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Targeted Disruption of the $GABA_A$ Receptor δ Subunit Gene Leads to an Up-regulation of γ_2 Subunit-containing Receptors in Cerebellar Granule Cells*

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$GABA_A$ receptors are chloride channels composed of five subunits. Cerebellar granule cells express abundantly six subunits belonging to four subunit classes. These are assembled into a number of distinct receptors, but the regulation of their relative proportions is yet unknown. Here, we studied the composition of cerebellar $GABA_A$ receptors after targeted disruption of the δ subunit gene. In membranes and extracts of δ−/− cerebellum, [^{3}H]muscimol binding was not significantly changed, whereas [^{3}H]Ro15-4513 binding was increased by 52% due to an increase in diazepam-insensitive binding. Immunocytochemical and Western blot analysis revealed no change in α_6 subunits but an increased expression of γ_2 subunits in δ−/− cerebellum. Immunoaffinity chromatography of cerebellar extracts indicated there was an increased coassembly of α_6 and γ_2 subunits and that 24% of all receptors in δ−/− cerebellum did not contain a γ subunit. Because 97% of δ subunits are coassembled with α_6 subunits in the cerebellum of wild-type mice, these results indicated that, in δ−/− mice, $\alpha_6\beta\gamma_2$ and $\alpha\beta$ receptors replaced δ subunit-containing receptors. The availability of the δ subunit, thus, influences the level of expression or the extent of assembly of the γ_2 subunit, although these two subunits do not occur in the same receptor.

γ-Aminobutyric acid A ($GABA_A$)[1] receptors are ligand-gated anion channels that mediate the majority of fast synaptic inhibition in the brain (1). A variety of drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants exert their pharmacologically and clinically important actions by modulating the function of these receptors (2).

$GABA_A$ receptors are composed of five subunits derived from different subunit classes. So far, six α, four β, three γ, one δ, one ε, one π, one θ, and three ρ subunits have been cloned from mammalian brain (3, 4). From possible permutations of these subunits, theoretically, an extremely large number of receptors with distinct subunit composition can be formed. Due to restrictions in the cellular expression of individual subunits and to mechanisms governing the assembly of receptors, however, only a limited number of receptor subtypes actually are present in the brain (5–7).

The cerebellum is an excellent brain area for studying the composition of $GABA_A$ receptors. The small number of cell types allows an almost complete account of which cerebellar cell types express which $GABA_A$ receptor subunit genes (8). For example, cerebellar granule cells express six subunit genes abundantly (α_1, α_6, β_2, β_3, γ_2, and δ), forming $\alpha_1\beta\gamma_2$, $\alpha_6\beta\gamma_2$, $\alpha_1\alpha_6\beta\gamma_2$, $\alpha_6\beta\delta$, and $\alpha_1\alpha_6\beta\delta$ $GABA_A$ receptor subtypes (9–13), and these receptors exhibit a distinct subcellular distribution (14, 15). The regulation of the expression of the different receptors relative to each other is unknown.

Targeted disruption of genes by homologous recombination is widely used to study the functional role of the respective gene product in the organism. In receptors composed of multiple subunits, the deletion of one subunit might also reduce the abundance of its subunit partners present in the same receptors. Indeed, after the disruption of the $GABA_A$ receptor α_6 subunit gene, not only were the α_6 subunits completely eliminated from cerebellar granule cells but also the δ subunits were dramatically reduced (16, 17). These results suggested that the α_6 subunit is necessary for the oligomerization and surface expression of the δ subunit.

The recent generation of δ−/− mice (18) allowed to investigate whether the δ subunit is also essential for the surface expression of the α_6 subunit. Therefore, in the present study, the abundance and subunit composition of $GABA_A$ receptors was compared in the cerebellum of δ+/+ and δ−/− mice. In contrast to α_6−/− mice (17), the total number of $GABA_A$ receptors was not reduced in the cerebellum of δ−/− mice, despite the complete loss of δ subunits. Instead, an up-regulation of the γ_2 subunit-containing receptors was observed. Furthermore, the composition of $GABA_A$ receptors in the cerebellum of δ−/− mice differed significantly from that of δ+/+ mice.

EXPERIMENTAL PROCEDURES

Generation of Mutant Mice—Targeted disruption of the δ subunit of the $GABA_A$ receptor in mouse embryonic stem cells and production of wild-type (δ+/+), and homozygous δ−/− mice is described in detail in a

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; NGS, normal goat serum; PB, phosphate buffer; TBS, Tris-buffered saline; DS, diazepam-sensitive; DIS, diazepam-insensitive.

previous study (18). The replacement-type DNA targeting construct placed a selectable marker gene in exon 4, upstream of the exons required for the putative transmembrane regions of this receptor subunit. This targeting event prevented the production of δ protein, *i.e.* produced a true null allele. The mice used for the present studies were the F2–F5 generation on a mixed C57BL/6J × strain 129Sv/SvJ genetic background. These mice were normal in their gross behavior. δ+/+ mice were the F3 generation on a mixed C57BL/6J × strain 129Sv/SvJ genetic background.

Antibodies—The generation of anti-peptide α_1-(1–9) (19), anti-peptide α_6-(317–371) (17), anti-peptide β_1-(350–404) (13), anti-peptides β_2-(351–405), β_3-(345–408) and γ_2-(319–366) (20), γ_1-(324–366) or γ_3-(322–372) antibodies (21), anti-peptide δ-(1–44) antibodies (16), or anti-peptide γ_2-(1–29) antibodies (22) has been described earlier. The antibodies were shown to precipitate recombinant $GABA_A$ receptors containing the respective subunit only and did not exhibit cross-reactivity with other $GABA_A$ receptors (13, 17).

