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Low level expression of glycine receptor β subunit transgene is sufficient for phenotype correction in *spastic* mice

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Mutations in inhibitory glycine receptor (GlyR) subunit genes are associated with neuromotor diseases in man and mouse. To use the potential of the mouse mutants as animal models of human disease, we altered GlyR levels in mutant mice and studied their phenotype. A transgene coding for the β subunit of the rat GlyR was introduced into the genetic background of the *spa* mutation, which is characterized by low endogenous expression levels of the β subunit and a dramatic neuromotor phenotype. The resulting transgenic mice expressed the β subunit mRNA at intermediate levels, and their phenotype was rescued. This provides formal proof for the causal relationship between GlyR β gene mutation and motor disease, and indicates that a low level of β gene expression (25% of normal) is sufficient for proper functioning of glycinergic synapses.

Keywords: glycine receptor/mouse mutant/neuromotor disease/transgenic rescue

Introduction

Synaptic inhibition in spinal cord and brain stem is mediated primarily by the inhibitory amino acid glycine. The post-synaptic glycine receptor (GlyR) is a ligand-gated chloride channel, whose activation is antagonized by the convulsant alkaloid strychnine (Becker, 1992; Kuhse *et al.*, 1995). In adult humans and rodents, the spinal GlyR is a heteropentamer of three copies of the ligand binding α_1 subunit and two copies of the β subunit (Pfeiffer *et al.*, 1982; Langosch *et al.*, 1988; Kuhse *et al.*, 1993). The β gene is expressed at high levels throughout most regions of the brain, whereas several isoforms of the α subunit create regional and developmental heterogeneity of the GlyR in development (Grenningloh *et al.*, 1990a; Malosio *et al.*, 1991; Betz, 1992; Matzenbach *et al.*, 1994).

GlyR deficits have been associated with hereditary motor diseases in man (Shiang *et al.*, 1993; Langosch *et al.*, 1994; Rajendra *et al.*, 1994; Rees *et al.*, 1994; Schorderet *et al.*, 1994), mice (White and Heller, 1982; White *et al.*, 1987; Buckwalter *et al.*, 1994; Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994; Ryan *et al.*, 1994; Saul *et al.*, 1994) and hoofed animals (Gundlach *et al.*, 1988, 1993). In particular, amino acid substitutions in the α_1 subunit have been identified in patients suffering from dominant and recessive forms of hereditary hyperekplexia or startle syndrome (Shiang *et al.*, 1993; Rees *et al.*, 1994), a disease characterized by a multitude of symptoms. Hyperekplexia patients develop startle-induced, transient, generalized muscle contractions in infancy, which are often accompanied by hypertonia. Related mouse mutants exist, which carry defects in either their GlyR α_1 or GlyR β subunits (White *et al.*, 1987; Buckwalter *et al.*, 1994; Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994; Ryan *et al.*, 1994; Saul *et al.*, 1994). The mutations *spd* and *spd^{ot}* represent partial or complete loss of function mutations of the α_1 subunit. The mutant *spa* has an intronic insertion of a repetitive element in the β subunit gene that leads to strongly reduced levels of functional β subunit mRNA. The now extinct mutant *spa^{alb}* was shown to be allelic to *spa* (White *et al.*, 1987) and may correspond to a complete loss of β subunit function. The phenotype of all four mutants develops at 2–3 weeks postnatally, i.e. around the time when the α_1 subunit containing GlyR reaches high levels of expression. This indicates that all these mutations affect the function of the adult form of the GlyR complex (Becker *et al.*, 1988), and that the other GlyR α isotypes cannot complement for the α_1 defect. In the *spa* mutant, which expresses only low levels of the β subunit, GlyR levels are strongly reduced, showing that the β subunit is required for the assembly (Kuhse *et al.*, 1993) and/or synaptic targeting (Kirsch *et al.*, 1995; Meyer *et al.*, 1995) of the adult receptor.

The severity of the disease phenotype differs in the known mouse mutants. Animals homozygous for *spd* and *spa* show an exaggerated startle reflex, inducible rapid tremor and rigidity and an impaired righting response (Chai, 1961; Chai *et al.*, 1962). While males are infertile, homozygous females can breed, and both sexes have a normal lifespan. Homozygotes of *spd^{ot}* and *spa^{alb}*, however, die within the first 25 days of life. These stronger phenotypes are consistent with a complete loss of GlyR function in these mutants.

