Investigation of the abundance and subunit composition of $GABA_A$ receptor subtypes in the cerebellum of α1-subunit-deficient mice

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Abstract

In cerebellum, 75% of all $GABA_A$ receptors contain α1 subunits. Here, we investigated compensatory changes in $GABA_A$ receptor subunit expression and composition in α1 subunit-knockout mice. In these mice the total number of cerebellar $GABA_A$ receptors was reduced by 46%. Whereas the number of receptors containing α6 subunits was unchanged, the total amount of α6 subunits was significantly elevated. RT-PCR showed no increase of α6 mRNA levels, arguing against increased biosynthesis of these subunits. Elevated levels of α6 subunits in α1 -/- mice might thus have been caused by an increased incorporation of unassembled α6 subunits, replacing α1 subunits in α1α6βγ2 or α1α6βδ receptors, thus rescuing α6 subunits from degradation. Elevated levels of α3 and α4 subunits in the cerebellum of α1 -/- mice possibly can be explained similarly. Finally, a small amount of receptors containing no γ or δ subunits was identified in these mice. Results suggest a total loss of $GABA_A$ receptors in cell types where α1 was the only α subunit expressed and a partial compensation for receptor loss in cell types containing other α subunits. Our results do not support a significant compensatory synthesis of other $GABA_A$ receptor subunits in the cerebellum of α1 -/- mice.

Keywords: α1 knockout, cerebellum, $GABA_A$ receptor, subunit composition, quantitative importance.

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$GABA_A$ receptors are ligand gated chloride ion channels that can be opened by GABA and are the site of action of a variety of psychoactive compounds such as benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants (Sieghart 1995). These receptors are composed of five subunits that can belong to different subunit classes. So far six α, three β, three γ, one δ, one ε, one π, one θ and three ρ subunits have been cloned and sequenced from the mammalian brain (Sieghart and Sperk 2002). Depending on their subunit composition, $GABA_A$ receptors have different pharmacological and electrophysiological properties (Sieghart 1995). Immunohistochemical studies have demonstrated that individual subunits exhibit a distinct and often widespread distribution throughout the nervous system (Fritschy and Mohler 1995; Pirker *et al.* 2000). The resulting expression of multiple subunits in the same neurons suggests the existence of a large variety of $GABA_A$ receptor subtypes in the brain. The majority of $GABA_A$ receptors, however, are composed of two α, two β and one γ subunit (Sieghart and Sperk 2002).

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Abbreviations used: DIS, diazepam insensitive binding; DOC, deoxycholate; DS, diazepam sensitive binding; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonylfluoride; RT-PCR, reverse transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Recently, several mouse strains have been generated with a targeted disruption of individual $GABA_A$ receptor subunits. Although the function of these receptors could not be determined by this approach due to possible changes in brain development and in the GABAergic or unrelated systems (Brickley *et al.* 2001), these mice provided information on possible subunit partnerships and compensatory reactions. Mice lacking the γ2 subunit exhibited an unchanged number of receptors that were composed only of α and β subunits, indicating that the γ2 subunit is dispensable for the assembly of functional $GABA_A$ receptors (Gunther *et al.* 1995). These mice died shortly after birth due to the absence of receptor clustering at the synapse mediated by γ2 subunits (Crestani *et al.* 1999). In contrast, mice lacking the α6 subunit of the $GABA_A$ receptor showed a strong reduction in the total number of receptors without major compensatory up-regulation of other $GABA_A$ receptor subunits. Interestingly, δ subunits were also dramatically reduced in α6 -/- cerebellum, suggesting a subunit partnership between α6 and δ subunits (Jones *et al.* 1997; Nusser *et al.* 1999). Surprisingly, these mice had no major phenotypic abnormalities (Jones *et al.* 1997), probably due to a compensatory up-regulation of potassium channels in this tissue (Brickley *et al.* 2001). Finally, mice lacking the δ subunit exhibited an unchanged total number of receptors but a compensatory increase in the number of α6- and γ2- subunit-containing receptors, suggesting that γ2 subunits could replace δ subunits in these mice. δ-/- mice were viable but exhibited attenuated sensitivity to neuroactive steroids (Mihalek *et al.* 1999).

The α1 subunit is the most abundant α subunit variant in the brain and is highly expressed throughout most brain regions. Recently, mice lacking the α1 subunit of $GABA_A$ receptors (α1 -/-) have been generated and characterized (Sur *et al.* 2001; Vicini *et al.* 2001). Interestingly, α1 -/- mice are viable, fertile and show no spontaneous seizures. These mice exhibit a marked loss of $GABA_A$ receptor numbers and compensatory increases in $GABA_A$ receptor α2 and α3 subunits in the forebrain (Sur *et al.* 2001; Kralic *et al.* 2002).

Recently, the quantitative importance and subunit composition of $GABA_A$ receptors has been determined in the cerebellum of rats and mice (Poltl *et al.* 2003; Jechlinger *et al.* 1998). In the present study, possible changes in the abundance and subunit composition of $GABA_A$ receptors were investigated in the cerebellum of α*1* -/- mice. Results indicate a dramatic reduction in the total number of $GABA_A$ receptors and an elevated level of α3, α4 and α6 subunits in this tissue.

Materials and methods

Generation of mutant mice

Production of $GABA_A$ receptor α1 +/+ and α1 -/- mice was previously described in detail (Vicini *et al.* 2001). Briefly, wild-type controls were homozygous for a floxed α1 allele that was functionally equivalent to the wild-type allele (Kralic *et al.* 2003), and $GABA_A$ receptor α1 -/- mice were homozygous for a cre-mediated global deletion of exon 8 of the α1 gene. These homozygous mice used for experimental analysis were obtained by interbreeding mice that were heterozygous for the wild-type floxed allele and the recombined allele. All mice were of a mixed C57BL/6 J, 129Sv/SvJ, and FVB/N genetic background and genotyped by PCR analysis of tail DNA.

Generation and purification of antibodies

The generation and purification of anti-peptide antibodies directed against $α_1$(1-9), $α_1$(328-382) $α_2$(416-424), $α_3$(338-385), $α_4$(379-421), $α_5$(337-380), $α_6$(1-15), $α_6$(317-371), $β_1$(350-404), $β_2$(351-405), $β_3$(345-408), $γ_1$(1-39), $γ_2$(1-33), $γ_2$(319-366), $γ_3$(1-35), $γ_3$(322-372) or δ(1-44) has been described previously (Jechlinger *et al.* 1998; Poltl *et al.* 2003).

All antibodies used for subunit specific immunoprecipitation were shown to precipitate recombinant $GABA_A$ receptors containing the respective subunit only and did not exhibit any cross-reactivity with other recombinant $GABA_A$ receptors. In addition anti-δ or anti-α6 antibodies did not precipitate $GABA_A$ receptors in δ- (Mihalek *et al.* 1999) or $α_6$- (Jones *et al.* 1997) knock out brains, respectively. Subunit specific antibody preparations were pooled in order to collect amounts of antibodies sufficient for all studies performed. The amount of antibody necessary for maximal precipitation of $GABA_A$ receptors was then determined in cerebellar extracts. The precipitation efficiency was determined by estimating the percentage of recombinant receptors precipitated under these conditions by the respective subunit specific antibody followed by a [^{3}H]muscimol binding assay. In these experiments the total amount of receptors in the extract was determined after precipitation with a mixture of antibodies directed against the α, β and δ subunit present in the recombinant receptor. The precipitation efficiency of antibodies directed against the α1, α6, γ2, or δ subunit was determined by comparing the amount of receptors precipitated by the respective antibody in cerebellar extracts to the total amount of these receptors present in the cerebellum as determined by immunoaffinity chromatography (Poltl *et al.* 2003). The precipitation efficiencies were then used to estimate the true percentage of receptors precipitated by the respective antibody in all subsequent experiments.