Quantitative Immunoblot Analysis—Membranes from a total of 12 δ+/+ and 12 δ−/− cerebella were isolated individually, and equal amounts (15 μg) of protein per slot were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the same 10% polyacrylamide gel (13). Proteins were blotted to polyvinylidene difluoride membranes and detected by subunit specific antibodies. Secondary antibodies (Fab'_2 fragments of goat anti-rabbit IgG coupled to alkaline phosphatase, Jackson ImmunoResearch Labs Inc.) were visualized by the reaction of alkaline phosphatase with CSPD (Tropix, Bedford, MA) and the chemiluminescence signal was quantified by densitometry of Kodak X-Omat S films with the Docu Gel 2000i gel documentation system using RFLP scan software (MWG biotech, Ebersberg, Germany). The linear range of the detection system was established by determining the antibody response to a range of antigen concentrations following immunoblotting. The experimental conditions were designed such that immunoreactivities obtained in the assay were within this linear range, thus permitting a direct comparison of the amount of antigen applied per gel lane between samples. Different exposures of the same membrane were used to ensure that the measured signal was in the linear range of the x-ray film.

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 Membrane Extracts, Affinity Chromatography, and Immunoprecipitation—$GABA_A$ receptors were solubilized from cerebellar membranes of δ+/+ and δ−/− mice using a deoxycholate buffer (0.5% deoxycholate, 0.05% phosphatidylcholine, 10 mM Tris/HCl, pH 8.5, 150 mM NaCl, 1 mM benzamidine, 200 μg/ml bacitracin, and 300 μM phenylmethylsulfonyl fluoride), and immunoaffinity chromatography was performed as described (13). Briefly, deoxycholate extracts were cycled three times through the affinity column (synthesized as described in Ref. 21) at a rate of 2 ml/h. To determine the percentage of receptors retained by the column, immunoprecipitations with subunit-specific antibodies and [^{3}H]muscimol binding assays were performed with the original extract and the column efflux in parallel.

For immunoprecipitation, 200 μl of the clear deoxycholate membrane extract was mixed with 30 μl of antibody solution (20–45 μg of antibody), and the mixture was incubated under gentle shaking at 4 °C overnight. Then 50 μl of pansorbin (Calbiochem, La Jolla, CA) plus 100 μl of a low salt buffer for immunoprecipitation (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, pH 8.0, 1 mM benzamidine, 200 μg/ml bacitracin, and 300 μM phenylmethylsulfonyl fluoride) containing 5% dry milk powder were added, and incubation was continued for 2 h at 4 °C. The precipitate was centrifuged for 10 min at 10,000 × *g*, and the pellet was washed twice with 500 μl of high salt buffer for immunoprecipitation (50 mM Tris/HCl, 600 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 8.3, 1 mM benzamidine, 200 μg/ml bacitracin, and 300 μM phenylmethylsulfonyl fluoride) and once with 500 μl of low salt buffer for immunoprecipitation.

For determination of the total amount of receptors present in the extract, solubilized receptors were precipitated by a mixture containing 10 μg of β_1-(350–404), 18 μg of β_2-(351–405), and 15 μg of β_3-(345–408) antibody per 200 μl of extract. This antibody mixture was used, because all functional $GABA_A$ receptors are supposed to contain at least one of these three β subunits. Precipitation with polyethylene glycol could not be used for the determination of total [^{3}H]muscimol binding due to the rapid dissociation of [^{3}H]muscimol from its binding site. The high viscosity of the polyethylene glycol solution causes relatively long filtration and washing times and, thus, significant losses of previously bound [^{3}H]muscimol. The amounts of β_1-(350–404), β_2-(351–405), and β_3-(345–408) antibodies used were sufficient to maximally precipitate $GABA_A$ receptors containing the respective β subunits from brain extracts. The total number of [^{3}H]muscimol binding sites measured in receptors precipitated by this antibody mixture was higher than that precipitated by polyethylene glycol, whereas the total number of [^{3}H]Ro15-4513 binding sites measured was identical whether receptors were precipitated by the antibody mixture or polyethylene glycol (13, 17).

Receptor Binding Studies—Cerebellar membranes were homogenized and washed three times with 50 mM Tris/citrate, pH 7.1. Extracted receptors were immunoprecipitated, and the precipitate was suspended in 1 ml of a solution containing 0.1% Triton X-100, 50 mM Tris-citrate buffer, pH 7.1. A total of 1 ml of a solution containing 100 μg of membrane protein or the resuspended immunoprecipitate was incubated with 2–50 nM of [^{3}H]Ro15-4513 (21.7 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 100 μM diazepam or 100 μM Ro15-1788, or with 2–50 nM [^{3}H]muscimol (20 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 1 mM GABA, for 90 min at 4 °C. Then the suspensions were filtered through Whatman GF/B filters, and the filters were washed and subjected to liquid scintillation counting.