Because of their molecularly defined defects and their graded phenotypes, the GlyR mouse mutants comprise unique experimental animal models of human glycine receptor deficiencies. As a first step to exploit this potential, we addressed the question of whether increased β subunit expression raises GlyR levels *in vivo*, as suggested by the phenotype and biochemistry of the *spa* mutation. We show that by introducing a rat GlyR β transgene into mice, intermediate GlyR expression levels can be generated that rescue the *spa* phenotype. These experiments prove that the GlyR β subunit deficiency is causal for the phenotype of the *spa* mouse and show that a partial restoration of β

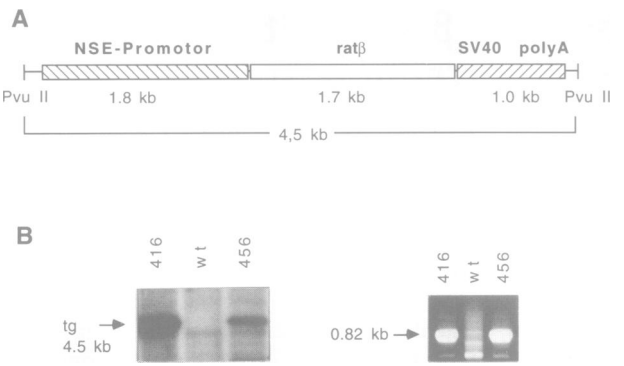

Fig. 1. Schematic representation of the transgene construct p18SNβ and its detection in animals. (**A**) The 4.5 kb *Pvu*II fragment used for injection is depicted. The fragments containing the rat NSE promoter, the rat GlyR β subunit transgene (rat β) and the SV40 splice and polyadenylation signals (SV40 polyA) are indicated. For details see Materials and methods. (**B**) Southern blot analysis. Genomic DNA from tail biopsies was digested with the restriction enzyme *Pvu*II. The blot was hybridized to a ^{32}P-labelled *Dra*II fragment of the rat GlyR β cDNA. 416 and 456 represent two different transgenic lines, wt denotes a non-transgenic control. PCR amplification: using a sense primer specific for the 3′-untranslated region of the GlyR β cDNA and an antisense primer positioned in the SV40 poly(A) region, a 0.82 kb fragment was amplified from DNA of transgenic animals.

gene expression is sufficient for the proper functioning of glycinergic pathways.

Results

Introduction of the rat GlyR β subunit gene into the germline of mice

The *spa* mutant mouse has been shown to carry a LINE 1 insertion within intron 5 of the GlyR β gene (Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994). To demonstrate that this insertion is indeed causal for the phenotype of homozygous *spa* mice, we introduced a functional GlyR β gene into the mutant genetic background by transgenesis. To this end, the rat GlyR β cDNA (Grenningloh *et al.*, 1990b) was cloned in an expression vector suitable for driving brain-specific expression under the control of the rat neuron-specific enolase promoter (NSE). This promoter has been used successfully in several transgenic expression approaches (Forss-Petter *et al.*, 1990). The β subunit minigene, which also made use of the SV40 small T antigen splice and polyadenylation signals (Gorman *et al.*, 1982; Hall *et al.*, 1983), is depicted schematically in Figure 1. The *Pvu*II fragment containing the expression construct was microinjected into the pronuclei of zygotes. To obtain transgenic strains, we used C57bl/6×DBA/2 F1 females as embryo donors after mating them with DBA/2 males. Four transgenic founder lines were derived, as shown upon screening of founder offspring by Southern blot and PCR, respectively (Figure 1). Once the founder lines had been identified, further monitoring was carried out by PCR only.

Introduction of the transgene into the spa genetic background rescues the mutant phenotype

To test whether phenotypic rescue of the *spa* mutation could be achieved by transgenesis, we bred several transgenic founders into the *spa/spa* homozygous background. All results described below were obtained with one of the