Preparation of receptor extracts

$GABA_A$ receptors were solubilized from mouse cerebellar membranes or total cerebella from adult mice by using per cerebellum 2–2.5 mL of a deoxycholate buffer [0.5% deoxycholate, 0.05%, phosphatidylcholine, 10 mM Tris/HCl, pH 8.5, 150 mM NaCl, 1 Complete Protease Inhibitor Cocktail Tablet (Roche Molecular Biochemicals, Mannheim, Germany) per 50 mL]. The suspension was homogenised using an Ultra-Turrax®and subsequently by pressing the suspension through a set of needles with increasingly smaller diameters using a syringe, followed by incubation under intensive stirring for 60 min at 4°C. After centrifugation at 150 000 g the clear supernatant was used either for subsequent immunoprecipitation, [^{3}H]muscimol and [^{3}H]Ro15–4513 binding studies or for affinity chromatography and subsequent immunoprecipitation and [^{3}H]muscimol binding studies in the column efflux. For the determination of the solubilisation efficiency of individual $GABA_A$ receptor subunits as well as of functional receptors, the 150 000 *g*

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pellet was re-dissolved in the same volume of deoxycholate buffer. Aliquots of this suspension as well as aliquots from the clear supernatant of the 150 000 *g* centrifugation were separately subjected to protein precipitation using the methanol/chloroform method (Wessel and Flugge 1984) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and quantitative western blot analysis (see below). Furthermore, aliquots of the supernatant as well as the re-dissolved pellet of the 150 000 *g* centrifugation were used for subsequent immunoprecipitation and [^{3}H]muscimol and [^{3}H]Ro15–4513 binding studies.

Quantification of $GABA_A$ receptors by immunoprecipitation and [^{3}H]muscimol binding assays

For immunoprecipitation, 175–300 μL of the clear deoxycholate membrane extract or column efflux were mixed with a solution containing 5 μg of α1(1–9), 10 μg of β1(350–404), 10 μg of β2(351–405), and 10 μg of β3(345–408) antibody. This antibody mixture was used because all functional $GABA_A$ receptors are supposed to contain at least one of the three β subunits, and most of them contain an α1 subunit (Tretter *et al*. 2001; Poltl *et al*. 2003). Additionally, the same volume of clear supernatant or column efflux was mixed with either 5 μg γ1(1–39), 15 μg of γ2(319–366), 5 μg γ3(1–35), 5 μg of δ(1–44), or 10 μg of α6(317–371) antibody in order to precipitate receptors containing the respective subunit (Poltl *et al*. 2003). The mixture was incubated under gentle shaking at 4°C overnight. Then 50 μL of pansorbin (Calbiochem, La Jolla, CA, USA) and 50 μL 5% dry milk powder, both in low salt buffer for immunoprecipitation (IP-low; 50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, pH 8.0) were added and incubation was continued for 2 h at 4°C. The precipitate was centrifuged for 5 min at 2300 *g*, washed twice with 500 μL of high salt buffer for immunoprecipitation (IP-high; 50 mM Tris/HCl, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 8.3) and once with 500 μL of IP-low.

The precipitated receptors were then suspended in 1 mL of a solution containing 0.1% Triton X-100, 50 mM Tris/citrate buffer pH 7.1 and 40 nM [^{3}H]muscimol (28.5 or 29.5 Ci/mmol, PerkinElmer Life Sciences, Boston, MA, USA) in the absence or presence of 1 mM GABA. After incubation for 60 min at 4°C the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 3.5 mL of 50 mM Tris/citrate buffer pH 7.1 and subjected to liquid scintillation counting (Filter-Count™, Packard; 2100 TR Tri-Carb® Scintillation Analyser, Packard®). Binding in the presence of 1 mM GABA (unspecific binding) was then subtracted from binding in the absence of GABA (total binding), resulting in specific binding to precipitated $GABA_A$ receptors.

Immunoaffinity chromatography

Immunoaffinity columns were prepared by coupling 3–5 mg of the purified antibodies γ1(1–39), γ2(319–366), γ3(322–372) or δ(1–44) to 1 mL of ImmunoPure® immobilized protein A using the ImmunoPure®Protein A IgG Orientation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The column was then washed once with PBS/NaCl (150 mM NaCl, 4 mM KH_2PO_4, 16 mM Na_2HPO_4, pH~7.5), then with glycine-elution buffer pH 2.45 (0.1 M glycine/HCl, pH 2.45, 150 mM NaCl, 0.1% Triton X-100), with IP-low buffer (see above), with alkaline elution buffer (100 mM Na_2HPO_4, 150 mM NaCl at pH 11,5) and finally with IP-low. Columns were stored protected from light in IP-low buffer containing sodium azide.

Immunoaffinity chromatography was performed at 4°C. The immunoaffinity columns were equilibrated in deoxycholate extraction buffer. The extract was chromatographed slowly up to two times on the same affinity column in order to completely remove the receptors containing the respective subunit from the extract, regenerating the respective column after each chromatography step by washing it with glycine-elution buffer pH 2.45, IP-low (see above), antibody elution buffer pH 11.5 (see above) and again with IP-low.

To determine the percentage of receptors containing a specific subunit or the total amount of receptors retained by the column, immunoprecipitations with subunit specific antibodies or with an antibody mixture containing antibodies β1(350–404), β2(351–405), β3(345–408) and α1(1–9) and subsequent [^{3}H]muscimol binding assays were performed in the original extract and column efflux in parallel.

Receptor binding studies

Cerebella from α1 +/+ and α1 -/- adult mice were homogenised using 2.5–3 mL per cerebellum of a 50 mM Tris/citrate buffer (pH 7.1, containing one complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) per 50 mL buffer) as described above. The homogenates were ultracentrifuged at 150 000 *g*, the pellets washed three times in 10 mL and finally resuspended in 2.5–3 mL of the same buffer. Extracted receptors were immunoprecipitated (see above), and the precipitate was suspended in 1 mL of a solution containing 0.1% Triton X-100, and 50 mM Tris-citrate buffer (pH 7.1).

For [^{3}H]Ro15–4513 binding studies, a total of 1 mL of a solution containing either 100 μL of resuspended membranes, 50 mM Tris/citrate buffer (pH 7.1), 150 mM NaCl, or the resuspended immunoprecipitate, and 1–50 nM [^{3}H]Ro15–4513 (20 or 28.3 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 1 μM diazepam or 100 μM Ro15–1788. After incubation for 90 min at 4°C, the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 5 mL of 50 mM Tris/citrate buffer (pH 7.1) and subjected to liquid scintillation counting. Binding in the presence of 10 μM Ro15–1788 (unspecific binding) was then subtracted from binding in the absence of Ro15–1788 (total binding), resulting in specific binding to $GABA_A$ receptors. Data were analysed using GraphPad Prism.

