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Spinal inhibitory synaptic transmission in the glycine receptor mouse mutant *spastic*

J. von Wegerer[a,*], K. Becker[b], D. Glockenhammer[b], C.-M. Becker[b], H.U. Zeilhofer[c], D. Swandulla[a]

[a]*Physiologisches Institut, Universität Bonn, Wilhelmstrasse 31, 53111 Bonn, Germany*
[b]*Institut für Biochemie, Universität Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany*
[c]*Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Universität Erlangen-Nürnberg, Fahrstrasse 17, 91054 Erlangen, Germany*

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Abstract

Inhibitory glycine receptor (GlyR) and $GABA_A$ receptor ($GABA_AR$)-mediated synaptic transmission was examined in two strains of the GlyR mutant mouse *spastic* and the respective wild types. The mutants display a mild and a severe neurological phenotype. Electrically evoked postsynaptic whole-cell currents were recorded from α-motoneurons in lumbar spinal cord slices. Amplitudes of GlyR-mediated IPSCs were significantly reduced in the severe phenotype in comparison to the respective wild type and the mild phenotype mutants. Surprisingly, amplitudes of $GABA_AR$-mediated IPSCs were also significantly reduced in both mutants. Fast time constants of the decay phase of IPSCs were slightly reduced for the GlyR-mediated IPSCs and significantly larger for the $GABA_AR$-mediated IPSCs in both mutant strains.

Keywords: Motoneuron; $GABA_A$ receptor; IPSC; Ventral horn; Slice; Patch clamp

Most ionotropic glycine receptors (GlyR) in the adult vertebrate spinal cord are a pentameric protein comprising three α_1 and two β-subunits. The spontaneous mouse mutant *spastic* carries an insertion of a long interspersed element (LINE)-1 element in intron 5 of the gene encoding the β-subunit of the GlyR. This leads to aberrant splicing of β-subunit pre-mRNA resulting in a strongly reduced number of functional GlyR [9]. The phenotype is characterized by muscle hypertonia, myoclonic seizures and an excessive startle response [2]. The severity of the phenotype additionally depends on the genetic background [2]. Homozygous mice (*spa/spa*) of the line C57BL/6J (in brief: B6) display strong symptoms, pups are severely hampered in growth and die at about 3 weeks after birth, while *spa/spa* animals of the backcross strain F1(C57BL/6J × C3H/HeJ) × C57BL/6J (in brief: C3H) show milder symptoms and most mutants survive the critical age. Here, we show that the reduction in GlyR-mediated neurotransmission in *spastic* mice correlates with the severity of the neurological symptoms and that the GlyR mutation affects also $GABA_A$ receptor ($GABA_AR$)-mediated neurotransmission. Part of the results have been published in abstract form [12].

Spinal cord slices were generated as described before [7]. In short, 14–21 day-old mice (both sexes) were anaesthetized with ether and decapitated. The lumbar segments of the spinal cord were isolated and transferred to ice-cold standard external solution which contained (in mM): NaCl 120, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, NaH_2PO_4 1.25, $NaHCO_3$ 26, HEPES 5 and glucose 15 (pH 7.40, 310 mosmol/l). The dorsal side of the spinal cord was glued onto a gelatine block and 250 μm thick transverse slices were cut with a vibratome (Campden, Loughborough, UK). Slices were incubated for 1.5–7 h after preparation in standard external solution at 32°C and bubbled continuously with carbogene (95% O_2/5% CO_2).

A Nissl staining in slices from a 14 day old mouse revealed that the largest class of neurons in the ventral horn, presumably α-motoneurons, had diameters between 20 and 30 μm. Electrophysiological recordings were made from

* Corresponding author. Tel.: +49-228-287-2662; fax: +49-228-287-2313.
E-mail address: jvw@physio.uni-bonn.de (J. von Wegerer).