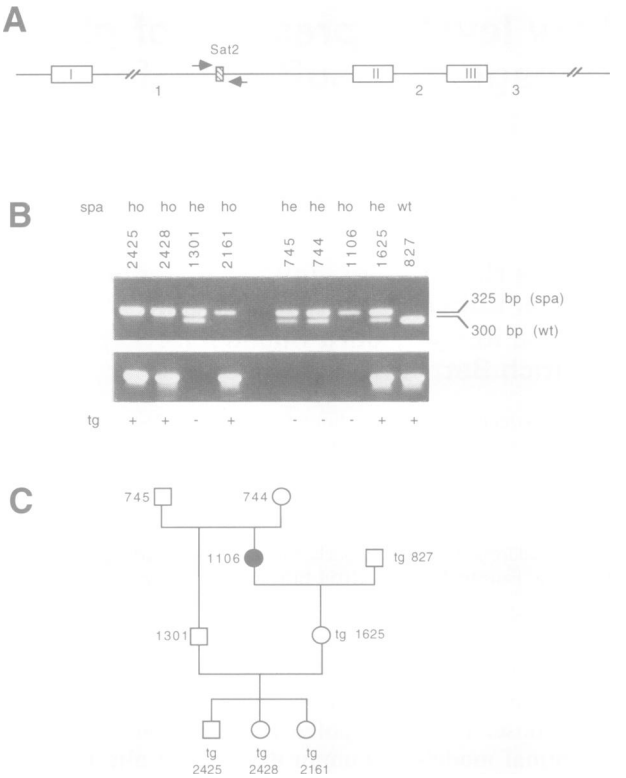

Fig. 2. Genotype analysis of rescue breedings. (**A**) Schematic representation of the partial exon–intron organization of the GlyR β gene (Mülhardt *et al.*, 1994) and location of the microsatellite sat2 (striped box). Exons are indicated by roman, and introns by arabic numerals. The positions of the PCR primers used are indicated by arrows. (**B**) PCR amplification of microsatellite- (upper panel) and transgene- (lower panel) specific fragments. Upper panel: the sat2 primers amplify a 325 bp fragment on DNA from homozygous (ho) *spa* mice; the wild-type (wt) allele generates a 300 bp fragment. In *spa* heterozygotes (he) both fragments are detectable. Numbers denote individual mice (see C). Lower panel: PCR amplification of the transgene-specific 0.82 kb fragment with primers β3′ UTR and SV40-2 (see Figure 1). (**C**) Pedigree of a cross, from which GlyR β transgene-carrying *spa/spa* mice were obtained. Males are indicated by squares, females by circles. The mouse 1106 (filled circle) displays the *spa* phenotype. The rescued mice 2425, 2428 and 2161 developed no detectable phenotype (open circles), although they are homozygous for the *spa* mutation.

lines, line 416, which showed the highest transgene expression (data not shown). To establish a molecular marker for the *spa* allele, we searched for microsatellites within the endogenous GlyR β locus. A $(CACA)_n$ sequence was identified between exons I and II (Sat2), which turned out to be polymorphic between the *spa* allele received from the Jackson Laboratory and all other strains in our mouse colony. This sequence was therefore used to follow the *spa* allele through breeding (Figure 2). Since *spa/spa* males are unable to breed, we mated transgenic males with homozygous *spa* females. All offspring of these crosses were heterozygous for *spa*, and transgenic individuals within this F1 generation, identified by PCR analysis, were used in further matings. Figure 2 gives an example of an analysis of a mating of a transgene-positive F1 animal (tg1625) with a heterozygous *spa* animal (1301), which generated *spa* homozygotes positive for the transgene. In this first experiment, the three animals representing this genotype appeared phenotypically normal.

Table I. Distributions of the *spa* allele, the rat GlyR β transgene and the tremor phenotype in the rescue breedings

Genotype	No. of F2 obtained	No. of animals developing tremor
tg/+ or tg/tg	54	0
+/+	28	0
spa/+	59	0
spa/spa	18	18
spa/spa-tg/+ or *spa/spa*-tg/tg	14	0
Total	119	18

To corroborate this finding, a number of transgene-positive F1 animals were intercrossed and the resulting F2 generation tested. In Table I the distribution of the different genotypes in 119 F2 individuals is given; 32 homozygous animals were found, and 14 of these carried a transgene (Table I). The animals from these and further breedings were then analysed for their phenotype. Notably, none of the transgene-carrying *spa/spa* homozygotes developed tremors, in contrast to their non-transgenic littermates (Table I). The righting response of these animals was tested by bringing them into a supine position and measuring the time they took to turn over (Figure 3A). Although there was considerable variation among the individual measurements, this simple test allows the severity of the phenotype in different individuals to be distinguished. For example, when *spa/spa* mice were compared with spd^{ot}/spd^{ot} homozygotes, a highly significant difference between the two phenotypes was seen. Whereas *spa/spa* mice took between ~0.6 and 50 s to turn over, spd^{ot}/spd^{ot} mice were completely unable to perform this task. *Spa*/+ or wild-type mice, in contrast, instantly right themselves (0.2–0.5 s). Interestingly, the transgene-containing *spa/spa* mice behaved indistinguishably from wild-type controls, regardless of whether the animals were heterozygous or homozygous for the transgene. Thus, the presence of a single copy of the transgene eliminated the disease phenotype. This rescue was highly penetrant, because all *spa* homozygous transgene carriers behaved indistinguishably from control animals. As a third parameter, we scored the hind feet clasping behaviour, which *spa/spa* animals display. This test also showed that the transgenic *spa/spa* animals behaved like wild-type animals (Figure 3B). The phenotypic rescue always appeared complete, since no intermediate phenotypes were detected. We therefore termed this spa/spa^{tg} genotype 'rescued'. Rescue was also seen with respect to male fertility, as all rescued males tested were fertile. Finally, to formally exclude phenotypic reversion of the *spa* phenotype in our breeding scheme, matings of rescued males with *spa* females were set up and gave rise to, among other genotypes, non-transgenic homozygous *spa/spa* mice, which exhibited the characteristic *spastic* phenotype (data not shown).

Expression of the transgene

In homozygous *spastic* mice the LINE 1 insertion in intron 5 of the GlyR β gene leads to an ubiquitous reduction of functional, full-length, stable GlyR β mRNA to ~10% of wild-type levels (Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994). In addition, low levels of aberrantly spliced GlyR