Immunocytochemistry—Two series of animals were processed. In the first series, five adult δ+/+ and five adult δ−/− mice were anesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg intraperitoneal) and perfused through the heart first with 0.9% saline, then with a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4; PB) for 7–15 min. After perfusion the brains were removed, and blocks from the vermis of the cerebellum were cut out and washed in PB before sectioning with a Vibratome. Normal goat serum (NGS, 20%) was used in 50 mM Tris-HCl containing 0.9% NaCl (pH 7.4, TBS) as the blocking solution for 1 h, before the sections were incubated in the primary antibody solution (in TBS containing 1% NGS and 0.05% Triton X-100) overnight. Primary antibodies were used at the following final protein concentrations (in μg/ml): α1 = 0.9–1.3 (P16); α6 = 0.5–0.6 (P24); β2 = 0.9 (β_2-(351–405)R23); β3 = 1.7–2.0 (β_3-(345–408)R1); γ2 = 1.0 (γ_2-(1–29), 22); and δ = 1.1 (δ-(1–44)R5). After washing, the sections were incubated in either biotinylated goat anti-rabbit IgG or biotinylated goat anti-guinea pig IgG (diluted 1:50 in TBS containing 1% NGS, Vector Laboratories, Peterborough, UK) for 2 h. The sections were then incubated in avidin biotinylated horseradish peroxidase complex (diluted 1:100 in TBS) for 1.5 h before the peroxidase enzyme reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride as chromogen and H_2O_2 as oxidant. Sections were then routinely processed for light microscopic examination. In the second series, three adult δ+/+ and three δ−/− mice were anesthetized and perfused with the same fixative as above for 10 min. After perfusion, 50-μm-thick sections were cut from the cerebellum on a Vibratome. All solutions contained 0.1% Triton X-100. Normal goat serum (NGS, 20%) in TBS was used as blocking solution, before the sections were incubated in the primary antibody solution in TBS containing 1% NGS overnight. Primary antibodies were used at the following final protein concentrations: α1(P16), 0.6 μg/ml; γ_2-(319–366, T12/20/B9), 0.9 μg/ml. After washing, the sections were incubated for 5 h in goat anti-rabbit IgG coupled to Alexa488, diluted 1:1000 (Molecular Probes, Eugene, OR) at room temperature. Sections were washed and mounted in Vectashield (Vector Laboratories).

Quantification of Immunoreactivity —The animals were coded and measurements were carried out according to a double-blind protocol. Neither the person taking the images nor the person making the subsequent measurements knew the identity of the animals. The code was opened only after completing the measurements. One digital image (cooled charge-coupled device camera, Xilix Microimager, OpenLab version 2.2.1, Improvision, Coventry, UK) was captured from each of three sections per animal using a 20× objective, 100-watt mercury lamp, and an L5 filter block (excitation filter, BP 480/40 nm; RKP 565 nm; suppression filter BP 610/75) of a Leitz DMRB fluorescence microscope. The same exposure time was used for images from all animals for a given antibody, and the images were treated in an identical way. The images were transferred to Adobe Photoshop (version 5.5) and the mean gray scale pixel value (12-bit) was determined separately for the molecular and granule cell layers for each section using the Histogram command. Each animal was characterized by the mean average gray scale pixel value obtained from the three sections. The values of control and δ−/− animals were compared with the unpaired *t* test.

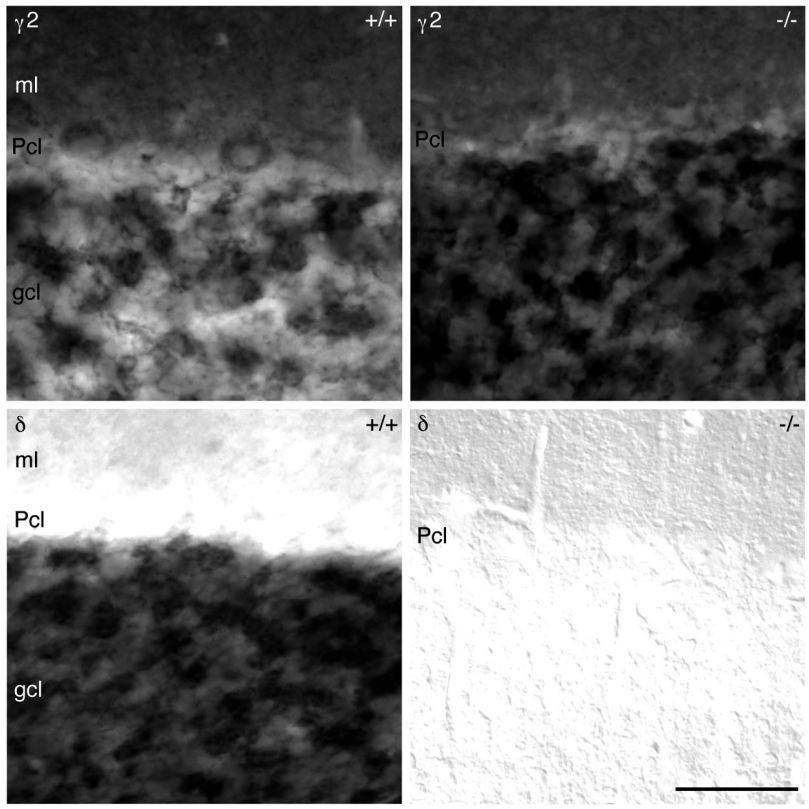

FIG. 1. **Changes in the expression of the γ_2 and δ subunits of the GABA$_A$ receptor in the cerebellum of δ−/− mice.** Pre-embedding immunoperoxidase reaction with antibodies γ_2-(1–29) and δ-(1–44)R5. An increase in the immunoreactivity for the γ_2 subunit was detected in the granule cell layer (*gcl*) of δ−/− mice, without any change in the molecular layer (*ml*). Immunoreactivity for the δ subunit completely disappeared in δ−/− mice. +/+, control mice; *Pcl*, Purkinje cell layer; *scale bar*, 50 μm.