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these large diameter neurons in lumbar spinal cord slices. The neurons were visualized by use of the infrared gradient contrast technique and monitored by a video microscope system mounted on an upright microscope (Zeiss Axioskop FS, Jena, Germany; Infracontrast, Luigs and Neumann, Ratingen, Germany; CF8/1 Kappa video camera, Gleichen, Germany). Slices were kept in position by a nylon mesh fixed to an *U*-shaped platinum wire. They were continuously superfused by standard external solution bubbled with carbogene. Experiments were carried out at room temperature (20 ± 2°C). Patch pipettes were pulled from borosilicate glass capillaries (Kimax 51, Kimble, USA) by a DMZ Universal puller (Zeitz, München, Germany) and tip-polished by default. The pipette resistance ranged between 3 and 5 MΩ, when filled with standard internal solution (in mM: K-gluconate 130, KCl 20, EGTA 0.05, Na_2ATP 3, Na_3GTP 0.1, HEPES 10, QX-314 5; pH = 7.30; 290 mosmol/l). Postsynaptic currents were elicited via an ipsilaterally placed glass pipette (cathode, KIMAX 51, inner tip diameter about 2 μm), at a circumradial distance of about 50 μm to the recording electrode, filled with 1 M NaCl. Electric stimulation (100 μs, 1–5 V, AM Systems, Everett, USA) occurred at intervals of 10 s. Recordings were performed in the whole-cell configuration of the patch-clamp technique with an EPC-7 patch-clamp amplifier (List Elektronik, Pfungstadt, Germany). Currents were monitored and stored with the Pulse Program (HEKA Electronik, Lambrecht, Germany) on a Macintosh Quadra 950 computer, linked to an ITC-16 interface (Instrutech, Great Neck, USA). Currents were sampled at a frequency of 20 kHz and filtered at 3 kHz. Recordings were analyzed using the IgorPro 2.01 programme (Wavemetrics, Lake Oswego, USA). Drug solutions were applied by bath perfusion at a rate of 3–5 ml min^{-1}.

For the semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) total RNA was extracted from spinal cord tissue of P20 animals by the acid guanidinium thiocyanate-phenol protocol [5]. cDNA fragments were generated using specific primers crossing borders of adjacent exons. Cycling conditions were 2′ at 94°C, 27 × (30″ at 94°C, 30″ at 57°C, 1′ at 72°C), 5′ at 72°C.

All inorganic salts, glucose, EGTA, Na_2ATP, Na_3GTP, K-gluconate, lidocaine N-ethyl bromide (QX-314), kynurenic acid, strychnine, and bicuculline were purchased from Sigma Chemie, Deisenhofen, Germany. HEPES was from Calbiochem-Novabiochem, Bad Soden, Germany. Bicuculline was solved in DMSO. Final concentration of the solvent in the experiments was 0.05%.

For each mouse strain the mean of current amplitudes was calculated under various conditions and SEM was determined. Different postsynaptic currents between wild type and mutants were compared using the Mann–Whitney rank test (*U*-test). The relative percentage of a component contributing to the total PSC or inhibitory postsynaptic current (IPSC) was determined for each neuron. Decay time analysis was performed by means of a custom written IgorPro macro. For these data a Student's *t*-test (one-tailed) was used.