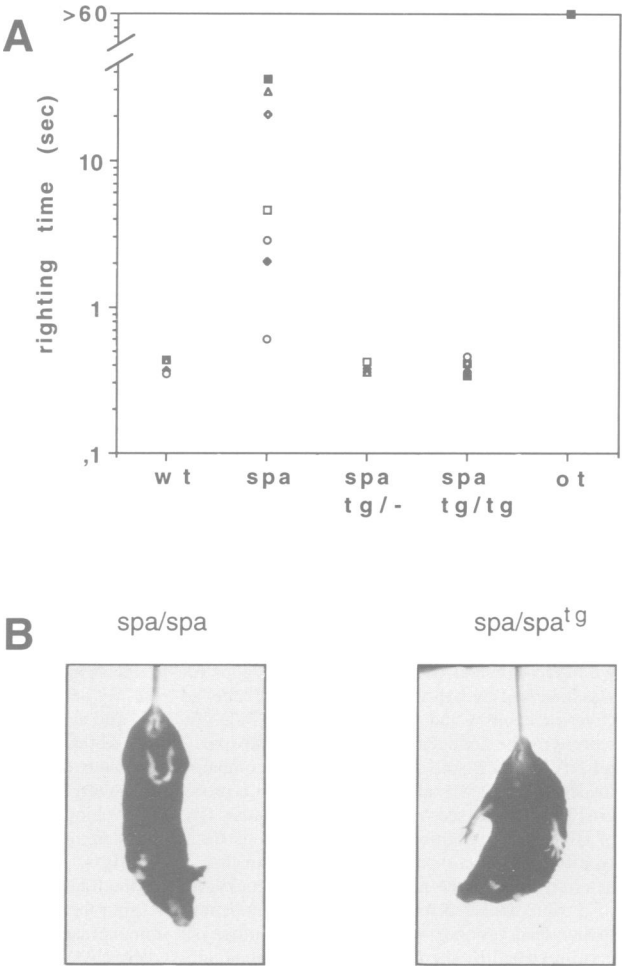

Fig. 3. Phenotypic rescue of the *spastic* phenotype in spa/spa^{tg} mice. (**A**) Righting response of *spa* mice (spa), rescued mice (tg/–, heterozygous for the transgene, tg/tg, homozygous for the transgene) and controls (wt: wild-type, ot: oscillator). Each point represents the mean of five independent measurements. Seven mice of each individual genotype were analysed. Each individual is represented by a data point. Note that oscillator mice never right themselves (>60 s). (**B**) Rescue of the 'hind feet clasping' phenotype by the transgene. When picked up by their tail, mice tend to move heavily, spreading out their legs (not shown). *Spa/spa* homozygotes in this situation clasp their hind feet (left panel) between episodes of tremor. Mice of the spa/spa^{tg416} genotype (right panel) behave like wild-type.

β transcripts lacking exons 4 and 5 have been demonstrated by PCR (Mülhardt *et al.*, 1994). Here, GlyR β mRNA levels in the brains of wild-type, *spa* homozygous and rescued mice were analysed by Northern blotting (Figure 4). As a probe, we used a fragment representing exons 4 and 5 to ensure that only full-length mRNA molecules of 3.5 kb were seen. Figure 4A shows that, in accordance with the published data (Mülhardt *et al.*, 1994), *spa/spa* mice only express ~8% of the full-length stable mRNA as compared with wild-type animals. In rescued animals, transgene expression was detected in addition to the endogenous mRNA as a band of ~2.5 kb, which corresponds to the size of mRNA transcribed from the introduced minigene. In addition, a larger band was seen at ~3.3 kb, which may be indicative of an alternative use of another polyadenylation signal in the flanking sequences. From scanning the intensity of the bands on the autoradiograph, we estimate that the transgene is expressed at ~16%

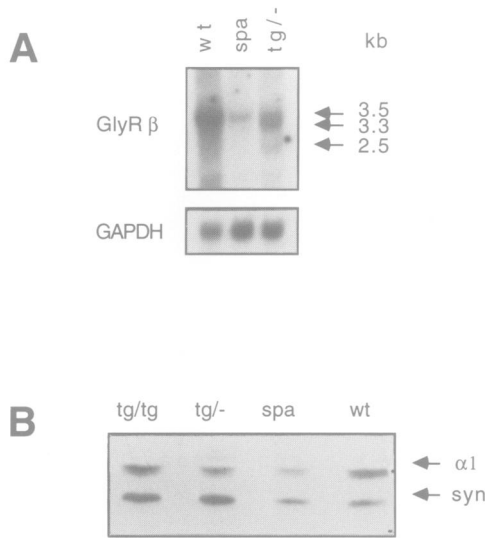

Fig. 4. Rat GlyR β subunit transgene expression and GlyR levels in rescued mice. (**A**) Northern blot analysis of transgenic and endogenous β subunit mRNAs. Total RNA from the brains of adult *spa/spa* (spa), wild-type (wt) and *spa/spa* mice heterozygous for the transgene (tg/–) was analysed by hybridization with a ^{32}P-labelled *Ssp*I–*Nco*I fragment covering exons 4 and 5 of the rat GlyR β cDNA. The band at 3.5 kb represents the endogenous mRNA (wt and spa). The 2.5 kb and 3.3 kb mRNA are transgene derived (tg/–). To compare the amounts of RNA loaded, the blot was also hybridized with a probe representing GAPDH cDNA (see Materials and methods). (**B**) Western blot analysis of GlyR levels. Membrane protein fractions from brain stem of wild-type (wt), *spa/spa* (spa) and transgene-carrying *spa/spa* (tg/–, heterozygous for the transgene, tg/tg, homozygous for the transgene) mice were analysed for α_1 subunit immunoreactivity using the monoclonal antibody 4a (mAb 4a). Synaptophysin immunoreactivity (syn) revealed by the simultaneously applied anti-synaptophysin antibody sy38 served as internal control.

of the endogenous level in wild-type mice. Therefore, given a similar spatial distribution of transgene expression to that in normal mice, these data suggest that only ~25% of normal GlyR β gene expression is sufficient to normalize GlyR function in mice. This is consistent with heterozygous *spa* mice expressing ~65% of normal GlyR β mRNA levels not displaying a phenotype (data not shown).