RESULTS

Immunoreactivity for the δ Subunit in Control and δ−/− Mice—In cerebellum, immunoreactivity for the δ subunit is restricted to the granule cell layer (15–17, 23) (Fig. 1). After targeted disruption of the δ subunit gene, staining of granule cells with the δ-(1–44) antibody (16, 17) completely disappeared (Fig. 1). In agreement with these immunocytochemical results, neither the complete δ subunit (54-kDa protein band) nor a truncated δ subunit protein were detectable in cerebellar membranes of δ−/− mice by Western blot analysis (18). Finally, in contrast to δ+/+ mice, where an immunoaffinity column containing the δ-(1–44) antibody was able to remove 28.8 ± 4.2% (n = 3, mean ± S.E.) of all [^{3}H]muscimol binding sites from cerebellar extracts, no [^{3}H]muscimol binding sites could be removed by this column from cerebellar extracts of δ−/− mice, confirming the absence of δ subunit-containing receptors in mice with a targeted disruption of the δ subunit.

Immunoreactivity for α_1, α_6, β_2, β_3, and γ_2 Subunits in Control and δ−/− Mice—In addition to the δ subunit, five other major subunits can be detected in cerebellar granule cells: α_1, α_6, β_2, β_3, and γ_2 (15, 17). We applied light microscopic immunolabeling to assess possible alterations in the expression of these subunits and to determine the cellular distribution of any change. Immunoperoxidase reactions indicated no change in immunoreactivity for the α_1, α_6, β_2, and β_3 subunits, but showed an up-regulation of immunoreactivity for the γ_2 subunit in the cerebellar granule cell layer of δ−/− animals (Fig. 1).

To quantify the change, light microscopic immunofluorescence labeling for the γ_2 and α_1 subunits was carried out and measured. For the γ_2 subunit, the mean gray scale pixel value increased by 82% (53.5 ± 7.9, mean ± S.D., n = 3, control; 97.2 ± 5.9, n = 3, δ−/−, $p < 0.01$, unpaired t test) in the granule cell layer of δ−/− mice. In addition, there was also a small (18%) but significant ($p < 0.01$, unpaired t test) increase in γ_2 subunit labeling in the molecular layer of the δ−/− mice (49.1 ± 1.8, n = 3, control; 58.2 ± 2.4, n = 3, δ−/−). In agreement with the results of the peroxidase reactions, no significant change ($p > 0.05$, unpaired t test) was detected in immunoreactivity for the α_1 subunit in either the granule cell (81.0 ± 6.7, n = 3, control; 91.5 ± 4.7, n = 3, δ−/−) or the molecular layers (75.2 ± 6.0, n = 3, control; 84.3 ± 13.0, n = 3, δ−/−).

Membranes from the cerebellum of δ+/+ or δ−/− mice were then subjected to SDS-polyacrylamide gel electrophoresis and quantitative Western blot analysis, using subunit-specific antibodies directed against α_1, α_6, β_2, β_3, γ_2, and δ subunits (13, 17). As shown in Fig. 2, the expression of α_1 subunits was increased by 25% in the cerebellum of δ−/− mice, whereas the levels of α_6 or β_2 subunits were not significantly different from those found in δ+/+ mice. The expression of β_3 subunits was increased by 23% and that of γ_2 subunits was increased by 45% (Fig. 2).

[^{3}H]Ro15-4513 Binding in Cerebellum of Control and δ−/− Mice—To investigate whether the additional γ_2 subunits expressed in δ−/− cerebellum were part of functional receptors, [^{3}H]Ro15-4513 binding studies were performed in membranes from δ+/+ and δ−/− cerebellum. [^{3}H]Ro15-4513 is a ligand for the benzodiazepine binding site of GABA$_A$ receptors, which are only formed by receptors containing a γ subunit (2). Scatchard analysis of specific [^{3}H]Ro15-4513 binding indicated that the total number (B_{max}) of sites was increased by 52% in δ−/− mice, whereas the binding affinity was not significantly different (Table I). In agreement with previous reports (24–26), 31% of total binding in the cerebellum of δ+/+ mice was not displaced by 100 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 α_6 subunits (27) was increased by 180% in δ−/− mice. The diazepam-sensitive (DS) [^{3}H]Ro15-4513 binding, however, was comparable in δ−/− and δ+/+ mice (Table I).