For [^{3}H]muscimol binding studies, extracted receptors were immunoprecipitated (see above), and the precipitate was suspended in 1 mL of a solution containing the resuspended immunoprecipitate and 1–40 nM [^{3}H]muscimol (29.5 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 1 mM GABA. After incubation for 60 min at 4°C, the suspensions were rapidly filtered through Whatman GF/B filters, washed twice with 3.5 mL of 50 mM Tris/citrate buffer (pH 7.1) and subjected to liquid scintillation counting. Binding in the presence of 1 mM GABA (unspecific binding) was then subtracted from binding in the absence of GABA (total binding), resulting in specific binding to $GABA_A$ receptors. Data were analysed using GraphPad Prism.

SDS–PAGE and western blot analysis

α1 +/+ and α1 -/- adult mouse cerebella were individually homogenised using an Ultra-Turrax® in a volume of 5 mL per

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cerebellum of a 50 mM Tris/citrate buffer (pH 7.1), containing one complete protease inhibitor cocktail tablet per 50 mL buffer (Roche Diagnostics, Mannheim, Germany). The suspensions were pressed through a set of needles with increasingly small diameters using a syringe.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to (Neville and Glossmann 1974), using 10% polyacrylamide gels in a discontinuous system. For estimation of the size of the proteins 'MagicMark^{TM} XP Western Standards' (Invitrogen, Carlsbad, CA, USA) were used in separate lanes. Equal amounts (containing 10 μg of protein) of the suspension were subjected to SDS–PAGE in different slots of the same gel. Proteins were blotted to polyvinylidene difluoride membranes and detected by antibodies to the following subunits: α1(amino acid residues 328-382); α_2(416-424), α_3(338-385), α_4(379-421), α_5(337-380), α_6(1-15), β_1(350-404), β_2(351-405), β_3(345-408), γ_1(1-39), γ_2(1-33), γ_3(1-35) or δ(1-44) (Jechlinger *et al*. 1998; Poltl *et al*. 2003). Secondary antibodies ($F_{(ab')2}$ fragments of goat anti-rabbit IgG, coupled to alkaline phosphatase, Axell (Westbury, NY, USA) were visualized by the reaction of alkaline phosphatase with CDP-Star (Applied Biosystems, Bedford, MA, USA). The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultImager (Bio-Rad Laboratories, Hercules, CA, USA) and evaluated using the Quantity One Quantitation Software (Bio-Rad Laboratories, Hercules, CA, USA) and GraphPad Prism (Graph Pad Software Inc., San Diego, CA, USA). Quantification was performed by an independent investigator blind to the identity of the samples. The linear range of the detection system was established by measuring the antibody generated signal to a range of antigen concentrations. Under the experimental conditions used, the immunoreactivities were within the linear range, and this permitted a direct comparison of the amount of antigen per gel lane between samples. The amounts of individual GABA$_A$ receptor subunits present in homogenates from α1 +/+ and α1 -/- mice were compared in the same gel. Data were generated from several different gels per subunit and per mouse, and expressed as mean ± standard error. Student's unpaired *t*-test was used for comparing groups, and significance was set at $p < 0.05$.

To test for equal protein loading, in some experiments a monoclonal anti-β-actin antibody was included in the antibody solution and the amounts of endogenous β-actin were quantitatively determined in a way analogous to GABA$_A$ receptor subunits. Protein loading was comparable in different slots and referring the data to the amounts of endogenous β-actin neither changed the results nor reduced variability.

Preparation of total RNA and multiplex RT-PCR analysis of α1, α6, δ, and GAPDH mRNA from α1 +/+ and α1 –/– cerebella

Total RNA was isolated from the cerebella of three *α1* +/+ and three *α1* -/- mice using the RNAqueous-4PCR kit (Ambion Inc., Austin, TX, USA). 1.5 μg of each RNA were reverse transcribed using the RETROscript kit (Ambion Inc.) and oligo $(dT)_{18}$ as first-strand primer according to the kit protocol. Aliquots of the cDNA containing identical amounts of total RNA were simultaneously PCR amplified with a combination of α_6 and δ primers:

α6-F: 5′-AAGATGGGCTATTTCATGATCCA-3′
α6-R: 5′-TGCTCACTTCCATTGTATCTTTGG-3′
δ-F: 5′-TGACCATATCTCAGAGGCAAACAT-3′
δ-R: 5′-CGGTAACTGGTGATAGTGAACTGG-3′

To verify that the target RNA used for RT-PCR was correctly derived from α1 +/+ and α1 -/- mice, PCR reactions were additionally performed in all samples using α1-specific primers:

α1-F: 5′-CAGCCCTCCCAAGATGAACTTA-3′
α1-R: 5′-CGCATACCCTCTCTTGGTGAAA-3′

To verify that equal amounts of RNA from each sample were used for cDNA synthesis a 940 bp fragment of glyceraldehyde-3-phosphate dehydrogenase was amplified as a positive control from all samples using GAPDH-specific primers:

GAPDH-f: 5′-ACAAAATGGTGAAGGTCGGTGT-3′
GAPDH-r: 5′-TACCAGGAAATGAGCTTGACAAAG-3′

PCR amplifications were performed in a total volume of 50 μL for 19 cycles (GAPDH), 23 cycles (α6 and δ), and 30 cycles (α1), respectively, using the QIAGEN Taq DNA polymerase (QIAGEN GmbH, Germany). All cycle numbers used for RT-PCR have been optimized to lie within the linear range. Following agarose gel electrophoresis, the density of the bands was quantified using a gel documentation system (Doc-Print, Vilber Lourmat, Marne-La-Vallee, France; software: Quantity One, Bio-Rad Laboratories, Hercules, CA, USA). The relative amounts of α6 subunit RNA were calculated using δ subunit RNA that was detected within the same agarose gel as an internal control. The amounts of individual GABA$_A$ receptor subunit or GAPDH mRNAs present in cerebella of three *α1* +/+ and three *α1* -/- mice were compared in the same gel. Data were expressed as mean ± standard error. Student's unpaired *t*-test was used for comparing data from *α1* +/+ and *α1* -/- mice, and significance was set at $p < 0.05$.

Results

GABA$_A$ receptor subunit expression in cerebellar membranes of *α1* -/- and litter mate control mice as determined by western blot analysis

Membranes from the cerebellum of *α1* -/- mice and litter mate controls (*α1* +/+) were subjected to SDS–PAGE and quantitative western blot analysis, using subunit-specific antibodies directed against α1, α2, α3, α4, α5, α6, β1, β2, β3, γ1, γ2, γ3, and δ subunits (Jechlinger *et al*. 1998; Poltl *et al*. 2003). The immunoreactive band for the α1 subunit (51 kDa) completely disappeared in *α1* -/- cerebella (Fig. 1). As shown in Table 1, the expression of α3, α4 and α6 subunits was increased by 64%, 102% and 54% in the cerebellum of *α1* -/- mice, whereas no significant difference was observed in the expression of α2 and α5 subunits between *α1* +/+ and *α1* -/- mice, respectively. The level of β2 subunit expression in *α1* -/- was decreased by 24%, but the expression of β1 and β3 subunits was not significantly different from that found in *α1* +/+ mice. The level of γ2 subunits was decreased by 50% compared to controls, whereas no significant difference was observed in the expression of γ1, γ3 or δ subunits, respectively.