The promoter of the rat NSE gene has been shown previously (Forss-Petter *et al.*, 1990) to drive transcription ubiquitously in nearly all neuronal cells, while the GlyR β subunit promoter has not been analysed with respect to its properties in transgenic mice. To test whether the NSE-driven GlyR β transgene was expressed ubiquitously and uniformly as expected from the published data, we performed *in situ* hybridizations on normal, *spa/spa* and rescued mice. As for the Northern analysis described above, a probe representing exons 4 and 5 of the transgene was used to detect only full-length, functional mRNA. Figure 5 (top panel) shows that in wild-type mice widespread expression of the endogenous GlyR β gene was observed. Particularly strong expression was observed in the regions representing the facial nucleus and the superior olivary complex. This is consistent with earlier data on β subunit mRNA (Fujita *et al.*, 1991) and GlyR expression in these areas in other vertebrate species (Wenthold *et al.*, 1987; Glendenning and Baker 1988). In contrast, in *spa/spa* brain, GlyR β mRNA expression was hardly detectable (Figure 5, centre panel). This is in agreement with

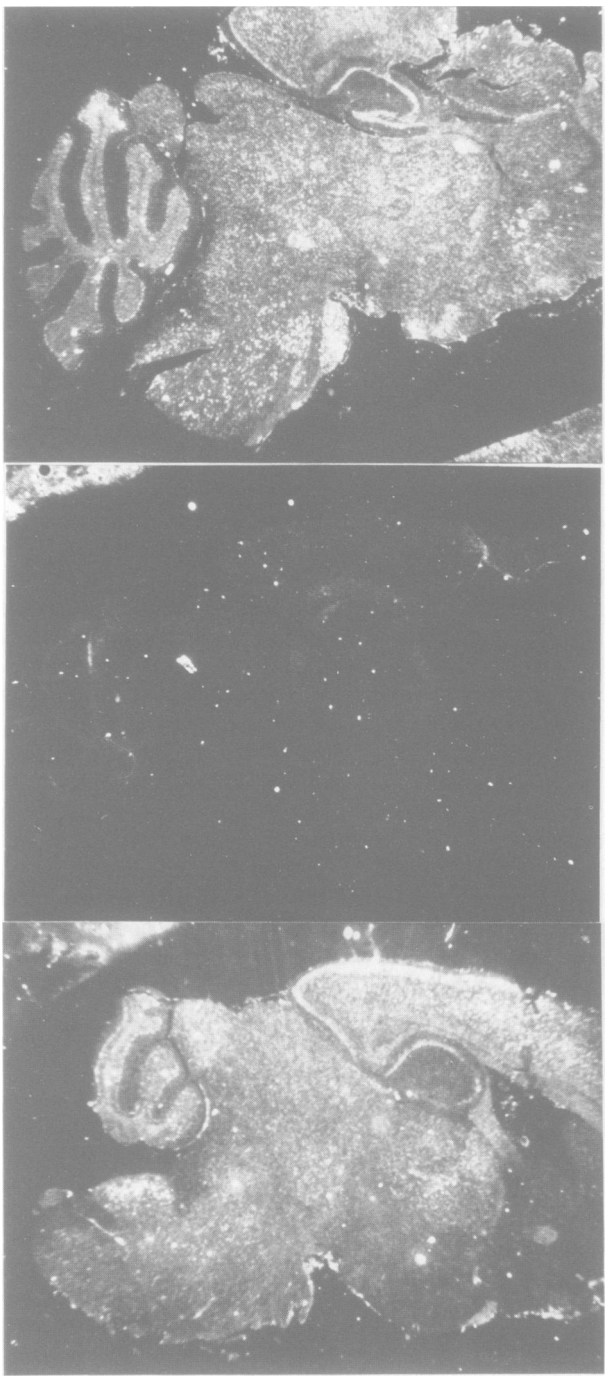

Fig. 5. *In situ* hybridization of sagittal sections of brains from wild-type (top), *spa/spa* (centre) and *spa/spa* tg/tg 416 (bottom) mice. The probe recognizes exons 4 and 5 and is therefore transgene specific on the *spa/spa* background. Equal exposure times were taken of the darkfield microscopic images. Magnification was 12.5-fold.

previously published reports (Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994). In the rescued mice, transgene expression was detected at intermediate levels throughout the brain, including the brain stem, where GlyR β subunit synthesis is thought to be essential for the proper functioning of glycine-mediated synaptic inhibition (Figure 5, bottom panel). Thus, with the exception of the hotspots within the wild-type pontine region, the similarity of the expression patterns of the transgene and the endogenous gene support our quantitative assessment of the GlyR β