These data were confirmed by binding studies using [^{3}H]flunitrazepam as a ligand. In contrast to [^{3}H]Ro15-4513, [^{3}H]flunitrazepam exclusively binds to DS sites (25). Scatchard

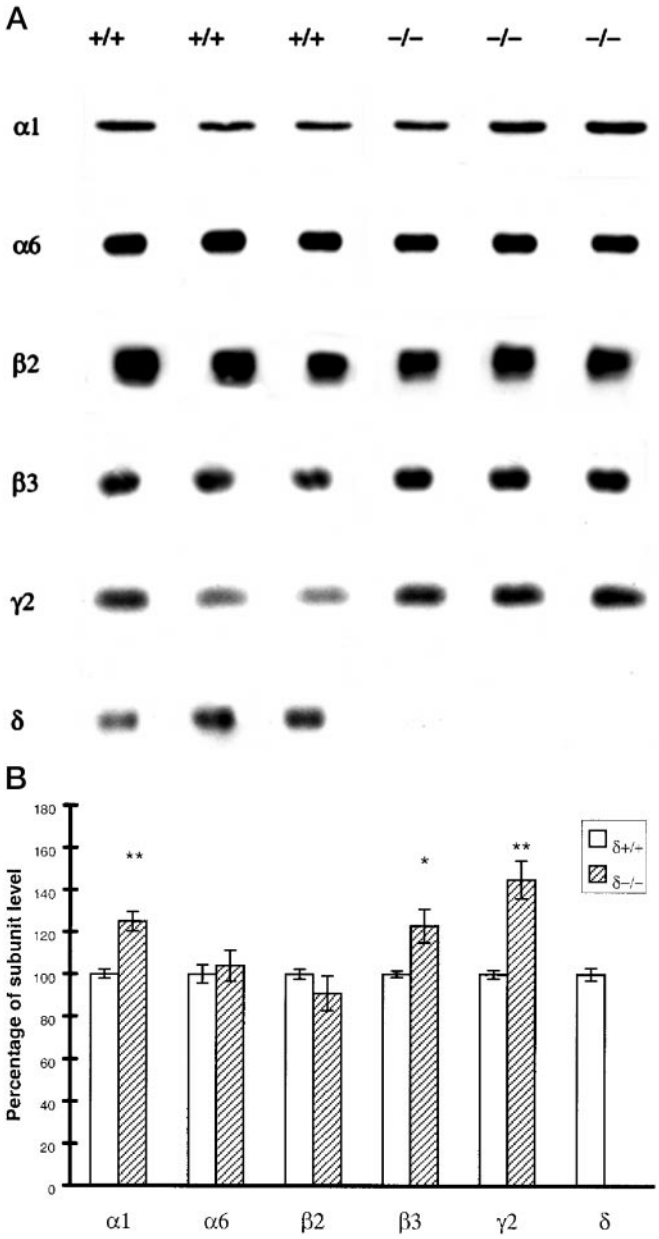

FIG. 2. **Quantification of GABA$_A$ receptor subunit proteins in cerebellar membranes by Western blot analysis.** Equal amounts of cerebellar membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subunit levels were compared in δ+/+ and δ−/− membranes by Western blot analysis. *A*, representative blot comparing immunoreactivity for α_1, α_6, β_2, β_3, and γ_2 subunits in three δ+/+ and three δ−/− cerebella. The chemiluminescence signals were quantified by densitometry. Results are expressed as percentage of the subunit level found in δ+/+ membranes ± S.E. *B*, results for α_1, α_6, β_2, β_3, and γ_2 subunits were obtained from 12 δ+/+ and 12 δ−/− mice. Results for δ subunits were from three δ+/+ and three δ−/− mice. No staining for the δ subunit could be detected in cerebellar membranes of δ−/− mice. For statistical comparisons unpaired Student's *t* test was used. * $p < 0.001$; ** $p < 0.0001$.

analyses from two separate experiments indicated that B_{max} values observed in membranes from δ+/+ cerebella (2072 and 1640 fmol/mg of protein) were comparable to those observed in δ−/− cerebella (2103 and 2126 fmol/mg of protein) and were also comparable to the B_{max} values of the DS [^{3}H]Ro15-4513 binding sites in these tissues (Table I).

In other experiments, GABA$_A$ receptors were extracted from cerebellar membranes by a deoxycholate buffer and then immunoprecipitated with a combination of antibodies directed against the β_1, β_2, and β_3 subunits of GABA$_A$ receptors (13, 17).

TABLE I

Comparison of benzodiazepine binding sites by Scatchard analysis of [^{3}H]Ro15–4513 binding

Cerebellar membranes from δ+/+ and δ−/− mice (A) or GABA$_A$ receptors extracted from these membranes and precipitated as described under "Experimental Procedures" (B) were incubated with various concentrations of [^{3}H]Ro15–4513 in the absence or presence of 100 μM diazepam or Ro15–1788. Membranes or precipitated receptors were then filtered through Whatman GF/B filters and washed as described. Scatchard analysis was performed from [^{3}H]Ro15–4513 binding data that could be displaced by 100 μM Ro15–1788 (total specific binding) or by 100 μM diazepam (diazepam sensitive = DS binding). Total specific binding minus DS binding resulted in diazepam insensitive (DIS) binding. Values are means ± S.E. from three separate experiments (A). For statistical comparisons unpaired Student's *t* test was used. In B only two experiments were performed due to the limited amounts of antibody available.

Parameter	δ+/+	δ−/−	*p*
A. Cerebellar membranes			
K_d (nM)	12.8 ± 4.5	8.2 ± 1.2	0.38
B_{max} (fmol/mg)	2803 ± 344	4255 ± 309	<0.05
DS (fmol/mg)	1934 ± 292	1818 ± 208	0.76
DIS (fmol/mg)	868 ± 80	2437 ± 401	<0.05
B. Cerebellar membrane extracts			
K_d (nM)	15/16	9/19	
B_{max} (fmol/mg)	1518/1606	2797/2033	
DS (fmol/mg)	1046/1160	1223/704	
DIS (fmol/mg)	472/446	1574/1329	

Scatchard analysis of [^{3}H]Ro15-4513 binding to the precipitated receptors indicated that the recovery of total, DS and DIS binding was 56%, 57%, and 53% in δ+/+ and 57%, 53%, and 60% in δ−/− cerebella, respectively. Receptors recovered in the extract, however, represented 93% of all binding sites detectable in cerebellar membrane homogenates treated with detergent, because part of the receptors present in the membranes became inactivated during solubilization. Total B_{max} values measured in cerebellar extracts from δ−/− mice were increased by 55% as observed in the corresponding membranes. Whereas DS binding was comparable in the extracts of δ+/+ and δ−/− cerebella, DIS binding in δ−/− extracts was increased by 216% relative to that in δ+/+ extracts (Table I).