Whole-cell patch-clamp recordings were performed in visually identified large diameter (>20 μm) neurons in the ventral horn of spinal cord slices. The holding potential was −80 mV throughout all experiments. Postsynaptic currents could be completely blocked in mutant as well as in wild type (wt) strains by a combination of 1 mmol/l kynurenic acid (KUA), 10 μmol/l bicuculline and 0.5 μmol/l strychnine, indicating that glutamatergic, $GABA_A$R-mediated and GlyR-mediated neurotransmission contributed to the postsynaptic currents recorded in this preparation. Control experiments showed that the relative contributions of IPSCs and excitatory postsynaptic currents (EPSCs) added up to 100 ± 5%. The total IPSC was isolated by bath application of 1 mmol/l KUA. For B6 mutants the total IPSC amounted to −134.9 ± 44.2 pA (range −55– −378 pA), while in the B6 wt −333.5 ± 89.0 pA were found (59.6% reduction; range −108– −581 pA; $P = 0.04$). In C3H mutants the total IPSC was −229.2 ± 27.3 pA (range −134– −314 pA), compared with −390.9 ± 24.5 pA in the wt (41.4% reduction; range −206– −454 pA; $P = 0.002$). The GlyR-mediated component was isolated by adding 10 μmol/l bicuculline to the bath solution (Fig. 1). It was significantly reduced by 66.3 ± 9.2% ($P = 0.02$) in B6 mutants (−85.8 ± 37.2 versus −254.4 ± 85.3 pA). In C3H mutants there was a smaller reduction of 44.9 ± 8.7%, which did not reach significance level in comparison to wild type ($P = 0.16$; −153.7 ± 36.0 versus 279.1 ± 76.1 pA), but is significantly different to B6 mutants ($P = 0.04$). The two wt strains did not differ in the GlyR-mediated IPSC amplitude ($P = 0.37$). To determine the relative contribution of the GlyR-mediated IPSC to the IPSC average peak amplitudes were normalized for each

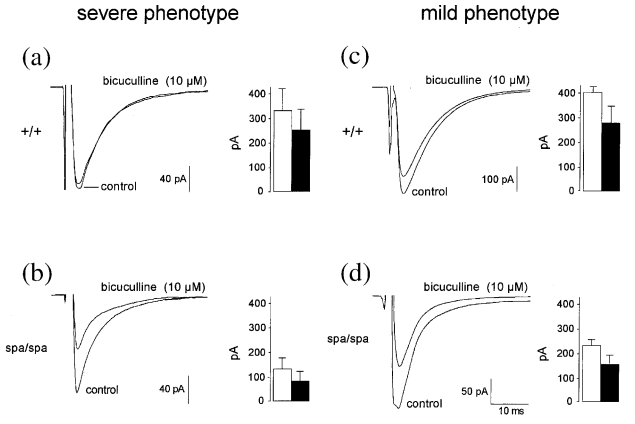

Fig. 1. GlyR-mediated IPSCs in *spastic* and wild type mice. Representative recordings of total IPSC and GlyR-mediated IPSC. Control currents were recorded in the presence of 1 mM KUA to block glutamatergic excitation. $GABA_A$R-mediated currents were blocked by 10 μM bicuculline. At the right, respectively: Amplitudes (mean ± SEM) of the total IPSCs (white bars) and the GlyR-mediated component (filled bars). Severe phenotype: B6; mild phenotype: C3H. +/+: wild type; spa/spa: mutants; *n*: number of experiments. (a) B6 wild type ($n = 7$); (b) B6 mutants ($n = 8$); (c) C3H wild type ($n = 6$); and (d) C3H mutants ($n = 6$).