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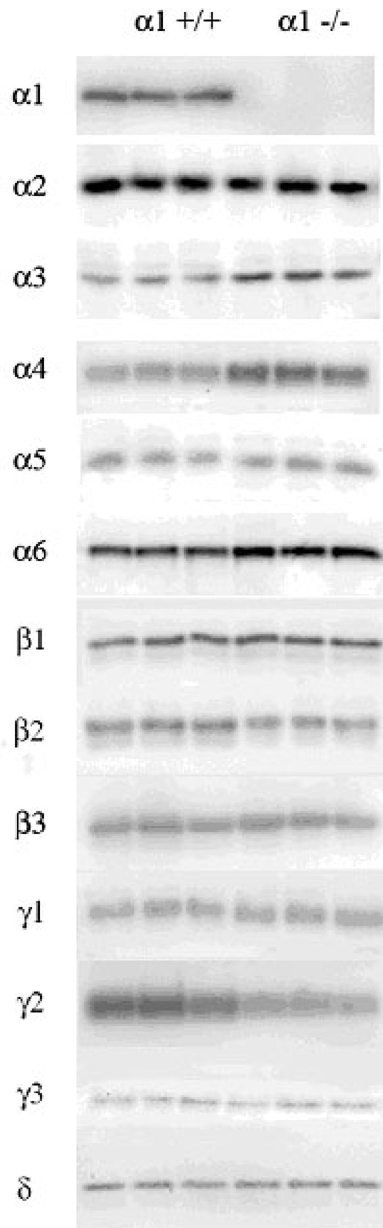

Fig. 1 Western blot analysis indicating changes in the abundance of the $GABA_A$ receptor subunits in *α1* +/+ and *α1* -/- cerebella. Immunoblot analysis of 13 $GABA_A$ receptor subunits present in cerebellar homogenates of three litter mate control (+/+) and three *α1* -/- mice. The immunoreactive band for the α1 subunit completely disappeared in *α1* -/- cerebella. There was a large increase in the intensity of the immunoreactive bands for the α3, α4 and α6 subunits, and a decrease in the intensity of the bands for β2 and γ2 subunist in *α1* -/- cerebella. No significant changes in the intensity of the bands for the α2, α5, β1, β3, γ1, γ3 or δ subunits were observed in *α1* -/- cerebella. Results of the quantification of these immunoblots are included in Table 1.

[^{3}H]Ro15–4513 binding in the cerebellum of control and *α1* -/- mice

In order to investigate the number of γ subunit containing $GABA_A$ receptors present in control and α1 -/- cerebella, receptor binding studies were performed with [^{3}H]Ro15–4513. [^{3}H]Ro15–4513 is a ligand of the benzodiazepine binding site of $GABA_A$ receptors that is formed by receptors containing a γ subunit (Sieghart 1995). Scatchard analysis of specific [^{3}H]Ro15–4513 binding in cerebellar membranes indicated that the total number of binding sites (B_{max}) in *α1* -/- mice was significantly decreased to 36.3% of that in control mice, whereas the Kd was not significantly different in these tissues (Table 2). In agreement with previous reports (Sieghart *et al.* 1987; Turner *et al.* 1991; Korpi *et al.* 1993), 44% of total binding in cerebellar membranes of *α1* +/+ mice could not be displaced by 1 μM diazepam, but could be displaced by 100 μM Ro15–1788, a benzodiazepine site antagonist. This diazepam-insensitive (DIS) binding, which is attributed to receptors containing α6 or α4 subunits (Luddens *et al.* 1990) was not significantly different in *α1* +/+ and *α1* -/- mice, but since the total number of [^{3}H]Ro15–4513 binding sites is reduced to 36.3% in *α1* -/- mice, DIS binding now contributes 84% of all [^{3}H]Ro15–4513 binding sites present in *α1* -/- mice. The diazepam-sensitive (DS) [^{3}H]Ro15–4513 binding, however, was significantly reduced in *α1* -/- mice and contributed to only 16% of total [^{3}H]Ro15–4513 binding sites in these mice (Table 2).

Table 1 Quantification of $GABA_A$ receptor subunit proteins by western blot analysis in cerebellar homogenates

	α1 +/+		α1 -/-		
Subunit	Percentage of $GABA_A$ receptors (mean ± SEM)	n	Percentage of $GABA_A$ receptors (mean ± SEM) as compared to *α1* +/+	n	Significance (p-value)
α_1	100.0 ± 2.8		nd		
α_2	100.0 ± 10.3	3	108.6 ± 12.2	3	ns
α_3	100.0 ± 2.9	3	163.7 ± 7.1	3	< 0.05
α_4	100.0 ± 6.6	3	202.3 ± 17.6	3	< 0.05
α_5	100.0 ± 6.8	3	108.4 ± 4.0	3	ns
α_6	100.0 ± 5.4	3	153.9 ± 16.8	3	< 0.05
β_1	100.0 ± 2.7	3	104.5 ± 3.7	3	ns
β_2	100.0 ± 2.9	3	76.3 ± 1.3	3	< 0.05
β_3	100.0 ± 4.0	3	81.1 ± 5.7	3	ns
γ_1	100.0 ± 11.2	3	92.8 ± 11.9	3	ns
γ_2	100.0 ± 4.0	3	49.7 ± 3.3	3	< 0.05
γ_3	100.0 ± 2.8	3	97.8 ± 12	3	ns
δ	100.0 ± 1.5	3	99.8 ± 2.0	3	ns

Equal amounts of protein containing total cerebellar homogenates were separated by SDS–PAGE and subjected to Western blot analysis. Results were obtained from three α1 +/+ and three α1 -/- mice that were investigated a total of six times each, and are expressed as percentages of the weighted average subunit level found in control mice ± SEM. For statistical comparison unpaired Student's *t*-test was used. (n = number of individual animals tested, nd = not detectable, ns = not significant).

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Table 2 Scatchard analysis of [^{3}H]Ro15-4513 binding in cerebellar membranes of α1 +/+ and α1 -/- mice

Parameter	α1 +/+ [^{3}H]Ro15-4513 binding sites (mean ± SEM)	n	α1 -/- [^{3}H]Ro15-4513 binding sites (mean ± SEM)	n	Significance (p-value)
Kd (nM)	7.3 ± 0.9	4	7.0 ± 1.3	3	ns
Bmax (fmol/mg)	4310 ± 807	4	1565 ± 138	3	< 0.05
DS (fmol/mg)	2397 ± 433	4	251 ± 24	3	< 0.05
DIS (fmol/mg)	1913 ± 509	4	1314 ± 114	3	ns

Cerebellar membranes from α1 +/+ mice and α1 -/- mice were incubated with various concentrations of [^{3}H]Ro15-4513 in the absence or presence of 1 μM diazepam or 100 μM Ro15-1788. Scatchard analysis was performed from specific [^{3}H]Ro15-4513 binding (that could be displaced by 100 μM Ro15-1788). Specific binding minus diazepam sensitive (DS) binding resulted in diazepam insensitive (DIS) binding. Data are mean values ± SEM from four (α1 +/+) or three (α1 -/-) experiments, each performed in triplicates. For statistical analysis unpaired Student's *t*-test was used. (n = number of individual animals tested, ns = not significant).

Extraction of GABA$_A$ receptors and determination of extraction efficiency

For all further studies, GABA$_A$ receptors had to be extracted from cerebellar membranes. For that, membranes were homogenized in a Tris/citrate buffer pH 7.1 (see Materials and methods) and an aliquot was taken for subsequent radioligand binding studies. The remaining homogenate was then extracted in a deoxycholate buffer using the same volume as before for homogenization. The suspension was ultracentrifuged at 150 000 *g* and receptors present in an aliquot of the clear extract and resuspended pellet were investigated by radioligand binding studies.