[^{3}H]Muscimol Binding in the Cerebellum of Control and δ−/− Mice—To investigate the abundance of GABA$_A$ receptors, Scatchard analysis of [^{3}H]muscimol binding to cerebellar membranes of δ+/+ and δ−/− mice was carried out. Comparable K_d values and a slight increase in the B_{max} value in δ−/− mice were found, but this increase in the B_{max} value did not reach statistical significance (Table II). Similar results were obtained from Scatchard analysis of [^{3}H]muscimol binding to receptors extracted and immunoprecipitated with a mixture of antibodies directed against the β_1, β_2, and β_3 subunits, which should precipitate the vast majority of GABA$_A$ receptors (13, 17): B_{max} values measured in δ−/− mice were higher than those found in δ+/+ mice, but the difference again did not reach statistical significance.

A comparison of B_{max} values in cerebellar membranes and solubilized receptors indicated that ~52% and 61% of GABA$_A$ receptors present in membranes of δ+/+ and δ−/− cerebella, respectively, could be recovered as binding sites in the membrane extract (Table II). The recovery of [^{3}H]muscimol binding sites was therefore comparable to that of total, diazepam-sensitive, or diazepam-insensitive [^{3}H]Ro15-4513 binding sites. The similar proportions of different binding sites recovered, as well as the parallel changes in membrane-bound and -extracted binding sites in δ−/− mice, indicate that the receptors recovered in the extract were representative of the entire functional GABA$_A$ receptor population.

Subunit Composition of GABA$_A$ Receptors in the Cerebellum

TABLE II

Comparison of total GABA$_A$ receptors by Scatchard analysis of [^{3}H]muscimol binding

Cerebellar membranes from δ+/+ and δ−/− mice (A) or GABA$_A$ receptors extracted from these membranes and precipitated as described under "Experimental Procedures" (B) were incubated with various concentrations of [^{3}H]muscimol in the absence or presence of 1 mM GABA. Membranes or precipitated receptors were then filtered through Whatman GF/B filters and washed as described under "Experimental Procedures." Scatchard analysis was performed from [^{3}H]muscimol binding data that could be displaced by 1 mM GABA (total specific binding). Data are mean values ± S.E. from three experiments performed in triplicate. For statistical comparisons unpaired Student's *t* tests were used.

Parameter	δ+/+	δ−/−	*p*
A. Cerebellar membranes			
K_d (nM)	25.8 ± 15.3	36.6 ± 18.2	0.67
B_{max} (fmol/mg)	4041 ± 572	5017 ± 827	0.38
B. Cerebellar membrane extracts			
K_d (nM)	9.6 ± 2.1	13.2 ± 1.2	0.09
B_{max} (fmol/mg)	2088 ± 418	3039 ± 487	0.21

of Control and δ−/− Mice—Cerebellar membrane extracts from δ+/+ and δ−/− mice were cycled on an α_6-(317–371) immunoaffinity column until α_6 subunits were no longer detectable in the efflux of the column using immunoprecipitation experiments or Western blot analysis. Total GABA$_A$ receptors present in the original membrane extract and in the column efflux were then determined by [^{3}H]muscimol binding studies after precipitation with a mixture of β_1, β_2, and β_3 subunit-specific antibodies. The total amount of GABA$_A$ receptors in the column efflux was reduced by 55.7% in δ+/+ and by 57.5% in δ−/− mice, indicating that the proportion of α_6 subunit-containing receptors present in the extracts from these tissues was similar (Table III).

In other experiments, the extent of reduction of α_1, β_2, β_3, γ_2, or δ subunit-containing receptors after chromatography on the anti-α_6 column was determined. For this, receptors containing these subunits were immunoprecipitated in the original extract and the anti-α_6 column efflux with α_1, β_2, β_3, γ_2, or δ subunit-specific antibodies and were quantitatively determined by [^{3}H]muscimol binding. In the absence of any cross-reactivity of the anti-α_6 antibodies with other GABA$_A$ receptor subunits (13, 17), the percentage of reduction indicates the percentage of these receptors containing α_6 subunits. As shown in Table III, the percentage of α_1, β_2, β_3, γ_2, or δ subunit-containing receptors containing α_6 subunits in the cerebellum of δ+/+ mice was similar to that reported previously (17) for the cerebellum of wild-type mice.

The percentage of α_1, β_2, and β_3 subunit-containing receptors removed by the anti-α_6 column from extracts of δ−/− cerebella was comparable, indicating that a similar proportion of these receptors contained α_6 subunits in control and δ−/− cerebella (Table III).

In contrast, the percentage of γ_2 receptors eliminated by the anti-α_6 column was significantly larger in the cerebellum of δ−/− mice, suggesting an increased formation of GABA$_A$ receptors containing both α_6 and γ_2 subunits. The actual increase in the number of receptors containing these subunits, however, was even larger, because of the increased expression of γ_2 subunits (Fig. 2) and the increased number of γ_2 subunit-containing receptors in δ−/− cerebellum (Table I).