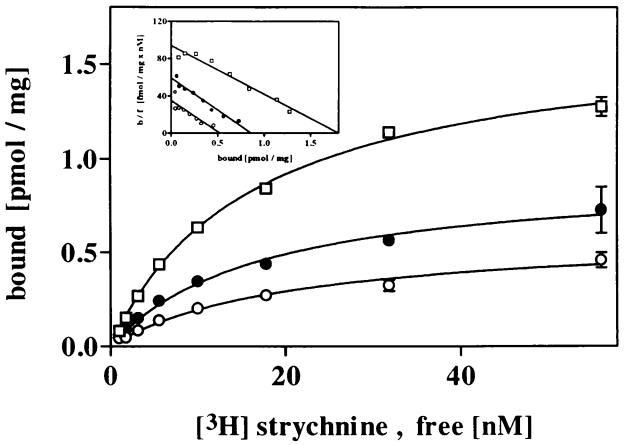

Fig. 6. Binding of [^{3}H]strychnine to spinal cord membranes from wild-type (□), *spa/spa* (○) and *spa/spa*-tg/-416 (●) mice. Binding assays were performed as described by Becker *et al.* (1986) and Saul *et al.* (1994). The insert depicts the Scatchard analysis of the data.

mRNA expression levels required for phenotypic rescue of the *spa* mutation.

The reduced GlyR β mRNA levels in *spa/spa* mice are associated with a severe reduction in GlyR protein, as revealed by a reduced binding of both α_1 subunit-specific antibodies and the specific antagonist [^{3}H]strychnine (White and Heller 1982; Becker *et al.*, 1986, 1992; Kingsmore *et al.*, 1994; Mülhardt *et al.*, 1994). An explanation for this would be a requirement for the β subunit in the GlyR complex in order to form and function *in vivo*. Thus, unlike in the *Xenopus* oocyte system (Schmieden *et al.*, 1989; Kuhse *et al.*, 1993), α_1 homo-oligomers would not be stable in the organism. We addressed this by investigating the quantities of immunoreactive α_1 subunit present in the brain stem of *spa/spa* mice carrying one or two copies of the strain 416-specific β subunit transgene. Figure 4B shows a Western blot analysis of membranes prepared from brain stem that compares mice of different genotypes. In control mice, a band at 48 kDa was detected with the GlyR-specific antibody mAb 4a, which represents the α_1 subunit of the GlyR complex (Pfeiffer *et al.*, 1984). In accordance with previous data (Becker *et al.*, 1986, 1992), *spa* homozygotes contain strongly reduced amounts of GlyR α_1 immunoreactivity. In the rescued animals, intermediate amounts of GlyR were seen: while *spa/spa* animals heterozygous for the transgene show—compared with non-transgenic *spa/spa* mice—only a slight enhancement, mice homozygous for the mutation and the transgene display higher levels of the GlyR α_1 subunit. These data imply that transgene-driven β subunit protein expression is indeed limiting the formation of GlyR complexes in the rescued strains. In addition, the amount of protein seen is roughly proportional to the gene dosage of the transgene, indicating that β subunit expression and complex formation are not limited by post-transcriptional control mechanisms. Finally, since a heterologous transgene was used, it seemed possible that the transspecies α_1–β GlyR heterooligomers in the transgenic strains are pharmacologically distinct from the endogenous mouse GlyR. We therefore performed [^{3}H]strychnine binding assays on spinal cord membranes from the rescued transgenics and control animals. Figure 6 summarizes these experiments, comparing measurements on control mice, *spa/spa* mice heterozygous for the transgene 416 and *spa/spa* mice without a transgene. Scatchard analysis of the binding data showed indistinguishable slopes for all three genotypes, indicating that the antagonist binding affinities were the same in all cases. Similar K_D values were determined for wild-type mice (16.6 ± 1.1 nM), *spa/spa* mice (20.7 ± 3.7 nM) and for rescued mice (17.6 ± 2.1 nM), respectively, and we therefore conclude that the GlyR complexes in the rescued mice appear pharmacologically normal. However, consistent with earlier findings (White and Heller 1982; Becker *et al.*, 1986), receptor numbers are reduced dramatically in the *spa/spa* mice (B_{max}: 595 ± 5 fmol/mg versus 1675 ± 4 fmol/mg in wild-type mice). Notably, in the 416 transgenics on the *spa/spa* background, intermediate receptor levels are found (B_{max}: 920 ± 5 fmol/mg). Thus, in agreement with the Northern data, an enhancement of binding activity to about half of the normal levels was seen. In summary, our data show that expression of the rat GlyR β gene in *spa/spa* mice can rescue them from developing their dramatic neurological phenotype, and that a low level of transgene-driven β subunit expression is sufficient for this functional complementation.