Scatchard analysis of specific [^{3}H]Ro15–4513 binding to membranes or extracted receptors of *α1* -/- mice resulted in a total number of binding sites (B_{max}) of 1565 or 884 fmol/mg protein, and a DIS binding of 1314 or 712 fmol/mg protein, respectively. The percentage of recovery in the extracts of *α1* -/- cerebella were thus similar for total binding and DIS binding (56% and 54%), supporting the conclusion that the extracted receptors were representative for the entire γ subunit-containing GABA$_A$ receptors present. The Kd of [^{3}H]Ro15–4513 binding (7.0 ± 1.3 nM or 12.7 ± 3.4 nM; means ± SEM, $n = 3$), however, was not significantly different between membranes or extracts of *α1* -/- cerebella, respectively. Recovery rates in *α1* -/- cerebellum were closely similar to those obtained by our group for the cerebellum of wild-type mice (Tretter *et al*. 2001; Poltl *et al*. 2003), indicating that there was no significant difference in the efficiency of solubilization of [^{3}H]Ro15–4513 binding sites in both tissues.

The total number of GABA$_A$ receptors usually is determined by [^{3}H]muscimol binding studies because it is assumed that most, if not all GABA$_A$ receptors in the brain contain [^{3}H]muscimol binding sites. As [^{3}H]muscimol binding to brain membranes is highly variable due to the presence of several binding sites with different affinities on the same receptor and the presence of variable amounts of endogenous GABA, [^{3}H]muscimol binding was performed in cerebellar extracts only. In the absence of membrane binding data, extraction efficiency was therefore determined by measuring the distribution of [^{3}H]muscimol binding sites between the clear supernatant (extract) and the re-dissolved pellet of the extraction step. Results indicated that 78% and 82% of all [^{3}H]muscimol binding sites present in the supernatant and re-dissolved pellet could be recovered in the clear supernatant of *α1* +/+ and *α1* -/- cerebella, respectively, and indicated that a comparable and large part of [^{3}H]muscimol binding sites was extracted from these tissues. These data are in agreement with results generated previously by our group (Jechlinger *et al*. 1998; Tretter *et al*. 2001; Poltl *et al*. 2003). The difference in the extraction efficiency of [^{3}H]Ro15–4513 binding sites (56%) and [^{3}H]muscimol binding sites (82%) in *α1* -/- cerebellum was due to differences in the experimental procedures and reference points used. Whereas in [^{3}H]Ro15–4513 binding experiments receptors still active after the extraction by detergent were referred to receptors in membranes that never had been in contact with detergent, in [^{3}H]muscimol binding experiments the detergent-extracted receptors were referred to the sum of receptors in the clear extract and the re-dissolved extraction pellet. Since receptors are not only extracted but also partially inactivated by the addition of detergent (in the present experiments by approximately 27%), in the latter case the reference point for calculation of the extraction efficiency was smaller, resulting in an apparently larger recovery rate.

To compare the extent of solubilization of individual GABA$_A$ receptor subunits in cerebellar membrane extracts, quantitative Western blot experiments were performed. Results from two separate experiments demonstrated that 69% and 73%, 67%, 70% and 60%, or 69% and 69% of α6, β3, γ2, or δ subunits could be recovered in the extract of *α1* +/+, and 78% and 75%, 71% and 63%, 72% and 50%, or 83% and 65% of α6, β3, γ2, or δ subunits in the extract of

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α1 -/- mice, respectively. As most $GABA_A$ receptors in the cerebellum of *α1* +/+ and *α1* -/- mice contain α6, β3, γ2 or δ subunits, these data support the conclusion that receptors containing these subunits were solubilised to a comparable extent.

[^{3}H]muscimol binding studies in cerebellar extracts show overall reduction of $GABA_A$ receptors in *α1* -/- mice

To estimate a possible change in the total number of $GABA_A$ receptors, receptors were immunoprecipitated from extracts of *α1* +/+ and *α1* -/- cerebella using a mixture of antibodies directed against α1-, β1-, β2- and β3 subunits. Subsequent [^{3}H]muscimol binding assays and Scatchard analysis of two independent experiments resulted in B_{max} values of 2415 and 2829 fmol/mg protein or 1121 and 1687 fmol/mg protein for *α1* +/+ and *α1* -/- cerebella, respectively. Due to the large amounts of antibodies necessary for these experiments, only two independent experiments were performed in triplicates. These data indicated that the average B_{max} value of [^{3}H]muscimol binding sites in *α1* -/- cerebellar extracts was only about 54% of that found in *α1* +/+ extracts. Kd values were comparable in *α1* +/+ and *α1* -/- cerebella (Table 3).

Quantitative importance of $GABA_A$ receptors containing α6, γ1, γ2, γ3 or δ subunits in cerebella of *α1* -/- mice

To determine the percentage of $GABA_A$ receptors formed by distinct subunits, quantitative immunoprecipitation of $GABA_A$ receptors was performed in cerebellar extracts of *α1* -/- mice using subunit-specific antibodies and subsequent [^{3}H]muscimol binding studies. Total $GABA_A$ receptors present in the extract of *α1* -/- cerebella were determined by precipitation with a mixture of antibodies directed against α1-, β1-, β2- and β3 subunits that could precipitate most if not all receptors. Since precipitation with that antibody mixture yielded a smaller total number of $GABA_A$ receptors present in the extract as in [^{3}H]muscimol binding studies, precipitation data were corrected by multiplying with the calculated factor 1.5, making a comparison of the two sets of experiments possible. Precipitation with the subunit-specific antibodies α6, γ1, γ2, γ3 and δ, subsequent [^{3}H]muscimol binding studies and correction of the results by the calculated factor 1.5 then yielded the amount of receptors containing the respective subunits.

Results from several independent experiments revealed that 1291 ± 59 fmol/mg protein of all [^{3}H]muscimol binding sites present in *α1* -/- cerebella contained α6 subunits, 85 ± 16 fmol/mg protein contained γ1 subunits, 512 ± 45 fmol/mg protein contained γ2 subunits, and 56 ± 24 fmol/mg protein contained γ3 subunits. Furthermore, 651 ± 46 fmol/mg protein of all [^{3}H]muscimol binding sites in *α1* -/- cerebella contained δ subunits. Similar experiments were performed in control mice, and the data are shown in Table 4. A comparison of data for *α1* +/+ and *α1* -/- cerebella indicated a total reduction of $GABA_A$ receptors by 46.5%. γ2 subunit-containing receptors were reduced by 71.4% in *α1* -/- mice, whereas α6- or δ subunit-containing receptors were not significantly reduced when compared to *α1* +/+ mice.

The extremely low numbers of γ1- and γ3 subunit-containing receptors in wild-type cerebellum (60 ± 21 and 8 ± 22 fmol/mg protein (Poltl *et al.* 2003) were at the border of detectability by immunoprecipitation and subsequent receptor binding studies and resulted in highly variable data. The fact that there was no significant change in the expression of γ1- or γ3 receptor subunit proteins in *α1* +/+ and *α1* -/- cerebella in western blot studies (Table 1) supports the conclusion that receptors containing these subunits were not significantly different in these tissues.

Table 3 Scatchard analysis of [^{3}H]muscimol binding data from cerebellar extracts of α1 +/+ and α1 -/- mice

	Specific [^{3}H]muscimol binding (mean ± SEM)			
	α1 +/+		α1 -/-	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Kd (nM)	2.4 ± 0.3	2.0 ± 0.5	2.8 ± 0.7	1.1 ± 0.3
B_{max} (fmol/mg)	2415 ± 64	2829 ± 152	1121 ± 55	1687 ± 119

$GABA_A$ receptors extracted from cerebellar membranes of α1 +/+ and α1 -/- mice were precipitated as described and incubated with various concentrations of [^{3}H]muscimol in the absence or presence of 1mM GABA. Scatchard analysis was performed from [^{3}H]muscimol binding data that could be displaced by 1mM GABA (total specific binding). Data are mean values ± SEM from two independent experiments performed in triplicates. For statistical analysis unpaired Student's *t*-test was used.