To further investigate the composition of γ_2-containing receptors, these receptors were completely eliminated from cerebellar extracts of δ+/+ and δ−/− mice using an immunoaffinity column containing the γ_2-(319–366) antibody. This column eliminated 68.3% of all receptors in δ+/+ and 76.5% of all receptors in δ−/− mice. These proportions were not significantly different (Table IV). It has to be kept in mind, however, that when comparing the number of γ_2-containing receptors present in δ+/+ and δ−/− mice, the total number of GABA$_A$ receptors in these tissues has to be considered (Table II).

TABLE III

Extent of copurification of GABA$_A$ receptor subunits with α_6 subunits from δ+/+ and δ−/− mice

GABA$_A$ receptors were extracted from the cerebellum of δ+/+ and δ−/− mice, and α_6 subunit-containing receptors were completely eliminated from the extract by chromatography on an immunoaffinity column containing the antibody α6-(317–371). GABA$_A$ receptors containing α_1, β_2, β_3, γ_2, or δ subunits were precipitated in the original extract and in the column efflux using the respective subunit-specific antibodies. Precipitated receptor subtypes were quantified using [^{3}H]muscimol binding. The percentage of reduction in the column efflux of receptors containing the respective subunit indicated their extent of colocalization with α_6 subunits in the same receptors. Data are mean values ± S.E. with the numbers of experiments given in parentheses. Results from δ−/− mice were statistically compared with results from δ+/+ mice using Student's unpaired *t* test.

Antibody for precipitation	Reduction by α_6 column		*p* value
	δ+/+	δ−/−	
	%		
$\beta_1 + \beta_2 + \beta_3$	55.7 ± 1.2 (3)	57.5 ± 0.2 (3)	0.21
α_1	30.6 ± 0.3 (4)	37.9 ± 4.2 (3)	0.09
β_2	52.9 ± 5.9 (3)	55.8 ± 0.3 (3)	0.65
β_3	65.6 ± 6.0 (4)	68.2 ± 2.9 (5)	0.69
γ_2	44.4 ± 1.6 (4)	55.5 ± 1.8 (6)	<0.005
δ	98.7 ± 0.8 (4)		

Quantification in the original membrane extract and column efflux of receptor subtypes precipitated with subunit-specific antibodies indicated that a comparable proportion of α_1 subunit-containing receptors was removed by the anti-γ_2 affinity column in δ+/+ and δ−/− extracts. In agreement with the results from the anti-α_6 column (Table III) a significantly higher percentage of α_6 receptors was associated with γ_2 subunits in extracts from δ−/− cerebella (Table IV). Although only 52.4% of α_6 receptors were eliminated by the anti-γ_2 subunit column from membrane extracts of δ+/+ mice, 70.2% of α_6 receptors were eliminated from those of δ−/− mice. The extent of association with γ_2 subunits was also slightly elevated for β_2 subunits: 67% of β_2 subunit-containing receptors contained γ_2 subunits in δ+/+ and 75.2% in δ−/− cerebella. The degree of association of β_3 and γ_2 subunits, however, was comparable in the cerebellum of δ+/+ and δ−/− mice (Table IV).

Supporting previous conclusions (13, 28, 29), the percentage of δ subunit-containing receptors present in cerebellar extracts from δ+/+ mice (28.8 ± 4.2% of all receptors) was not reduced in the efflux of the anti-γ_2 subunit column (Table IV). This demonstrates that GABA$_A$ receptors containing both γ_2 and δ subunits are not present in the cerebellum.

Summing of the proportions of δ (28.8%)- and γ_2 (68.3%)-containing receptors in δ+/+ mice indicates that 97.1% of all receptors in the cerebellum are assemblies of $\alpha\beta\gamma_2$ or $\alpha\beta\delta$ combinations. Interestingly, despite the absence of δ subunits, only 76.5% of all receptors in δ−/− mice contained γ_2 subunits. This raised the possibility that the remaining GABA$_A$ receptors contained up-regulated γ_1 or γ_3 subunits. However, no significant amounts of γ_1 or γ_3 subunit-containing GABA$_A$ receptors could be immunoprecipitated in cerebellar extracts from δ+/+ and δ−/− mice using γ_1-(324–366) or γ_3-(322–372) antibodies under conditions where these antibodies precipitated significant amounts of receptors in forebrain extracts (experiments not shown, Ref. 21). This indicated that 23.5% of all GABA$_A$ receptors in cerebellum extracts from δ−/− mice did not contain γ_1, γ_2, γ_3, or δ subunits. Because significant amounts of α_1, α_6, β_2, or β_3 subunit-containing receptors remained in the extract after complete elimination of γ_2 subunit-containing

TABLE IV
Extent of copurification of GABA$_A$ receptor subunits with γ_2 subunits from δ+/+ and δ−/− mice

GABA$_A$ receptors were extracted from the cerebellum of δ+/+ and δ−/− mice, and γ_2 subunit-containing receptors were completely eliminated from the extract by chromatography on an immunoaffinity column containing the antibody γ_2-(319–366). GABA$_A$ receptors containing α_1, α_6, β_2, β_3, or δ subunits were precipitated in the original extract and in the column efflux using the respective subunit-specific antibodies. Precipitated receptor subtypes were quantified using [^{3}H]muscimol binding. The percentage of reduction in the column efflux of receptors containing the respective subunit indicated their extent of colocalization with γ_2 subunits in the same receptor. Data are mean values ± S.E. with the numbers of experiments given in parentheses. Results from δ−/− mice were statistically compared with results from δ+/+ mice using Student's unpaired *t* test.