Discussion

Mutations in the GlyR genes have been found to be associated with neuromotor disorders in different mammalian species. So far, in man, only mutations in the gene coding for the ligand binding α_1 subunit have been identified, while in mice mutations also exist in the β subunit gene. Thus, whereas mutations of the α_1 subunit lead to an altered pharmacology (Langosch *et al.*, 1994; Rajendra *et al.*, 1994), the *spa* mutation in the β subunit gene reduces the amount of pharmacologically normal receptor molecules. Such a reduction to ~20% of normal levels results in a strong neuromotor phenotype, similar to those observed in the case of some α_1 subunit mutations. Thus, the β subunit, which is normally expressed at high levels in most, if not all, neurons (Malosio *et al.*, 1991), is a neccessary structural component of the GlyR. Consistently, variations in its expression in the presence of normal amounts of α_1 subunit may well determine the amount of functional GlyR in the post-synaptic membrane. We have generated such variations by introducing a functional β subunit into *spa* mice via transgenesis. This experiment revealed that the mutation in the β gene in these mice is indeed causal to the motor disorder, as transgene expression rescued the phenotype. Quantitation of the mRNA levels in the brain of the rescued mice showed that ~25% of the normal mRNA expression caused an increase in strychnine binding from 20 to 50%, and thereby rescued the phenotype. Most probably, this rescue is dependent upon an observed increment of interspecies mouse α_1–rat β receptor complexes. However, we presently cannot exclude that the β subunit has another, α_1-independent function. The widespread expression of the β subunit gene, compared with α_1, in normal mice points in this direction. Future experiments targeting β transgene expression to specific regions of the brain using selective promoters could address this question.

The activity of the NSE promoter apparently is sufficient to produce ~25% of normal GlyR β mRNA expression

levels in transgenic *spa/spa* mice. Of the four transgenic strains we derived, strain 416 described here displayed complete rescue of the phenotype when heterozygous for the transgene. The other strains showed lower transgene expression and, interestingly, in one of them complete rescue was observed in animals homozygous for the transgene (B.Hartenstein and H.Weiher, unpublished data). At present, we are studying these low expression strains with respect to the development of intermediate or partial phenotypes. A difficulty in these studies is the valid quantification of such phenotypes, since they vary substantially with respect to their different aspects. More quantitative methods of phenotype analysis will be required to clarify whether transgene expression indeed correlates with a gradual disappearance of the phenotype. It would be particularly interesting to analyse whether, at low GlyR expression, phenotypic changes correlate with the age of the animals. In human hyperekplexia patients, an age-dependent decrease in the severity of the symptoms is indeed observed.

Mutations in the α_1 subunit of hyperekplexia patients result in altered pharmacology, as measured in the *Xenopus* oocyte expression system (Langosch *et al.*, 1994; Rajendra *et al.*, 1994). However, this and other non-neuronal test systems appear to have limitations regarding GlyR assembly and localization functions. For example, whereas α_1 homooligomeric GlyRs form readily in *Xenopus* oocytes (Schmieden *et al.*, 1989) and transfected cells (Sontheimer *et al.*, 1989), such complexes seem not to exist stably *in vivo*, as demonstrated by the analysis of homozygous *spa/spa* mice and the β subunit-underexpressing transgenics described in this study. Particularly in light of the dominant nature of some of the known disease mutations, it is possible that the altered pharmacology seen *in vitro* might not reflect the *in vivo* situation, but that GlyR assembly and stability are affected also. Moreover, recent biochemical studies indicate that the synaptic localization of the GlyR mediated by binding to the synaptic anchoring protein gephyrin (Kuhse *et al.*, 1995) requires a direct interaction of the β subunit with gephyrin (Kirsch *et al.*, 1995; Meyer *et al.*, 1995). Thus, both the functional properties and the cellular distribution of the GlyR may be affected by disease mutations. It seems appropriate, therefore, to examine human hyperekplexia mutations in transgenic mice to further unravel the pathogenic mechanisms involved. As intraspecific human α_1–murine β GlyR complexes form readily in heterologous expression systems, a transgenic analysis following the protocol outlined here is likely to prove successful. Furthermore, test mice expressing completely humanized GlyR could be created by introducing the human subunit genes into a spd^{ot}/spd^{ot} – *spa/spa* double mutant background. Following these approaches, precise models of human hyperekplexia and related diseases should emerge soon.

Materials and methods

Cloning

A 1.8 kb *Hin*dIII fragment including the rat NSE promotor from pNSE 2K luc (Forss-Petter *et al.*, 1990) and a 1 kb *Bam*HI–*Eco*RI fragment containing the poly(A) site of the SV40 small T antigen with its splice site (Gorman *et al.*, 1982) were cloned into pUC18 (Pharmacia) generating p18SN. A 1.7 kb DNA fragment coding for rat GlyR β subunit (Grenningloh *et al.*, 1990b) was released from the plasmid pCIS β (Bormann *et al.*, 1993) by digestion with the restriction enzyme *Xho*I. The *Xho*I fragment was then cloned into the unique *Sal*I site of the plasmid p18SN, generating p18SNβ.

Production of transgenic mice

*Pvu*II fragments of the p18SNβ DNA were microinjected into pronuclei of zygotes generated from F1 hybrid female mice (C57bl/6×DBA/2) mated to DBA/2 males. One or two cell embryos surviving micromanipulation were transferred into oviducts of pseudopregnant foster mice according to published methods (Hogan *et al.*, 1994).