Identification and quantification of $GABA_A$ receptor subtypes containing no γ or δ subunits

The sum of γ1-, γ2-, γ3- and δ subunit-containing receptors in *α1* -/- cerebella added up to only 93% of total receptors (Table 4). As in previous investigations (Jechlinger *et al.* 1998; Bencsits *et al.* 1999; Poltl *et al.* 2003) the sum of δ and γ amounts reproducibly added up close to 100%, the possible existence of receptors containing only α- and β subunits was investigated. For that, cerebellar membrane extracts were chromatographed consecutively on γ1-, γ2-, γ3- and δ immunoaffinity columns under conditions were receptors containing these subunits could be removed as efficiently as possible. Results indicated that after affinity chromatography of extracts containing 1404 ± 70 fmol/mg protein (mean ± SEM, $n = 8$), $GABA_A$ receptors amounting to 441 ± 27 fmol/mg protein could still be found in the column efflux, and all of those (477 ± 37 fmol/mg protein) seemed to contain α6 subunits. 141 ± 25 fmol/mg protein or 78 ± 29 fmol/mg protein of receptors found in the column efflux still contained γ2- or δ subunits (Table 5), indicating

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Table 4 Quantification of GABA$_A$ receptor subtypes by immunoprecipitation with subunit specific antibodies and subsequent [^{3}H]muscimol binding

	α1 +/+		α1 -/-			
Antibodies for precipitation	fmol/mg (mean ± SEM)	n	fmol/mg (mean ± SEM)	n	Reduction (%)	Significance (p-value)
$\alpha_1+\beta_1+\beta_2+\beta_3$	2622 ± 108	6	1404 ± 70	8	46.5	< 0.05
α_1	1946 ± 29	6	–	–	100	< 0.05
α_6	1460 ± 132	6	1291 ± 59	6	11.6	ns
γ_1	60 ± 21	6	85 ± 16	4	nd	ns
γ_2	1791 ± 145	6	512 ± 45	6	71.4	< 0.05
γ_3	8 ± 22	6	56 ± 24	4	nd	ns
δ	755 ± 110	6	651 ± 46	6	13.8	ns

GABA$_A$ receptors were extracted from cerebellar membranes of α1 +/+ and α1 -/- mice and precipitated by subunit-specific antibodies or by a mixture of antibodies directed against α1-, β1-, β2- and β3 subunits. Receptor subtypes containing the respective subunit were quantified using [^{3}H]muscimol binding. In the second and fourth columns, the amount of receptors containing a given subunit was expressed in absolute numbers (fmol/mg protein) for α1 +/+ and α1 -/- mice, respectively. Data were calculated after normalization for precipitation efficacy. The percentage of reduction of receptors in α1 -/- cerebella (sixth column) was calculated from a direct comparison of immunoprecipitation data from α1 +/+ and α1 -/- cerebella (second and fourth columns). A comparison of these columns furthermore indicated that the difference between the two sets of animals was significant (p-value < 0.05) only for α1- and γ2 subunit containing receptors. Data are expressed as mean values ± SEM. For statistical comparison, unpaired Student's *t*-test was used. (n= number of individual experiments, nd = not determined; ns = not significant).

that the anti-γ2 and anti-δ column could not completely retain all γ2- or δ subunit-containing receptors. In contrast, γ1- and γ3 subunit-containing receptors had been successfully removed by the anti-γ1 and anti-γ3 columns. Subtracting the 141 ± 25 fmol/mg protein γ2 and the 78 ± 29 fmol/mg protein δ subunit-containing receptors from the 441 ± 27 fmol/mg protein found in the column efflux, a number of 222 ± 28 fmol/mg protein seemed to contain neither γ1-, γ2-, γ3- nor δ subunits (Table 5). These receptors represented approximately 16% of total receptors present in the extract of *α1* -/- cerebellum.

Quantification of α6 subunit mRNA levels in *α1* -/- and litter mate control cerebella

In order to investigate whether the elevated level of α6 subunit protein in the cerebellum of *α1* -/- mice was reflected by an increased mRNA concentration, RT-PCR was performed. To verify that the RNA was correctly derived from *α1* +/+ or *α1* -/- mice and that equal amounts of target RNA were used for RT-PCR, PCR reactions were performed in parallel in all samples using α1-specific and GAPDH-specific primers, respectively. As shown in Fig. 2, the α1-specific 945 bp fragment was only amplified in target RNA from *α1* +/+ mice and there was no significant difference in the intensity of the GAPDH band in *α1* +/+ and *α1* -/- mice.

To compare the amounts of α6 mRNA in *α1* +/+ and *α1* -/- cerebella, δ subunit mRNA levels were simultaneously determined in the same reaction as internal control since neither the amount of δ subunit protein nor the number of δ subunit-containing receptors was significantly different in *α1* +/+ and *α1* -/- mice. As shown in Fig. 2, α6 or δ subunit mRNA levels were comparable in *α1* +/+ and *α1* -/- cerebella. Normalization to δ subunit mRNA indicated that α6 subunit mRNA levels in *α1* -/- mice were 96 ± 5% (mean ± SEM, $n = 3$) of those in *α1* +/+ mice and were therefore not significantly different ($p > 0.05$). Results did not change when different numbers of cycles were used for amplifying mRNA, indicating that the reaction was in its linear range. Similar results were obtained when the relative amounts of α6 subunit mRNA were referred to that of GAPDH.

Discussion

GABA$_A$ receptors in *α1* -/- cerebellum are significantly reduced

GABA$_A$ receptor composition has been extensively investigated in the cerebellum of wild-type and mutant mouse strains. In the present study the abundance, subunit expression, and subunit composition of GABA$_A$ receptors were investigated in the cerebellum of mice with a targeted disruption of the α1 subunit gene. Scatchard analysis of [^{3}H]muscimol binding data indicated that the number of GABA$_A$ receptors was reduced by 46% in membrane extracts of *α1* -/- cerebellum as compared to wild-type cerebellum. These data are in good agreement with other studies (Sur *et al.* 2001; Kralic *et al.* 2002) that indicated a loss of 50–60% of all GABA$_A$ receptors in the forebrain of

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Table 5 Abundance of $GABA_A$ receptor subtypes devoid of γ1-, γ2-, γ3- or δ subunits in cerebellar extracts of α1 -/- mice

Antibodies for precipitation	α1 -/- fmol/mg receptors found after affinity-chromatography (mean ± SEM)	α1 -/- % of total receptors found after affinity-chromatography (mean ± SEM)	(n)
α1+β1+β2+β3	441 ± 27	31.4 ± 1.9	7
α6	477 ± 37	31.8 ± 2.5	5
γ1	nd	nd	
γ2	141 ± 25	10 ± 1.8	5
γ3	nd	nd	
δ	78 ± 29	5.6 ± 2.1	5
Receptors devoid of γ or δ subunits	222 ± 28	15.8 ± 2.0	5

$GABA_A$ receptors were extracted from cerebellar membranes of α1 -/- mice, and extracts were chromatographed on consecutive immuno-affinity columns containing antibodies directed against γ1-, γ2-, γ3- and δ subunits. To determine the percentage of the total amount of receptors or of receptors containing a specific subunit in the efflux of the columns, immunoprecipitation with an antibody mixture or with subunit-specific antibodies and subsequent [^{3}H]muscimol binding were performed in the original extract and in column efflux in parallel. After subtracting all γ2- and δ subunit-containing receptors that remained in the column efflux of α1 -/- cerebella from the total amount of receptors present in the column efflux, a significant amount ($p <$ 0.05) of receptors could be identified that contained neither γ- nor δ subunits. All experiments were performed in quadruplicates. Data are mean values ± SEM and were calculated after normalization for precipitation efficiency, using Prism (Graph Pad Software Inc., San Diego, CA, USA). For statistical analysis, student's *t*-test was used. (n = number of individual experiments, nd = not detectable).

