committee and were in accordance with the German law on animal experimentation.

Spinal Cord Cultures and Immunocytochemistry

Spinal cord cultures were prepared from E13 mice and kept in culture for 3 weeks as described (Laube, 2002). Immunolabeling of spinal cord sections was performed as detailed previously (Kneussel et al., 1999), using primary antibodies to the postsynaptic GlyR clustering protein gephyrin (1:100) and the GlyR subunits α1 and α2 (1:100). MAb2b is specific for the GlyR α1 subunit; mAb4a recognizes all GlyR α subunits. A polyclonal goat antibody against the N-terminal 18 residues of the GlyR α2 subunit was purchased from Santa Cruz Biotechnology. Confocal microscopy was performed using a confocal laser-scanning microscope Leica TCS-SP equipped with the image software Leica-TCS-NT version 1.6.55. To obtain spinal cord sections, tissue was cut in blocks of 5 mm and immediately frozen. Cryostat sections (12 μm) were fixed for 2 min in 4% (w/v) paraformaldehyde and processed for immunofluorescence. Punctate immunofluorescence was analyzed from confocal images taken with a 63× objective. The number of puncta per 100 μm² was counted from three different sections. Between 400 and 600 individual puncta were analyzed for the α1 GlyR subunit in WT and Glra1(D80A) samples.

Electrophysiological Recording of Agonist-Evoked and mIPSCs

Whole-cell currents of transfected HEK293 (ATCC CRL 1573) were analyzed as described previously (Laube et al., 2000). Cultured cells were continuously superfused (0.5 ml/min) at room temperature with an extracellular bathing solution containing 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 20 mM glucose, and 10 mM HEPES; adjusted to pH 7.2 with NaOH. Patch pipettes contained 120 mM CsCl, 20 mM tetraethylammonium chloride, 1 mM CaCl2, 2 mM MgCl2, 1 mM ATP, 11 mM EGTA, and 10 mM HEPES (pH 7.2). Agonist-induced currents and mIPSCs in cultured spinal neurons were recorded from neuronal somata in the whole-cell configuration of the patch-clamp technique (Laube, 2002).

Electrophysiological Recordings from Acutely Isolated Hypoglossal Slices


References