Animal care

Homozygous *spa/spa* mice were bred by intercrossing heterozygous *spa* breeders (B6C3Fe-*a/a-spa/*+) purchased from the Jackson Laboratory (Bar Harbor, ME). For the rescue experiments, homozygous *spa/spa* females were crossed with β-transgenic males. Transgenic littermates of the F1 generation were then intercrossed to obtain the transgenic *spa/spa* mice (among other genotypes).

Isolation of DNA from mouse tails and Southern blot analysis

Tail biopsies were taken and digested overnight in 800 µl of tail buffer [50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, 0.5 mg/ml proteinase K] at 55°C. Proteins were then precipitated by adding 300 µl 5 M NaCl and removed by centrifugation. The DNA extracted from the supernatant by isopropanol precipitation was digested with restriction enzymes and separated on a 0.8% agarose gel (20 µg/lane). Products were transferred to Hybond N+ membrane (Amersham) and probed with the ^{32}P-labelled *Dra*II fragment of the rat GlyR β cDNA. Hybridization was performed at 65°C in hybridization buffer [250 mM sodium phosphate buffer, 1 mM EDTA, 7% (w/v) SDS] overnight, followed by sequential washing with 2× SSC, 1% (w/v) SDS, with 1× SSC, 0.1% (w/v) SDS and with 0.1× SSC, 0.1% (w/v) SDS. All washing steps were performed for 15 min at 65°C.

Oligonucleotides and PCR

Tail DNA (300 ng) served as template for PCR in 30 µl of PCR mix, containing 1× PCR buffer [25 mM Tris–HCl, pH 9.5, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin (BSA)], deoxynucleoside triphosphates (250 µM each) and 50 pmol of each of the oligonucleotide primers. One unit of *Taq* polymerase (Pharmacia) was used per reaction. Cycle conditions were as follows: denaturing at 94°C for 1 min, annealing at 55°C for 1 min and synthesis at 72°C for 45 s.

The following oligonucleotides (synthesized by MWG Biotech, Ebersberg) were used: β3′ UTR (rat GlyR β cDNA, nucleotides 1817–1833; see Grenningloh *et al.*, 1990b): 5′-GCA TTT TGG ATG CCA CT-3′; SV40-2 (SV40 sequence, early RNA, nucleotides 2739–2755; see Tooze, 1980): 5′-GTG GTA TGG CTG ATT AT-3′. Oligonucleotides derived from the sequence flanking the polymorphic satellite sequence were used to follow the *spa* allele: Pex 1: 5′-CCA GAC TTC ATG CCT ATG-3′; Sat2.2b: 5′-TAT TCC CCT TAG AGT CAG-3′.

Preparation and analysis of total RNA

Tissue RNA was prepared by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). Fifteen micrograms of total RNA were loaded on a 1% agarose gel supplemented with 3.3% (w/v) formaldehyde, which was run in MOPS buffer (20 mM morpholinopropyl sulfonate, MOPS, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.0). The RNA was transferred to a nylon membrane (Hybond N+, Amersham), which was pre-hybridized in hybridization buffer (see above) for 2 h at 65°C. The randomly ^{32}P-labelled probe (Feinberg and Vogelstein, 1983) was subsequently added and hybridized overnight at 65°C. The filter was washed for 15 min at 65°C, once with 2× SSC, 0.1% (w/v) SDS and twice with 0.1× SSC, 0.1% (w/v) SDS. As a probe, a 264 bp *Ssp*I–*Nco*I fragment representing exons 4 and 5 of rat GlyR β cDNA (Grenningloh *et al.*, 1990) was used. To quantify the amount of mRNA loaded on the gel, the blot was hybridized to a ^{32}P-labelled 1 kb fragment of the rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA (Fort *et al.*, 1985).

In situ hybridization

In situ hybridization was performed on sagittal sections of brains from mice of different genotypes essentially as described in Gack *et al.* (1995). *In vitro* ^{35}S-labelled RNA transcripts from a 264 bp *Ssp*I–*Nco*I fragment representing exons 4 and 5 of rat GlyR β cDNA were used as a probe. For *in vitro* transcription, this fragment was cloned in a pT3-

T7 type vector (Gibco/BRL). Autoradiographic exposure was carried out for 10 days. Photographs of darkfield images were taken on a Zeiss microscope using Kodak Ektachrome 160T film. Exposure time was 30 s.

Western blot analysis

The preparation of membranes from spinal cord and brain stem and the solubilization and extraction of membrane proteins was done as described in Becker *et al.* (1986). Membrane protein extract pellets were suspended in Laemmli loading buffer and heated to 95°C for 5 min; 80 μg of total protein were then electrophoresed on a 10% SDS–PAGE (Laemmli, 1970) and transferred to an Immobilon-P membrane (Millipore) using a semi-dry electroblotting apparatus (Bio-Rad). The filter was blocked in 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS) containing 0.3% (w/v) Tween 20 for 1 h and incubated with the monoclonal antibodies GlyR mAb 4a and anti-synaptophysin (sy38). Bound immunoglobulins were visualized with horseradish peroxidase-conjugated anti-mouse IgG (Amersham) and signals were detected using the ECL system (Amersham).

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