α1 -/- mice. Surprisingly, mice lacking the α1 subunit do not exhibit major phenotypic abnormalities or spontaneous seizures except some signs of intention tremor (Kralic *et al.* 2005), and are viable and fertile (Sur *et al.* 2001).

Receptors containing γ2 subunits are strongly reduced in *α1* -/- cerebellum

[^{3}H]Ro15–4513 binding to cerebellar membranes which is representative for γ subunit-containing receptors (Sieghart 1995), was decreased by 64% in *α1* -/- mice. Furthermore, immunoprecipitation of receptors from cerebellar extracts using γ2-specific antibodies and subsequent [^{3}H]muscimol binding studies indicated that the number of γ2 subunit-containing receptors was reduced by 71% in *α1* -/- mice. A loss of 64–71% of γ2-receptors corresponds to a loss of 44–48% of total receptors, considering that 68% of all receptors in the cerebellum of wild-type mice contained γ2 subunits (Poltl *et al.* 2003). This loss in γ2-receptors is identical with the loss of [^{3}H]muscimol binding sites in *α1* -/- cerebellum

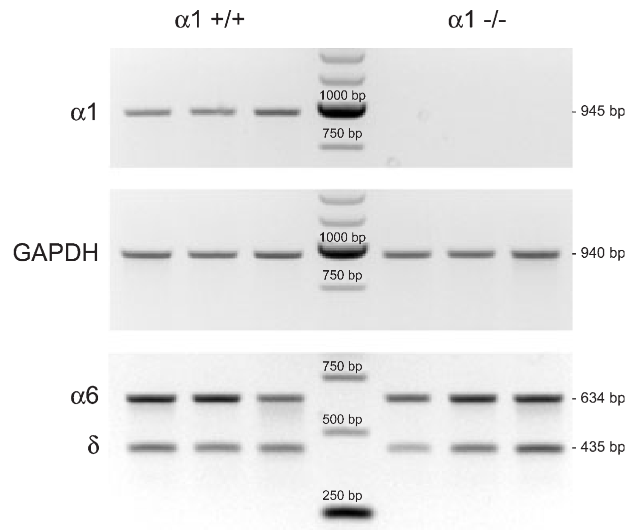

Fig. 2 RT-PCR analysis indicating no change in the amounts of α6 and δ subunit mRNA in *α1* -/- cerebella. mRNA levels of α6 and δ subunits were amplified simultaneously by RT-PCR in six parallel reactions using individual total RNA from the cerebella of three litter mate control (+/+) and three knockout (–/–) mice. For estimation of the correct length of the PCR fragments, a 250 bp DNA Ladder (Invitrogen GmbH, Lofer, Austria) was used in a separate lane. To verify that the target RNA was correctly derived from *α1* +/+ and *α1* -/- mice, or that equal amounts of the target RNA were used for RT-PCR, PCR reactions were additionally performed in all samples using α1- or GAPDH-specific primers, respectively. Results indicated that there was no significant difference in the amounts of α6 and δ subunit mRNA in *α1* +/+ and *α1* -/- cerebella.

determined in this study, suggesting that only γ2 subunit-containing receptors were lost in these mice. As 41% of all receptors in wild-type cerebellum are composed of α1βγ2 subunits (Poltl *et al.* 2003), it is reasonable to assume that most of the receptors lost in *α1* -/- mice had this subunit composition.

Although 64–71% of γ2 subunit-containing receptors were lost in *α1* -/- cerebellum, western blot analysis indicated that γ2 subunit expression was reduced by only 50%. This discrepancy might have been due to the fact that western blot analysis not only detects functional receptors, but also newly synthesized subunits, assembly intermediates or inactivated receptors. Thus, it is not surprising that the reduction in the number of functional receptors could be more prominent than that of the subunit protein.

Abundance of receptors containing α6 and δ subunits is not changed in *α1* -/- cerebellum

The number of α6 subunit-containing receptors was not significantly changed in the cerebellum of *α1* -/- mice as determined by immunoprecipitation with α6-specific antibodies from membrane extracts and subsequent [^{3}H]muscimol binding studies. These results can only be explained if it

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is assumed that α1α6βγ2- or α1α6βδ receptors that are present in wild-type but not in *α1* -/- cerebellum were largely converted to α6βγ2 or α6βδ receptors in *α1* -/- mice. Part of this conclusion is supported by studies investigating diazepam-insensitive [^{3}H]Ro15–4513 binding sites that are formed by receptors containing α6βγ2- or α1α6βγ2 subunits (Luddens *et al.* 1990). The number of these sites was not significantly altered in *α1* -/- cerebellum, suggesting a conversion of α1α6βγ2- into α6βγ2 receptors. Interestingly (Sur *et al.* 2001) found a significant reduction in the amount of α6 subunit-containing receptors in *α1* -/- cerebellum as determined by radioligand binding studies. However, the *α1* -/- mouse strain (Sur *et al.* 2001) used for their studies was generated by a different targeting strategy than the knockout mouse strain we used for the present data (Vicini *et al.* 2001). As both lines already differ in the expression pattern of α2 and α3 subunits (Sur *et al.* 2001; Kralic *et al.* 2002), it is reasonable to assume that differences in α6 subunit expression might be due to distinct gene-targeting methods or breeding strategies used.

If there was no change in the total number of α6- as well as in α6- and γ2 subunit-containing receptors, it was to be expected that there was also no change in α6- and δ subunit-containing receptors. This actually was confirmed by immunoprecipitation of solubilized receptors with δ-specific antibodies and subsequent [^{3}H]muscimol binding studies, and supported by western blot analysis that indicated that there was also no significant change in the expression of δ subunits in this tissue. This actually was expected from the finding that there were no significant amounts of α1βδ receptors in wild-type that could have been lost in *α1* -/- mice (Poltl *et al.* 2003). Despite the finding of an unchanged number of α6- and γ2- or α6- and δ-subunit-containing receptors, there was a 54% increase in the expression of α6 subunits in western blot analysis, supporting the conclusion that α1 subunits in α1α6βγ2- and α1α6βδ receptors had been replaced by α6 subunits in *α1* -/- mice.

GABA$_A$ receptors devoid of γ or δ subunits are formed in the cerebellum of *α1* -/- mice

In wild-type cerebellum the total number of γ- and δ subunit-containing receptors adds up to 100% (Poltl *et al.* 2003), supporting the conclusion that all GABA$_A$ receptors in the cerebellum contain either a γ- or a δ subunit, and that the γ- and δ subunits are not present together in the same receptor (Araujo *et al.* 1998; Jechlinger *et al.* 1998; Bencsits *et al.* 1999; Poltl *et al.* 2003). In *α1* -/- cerebellum, however, the γ2- (29–36%) and the δ-receptors (48%) sum up to only 77–84% of all receptors. As the number of γ1- or γ3-receptors present in the cerebellum of these mice was not significantly changed, this indicates that a significant part of all receptors in the cerebellum of *α1* -/- mice might contain neither γ- nor δ subunits.