experiment. The contribution of the GlyR-mediated component to the IPSC was determined for each strain. It yielded 63.9 ± 8.6% in B6 mutants and 65.9 ± 15.3% for C3H mutants, respectively, while in the wild type strains 87.6 ± 27.0 and 75.5 ± 22.4% were mediated by GlyR. No significant reduction, could be detected ($P = 0.35$ and $P = 0.42$, respectively). In a further series of experiments the $GABA_A$R-mediated component was isolated by applying a combination of 1 mmol/l KUA and 0.5 μmol/l strychnine (Fig. 2). Interestingly, also this component was significantly reduced by 60.6 ± 7.4% in B6 mutants ($P = 0.04$; −23.9 ± 6.6 versus 60.7 ± 14.6 pA) and by 62.4 ± 13.4% in C3H mutants ($P = 0.03$; −37.8 ± 32.6 versus −100.4 ± 33.2 pA). No difference was found between the two mutant strains as well as the wild types for the $GABA_A$R-mediated IPSC. The contribution of the $GABA_A$R-mediated component to the total IPSC amounted to 39.5 ± 8.3 (B6) and 29.1 ± 13.2% (C3H) in the mutants and to 14.4 ± 5.6 (B6) and 30.9 ± 7.1% (C3H) in the wild type strains. This shows that also the $GABA_A$R-mediated component was reduced in amplitude in both mutant strains. In B6 mutants the relative contribution of $GABA_A$R-mediated IPSCs to the total IPSC was significantly increased ($P = 0.03$), while in C3H the percentage remained constant ($P = 0.41$). A decay time analysis was performed on the GlyR- and $GABA_A$R-mediated IPSCs for each experiment. The curves were fitted by a bi-exponential function. The relative weight of the amplitudes of the fast component τ_1 was between 68 and 86% in the different strains. In most neurons the current decay could have been satisfactorily fitted with a monoexponential function. Therefore our analysis is focussed on τ_1. At a holding potential of −80 mV $GABA_A$R-mediated IPSCs show a

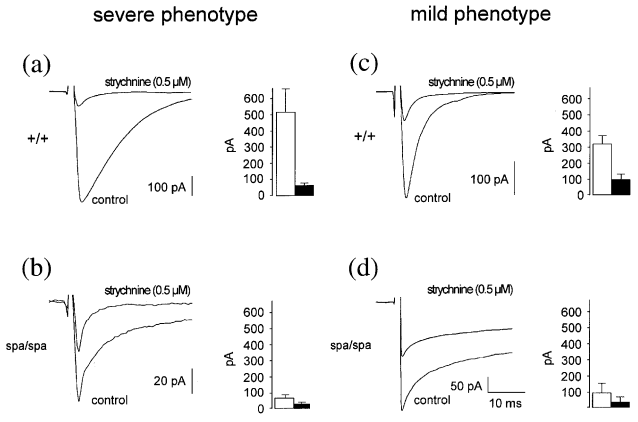

Fig. 2. $GABA_A$R-mediated IPSCs in *spastic* and wild type mice. Representative recordings of total IPSC and $GABA_A$R-mediated IPSC. Control currents were recorded in the presence of 1 mM KUA to block glutamatergic excitation. GlyR-mediated currents were blocked by 0.5 μM strychnine. At the right, respectively: Amplitudes (mean ± SEM) of the total IPSCs (white bars) and the $GABA_A$R-mediated component (filled bars). Severe phenotype: B6; mild phenotype: C3H. +/+: wild type; spa/spa: mutants; and *n*: number of experiments. (a) B6 wild type ($n = 8$); (b) B6 mutants ($n = 6$); (c) C3H wild type ($n = 6$); and (d) C3H mutants ($n = 6$).

faster decay time constant than GlyR-mediated IPSCs in juvenile mice [1]. A slight reduction in the fast time constant τ_1 in the GlyR-mediated IPSCs of both mutant strains did not reach significance level. In contrast, τ_1 for the $GABA_A$R-mediated IPSC was significantly slower in both mutants (Table 1). The reduction in IPSC amplitudes may involve modifications on the presynaptic site. To identify possible changes in the amount of presynaptically released transmitter, a variance analysis of synaptic current amplitudes was performed for single stimulations [8]. It was found that the coefficient of variation (V) to the −2 power increases significantly with the mean amplitude of IPSCs pooled for the respective mouse strains. This suggests that in *spastic* there may be additionally a reduced transmitter release from glycinergic terminals in B6 mutants and GABAergic terminals in both mutant strains (not shown). A possible explanation for the changes in the $GABA_A$R-mediated neurotransmission could be an altered expression of $GABA_A$R subunits. Thus, total RNA was isolated from entire spinal cords of P20 animals ($n = 5$ animals/group, pooled samples) and tested for the most frequent subunits in spinal cord by RT-PCR. No difference in overall level and pattern of expression between wild-type and mutant lines was detectable for the $GABA_A$R subunits α_1, α_2, α_3, β_2 and γ_2 (not shown).