This possibility was investigated by removing γ1-, γ2-, γ3- and δ subunit-containing receptors by immunoaffinity chromatography. Results indicated that a total of 15.8 ± 2.0% (mean ± SEM, $n = 5$) of all receptors present in the original extract did not contain γ- or δ subunits and could be found in the efflux of these immunoaffinity columns. These receptors seemed to be composed of α- and β- subunits, but the possibility that they might have additionally contained yet unidentified subunits cannot be excluded. Most, if not all of the receptors in the efflux contained α6 subunits, but the exact composition of these receptors could not be determined due to their low abundance.

Cell type-specific changes in GABA$_A$ receptor composition in *α1* -/- cerebellum

In cerebellar Purkinje cells, only α1, β2, β3 and γ2 subunits have been identified (Laurie *et al.* 1992). In the absence of any other α subunit it is reasonable to assume that the loss of α1 subunits in *α1* -/- mice prevented the formation of α1βγ2 receptors. Although the formation of minor amounts of receptors composed of the remaining subunits (homooligomeric β3 receptors, β2β3 and βγ2 receptors) cannot be excluded, such receptors are formed to a minor extent (Taylor *et al.* 2000). Consistent with the observation that GABA currents are almost absent in Purkinje cells of *α1* -/- mice (Sur *et al.* 2001), it can thus be assumed that Purkinje cells lost most, if not all of their GABA$_A$ receptors. This assumption is supported by (Kralic *et al.* 2005) who additionally demonstrated that in spite of the loss of all responses to synaptic or exogenous GABA in *α1* -/- Purkinje cells, these cells seem to exhibit no differences in abundance, gross morphology or spontaneous synaptic activity, and also no GABAergic compensation. However, loss of GABAergic inhibition by Purkinje cells partly seems to induce the permanent postural and kinetic tremor that could be observed in *α1* -/- mice and might therefore impair communication among different regions of the motor pathway (Kralic *et al.* 2005).

In contrast, cerebellar granule cells contain α1, α6, β1, β2, β3, γ2 and δ subunits (Laurie *et al.* 1992; Persohn *et al.* 1992; Pirker *et al.* 2000). In addition, small amounts of α4 and α5 subunits seem also to be present in these cells (Laurie *et al.* 1992; Persohn *et al.* 1992). In the cerebellum of *α1* -/- mice α significantly increased amount of α6 subunits was detected by western blot analysis that partially compensated for the loss of α1 subunits. RT-PCR performed in *α1* +/+ and *α1* -/- cerebella indicated no significant difference in the amounts of α6 subunit mRNA between the two tissues, suggesting that the elevated level of α6 subunit protein in *α1* -/- mice was not caused by an increased biosynthesis of this subunit. Recently, it was demonstrated that not only misfolded but also unassembled subunits are continuously degraded by an endoplasmatic reticulum-associated degradation system (Christianson and Green 2004). The authors

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estimated that 70% of all α subunits of the nACh receptor synthesized are degraded by this system and that not only the lifetime of the unassembled subunits, but also the number of nACh receptors on the cell surface could be increased by proteasome inhibitors, suggesting that the additional nACh receptors were assembled from subunits that would have normally be degraded (Christianson and Green 2004). It is therefore possible that the increased α6 subunit level was caused by an increased incorporation of unassembled α6 subunits into receptors due to the absence of competition with α1 subunits, thus rescuing them from being degraded. Receptors containing α1 as well as α6 subunits in wild-type cerebellum are the prime candidates for such an α1 subunit replacement, because in order to assemble these receptors both subunits have to be expressed at the same time in the same subcellular compartment (Connolly *et al*. 1996). This conclusion is supported by the fact that 56% of all receptors in cerebellum contain α6 subunits and about 59% of α6 subunit containing receptors contain α1 and α6 subunits (Poltl *et al*. 2003). Replacement of α1 by α6 subunits thus requires a second α6 subunit for 59% of all α6 subunit-containing receptors. This is in excellent agreement with the finding that α6 subunits are increased by about 54% in *α1* -/- mice.

In addition, an elevated level of α4 subunits was detected in *α1* -/- cerebellum, and it is tempting to speculate that α4 and α1 subunits also were coexpressed in granule cells of wild-type mice, and by this allowed an easy replacement of α1 by α4 subunits. The formation of receptors containing α1 as well as α4 subunits has been demonstrated previously in rat cerebral cortex (Bencsits *et al*. 1999). As α4 subunit-containing receptors represent only 1.8 ± 0.8% of all receptors in cerebellum (Poltl *et al*. 2003) these receptors did not significantly contribute to total receptors in this tissue and were not further studied.

In contrast, no change in the expression level of α5 subunits was observed in *α1* -/- mice. It therefore can be assumed that α1 and α5 subunits either do not occur within the same receptor, that biosynthesis of receptors containing these subunits is located in different subcellular compartments, or that they form a minor part of the receptor population in granule cells.

Cerebellar basket and stellate cells seem to contain only α1, α3, β2 and γ2 subunits in wild-type mice (Laurie *et al*. 1992; Persohn *et al*. 1992). Upon loss of α1 subunits in *α1* -/- mice, a significantly elevated level of α3 subunits was observed, possibly again suggesting that α1 and α3 subunits are coexpressed in the same subcellular compartment in these cells, and that α3 subunits could partially replace the lost α1 subunits during formation of $GABA_A$ receptors. Similar changes in the level of α3 subunit-containing receptors in *α1* -/- cerebellum have already been demonstrated by other studies (Sur *et al*. 2001). Elevated levels of α3 as well as α5 subunits might again be due to a decreased protein degradation rather than to an increased protein biosynthesis as already shown for α6 subunits.

The α2 subunit is the most abundant minor subunit in the cerebellum and together with β1 and γ1 subunits is predominantly located in Bergmann glia cells (Laurie *et al*. 1992; Persohn *et al*. 1992; Poltl *et al*. 2003). As no α1 subunits seem to be expressed in these cells, it is not surprising that there was no significant change in the expression of α2 subunits in *α1* -/- cerebellum. However, α2 subunits can also be found in the granule and molecular cell layer of the cerebellum (Pirker *et al*. 2000), but the absence of a significant change in the expression of this subunit seems to suggest that α2 subunits are not significantly coexpressed with α1 subunits in the same cells.

In summary, the present results can be explained by the assumption that upon targeted disruption of the $GABA_A$ receptor α1 subunit there is no increased biosynthesis of α6 subunits in the brain but rather a decreased protein degradation of surplus α6 subunits. Although elevated levels of α3-, α4- or α6 subunits can be observed in the cerebellum of *α1* -/- mice, this might be explained by the presence of these subunits in the compartment in which α1 subunit-containing receptors are assembled. Replacement of α1 subunits by α3-, α4- or α6 subunits might then rescue part of these subunits that would otherwise have been degraded due to a lack of assembly partners. α1 subunit-containing receptors will only be lost completely if no other α subunit is expressed in the respective cell that can compensate for the loss of α1 subunits. Whether ion channels or other receptors also compensate for the loss of α1 subunits as demonstrated for α6 -/- mice (Nusser *et al*. 1999) will have to be investigated in the future.

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