The electrophysiological changes in inhibitory synaptic transmission in the ventral horn of the spinal cord in two different strains of a mouse mutant with a glycine receptor defect were investigated. Total IPSCs were significantly reduced in both mutant strains, due to reductions in the GlyR- and $GABA_A$R-mediated IPSC amplitudes. As expected, the GlyR-mediated IPSC amplitudes were reduced in both mutant strains. The degree of the reduction of the IPSCs depends on the genetic background. In B6 mutants the reduction was about 50% larger than in C3H mutants. These results correspond closely to the severity of the neurological phenotype.

The fast decay time constant of the GlyR-mediated IPSCs was slightly reduced to the same degree in both mutant strains. This might be a consequence of an altered intracellular regulation of glycine receptors such as by receptor phosphorylation [10]. An increased maximum transport rate for glycine reuptake in *spastic* was described

Table 1
Decay time constants τ_1 [ms] for the GlyR-mediated and the $GABA_A$R-mediated IPSCs in the respective mouse strains

	Gly-R	*n*	$GABA_A$-R	*n*
B6-M	5.28 ± 0.94	8	11.59 ± 4.28	6
B6-WT	6.35 ± 1.23	7	3.78 ± 0.74	7
C3H-M	5.01 ± 1.09	6	5.88 ± 1.26	6
C3H-WT	6.45 ± 0.48	6	3.29 ± 0.38	6

M, mutant; and WT, wild type. Mean ± SEM. *n* = number of experiments.

[14]. This could lead to a shorter decay time of GlyR-mediated IPSCs.

Also the $GABA_AR$-mediated IPSCs were reduced in amplitude in both mutant strains. This indicates that in C3H mutants the GABAergic system does not compensate the GlyR defect, as might have been expected from the finding of increased radioligand binding of GABA in the spinal cord of *spastic* [13]. Interestingly, substances that increase GABAergic transmission can alleviate the symptoms of *spastic* [3].

In rat spinal motoneurons the mainly found $GABA_AR$ subunits are α_2/γ_2 and $\alpha_2/\alpha_5/\gamma_2$ [4]. In our PCR-amplification of the most frequent $GABA_AR$ subtype cDNAs we did not find evidence for a changed expression pattern. However, changes smaller than 50% might have escaped detection.

The variance analysis of IPSC amplitudes shows a covariation of CV and IPSC amplitudes for GlyR-mediated IPSCs in B6 mutants and $GABA_AR$-mediated IPSCs in both mutant strains. These results can be explained by a diminished transmitter release, resulting at least in a reduced $GABA_AR$-mediated IPSC amplitude. Recently, presynaptic glycine receptors were identified in the brain stem [11]. These receptors were shown to be depolarizing, thus increasing glutamatergic postsynaptic potentials significantly. Depolarizing presynaptic glycine receptors might also exist on synaptic boutons projecting on α-motoneurons. In this case one would expect highly less effective presynaptic glycine receptors in the mutant *spastic* which would result in diminished transmitter release. A corresponding effect was found for presynaptic depolarizing $GABA_AR$ which control the glycinergic transmission onto rat sacral dorsal commissural neurons [6]. It is conceivable that there is a cross modulation of presynaptic GlyR and $GABA_AR$ on spinal cord interneurons projecting on α-motoneurons. Thus, presynaptic GlyR could contribute to the reduction in GlyR- and $GABA_AR$-mediated IPSCs and might explain the results of the variance analysis. This could also explain the augmented relative IPSC(GABA)/IPSC(Gly) ratio in B6 mutants. If it is assumed that in B6 mutants relatively less glycine is released, there will be less activation of presynaptic GlyR resulting in a relatively larger reduction of glycine.

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