Antibody for precipitation	Reduction by γ_2 column		*p* value
	δ+/+	δ−/−	
	%		
$\beta_1 + \beta_2 + \beta_3$	68.3 ± 5.6 (3)	76.5 ± 1.3 (3)	0.23
α_1	82.5 ± 2.5 (4)	76.3 ± 5.4 (3)	0.3
α_6	52.4 ± 1.4 (4)	70.2 ± 1.1 (4)	<0.0001
β_2	67.0 ± 1.1 (3)	75.2 ± 1.7 (4)	<0.05
β_3	63.3 ± 7.7 (3)	64.6 ± 2.7 (4)	0.86
δ	0		

receptors (Table IV), it can be concluded that the remaining 23.5% of GABA$_A$ receptors were composed of combinations of α_1, α_6, β_2, and β_3 subunits, or additionally contained as yet unidentified subunits.

DISCUSSION

No Reduction in the Number of GABA$_A$ Receptors in δ−/− Cerebellum—In the present study possible changes in the abundance and subunit composition of GABA$_A$ receptors were investigated in the brain of mice with a targeted disruption of the δ subunit expression. Because the δ subunit is much more abundant in the cerebellum than in the forebrain (30, 31), we focused our investigation on this brain region. Despite the loss of δ subunit-containing receptors, which comprise nearly 30% of all GABA$_A$ receptors in the cerebellum, the number of GABA$_A$ receptors was not reduced in membranes or membrane extracts of δ−/− cerebellum, as demonstrated by Scatchard analysis of [^{3}H]muscimol binding. These data are in contrast to those of a previous study (18) in which a significant reduction in [^{3}H]muscimol binding was observed in whole brain homogenates of δ−/− mice. The different tissues used, as well as differences in the tissue preparation and [^{3}H]muscimol binding assay conditions, might have contributed to the different results.

Increase in GABA$_A$ Receptors Containing γ_2 Subunits in δ−/− Cerebellum—Western blot analysis demonstrated a 45% increase in the expression of γ_2 subunits in the cerebellum of δ−/− mice, which corresponded to a 52% increase in GABA$_A$ receptors containing [^{3}H]Ro15-4513 binding sites. This finding is consistent with the elevated [^{3}H]Ro15-4513 binding in cerebellum and several other brain tissues of δ−/− mice observed in autoradiographic studies (18). The increase in [^{3}H]Ro15-4513 binding sites was due to a dramatic increase in DIS binding sites in membranes and extracts from δ−/− cerebellum. Because DIS binding sites are formed by receptors containing $\alpha_6\beta\gamma_2$ or $\alpha_1\alpha_6\beta\gamma_2$ subunits (12, 25, 27), these data suggested a significant increase in the formation of receptors containing α_6 as well as γ_2 subunits in the cerebellum of δ−/− mice.

This conclusion was supported by studies investigating subunit partnerships in GABA$_A$ receptors: Immunoaffinity chromatography using an anti-α_6 subunit column showed that the proportion of γ_2 receptors containing α_6 subunits was significantly larger in membrane extracts from δ−/− than in those from δ+/+ mice. Similarly, the use of an anti-γ_2 subunit column showed the increased coassembly of the γ_2 and α_6 subunits but not the γ_2 and α_1 subunits in extracts from δ−/− cerebella. These data indicated an increased formation of $\alpha_6\beta\gamma_2$ but not of $\alpha_1\alpha_6\beta\gamma_2$ receptors in δ−/− cerebellum.

Increase in GABA$_A$ Receptors Composed of αβ Subunits in δ−/− Cerebellum—In wild-type cerebellum, 68.3% and 28.8% of GABA$_A$ receptors contained γ_2 and δ subunits, respectively. Because γ_2 and δ subunits are not colocalized in the same GABA$_A$ receptors in this tissue (13, 28) 97% of all cerebellar GABA$_A$ receptors contained γ_2 or δ subunits. This is consistent with the finding that receptors containing γ_1 or γ_3 subunits are not very abundant in the cerebellum (30, 31). After the complete loss of δ receptors, most of the remaining receptors might be expected to contain γ_2 subunits. However, only 76.5% of all receptors in the extract of δ−/− cerebella contained γ_2 subunits. Because γ_1 or γ_3 receptors were not up-regulated in the cerebellum of δ−/− mice, the remaining receptors must have been composed of either α and β subunits only, or these subunits may have been associated with as yet unidentified subunits. Because comparable proportions of α_1, α_6, β_2, or β_3 subunit-containing receptors remained in the extracts of δ−/− cerebellum after the removal of γ_2 subunit-containing receptors, the remaining receptors were probably composed of various combinations of α_1, α_6, β_2, and β_3 subunits. The exact composition of receptors formed, however, cannot be deduced from the data available.

Changes in the Subunit Composition of GABA$_A$ Receptors in δ−/− Cerebella Mostly Occur in Granule Cells—Immunohistochemical observations indicated a large increase in labeling for the γ_2 subunit in the cerebellar granule cell layer and a small increase in the molecular layer of δ−/− mice. Combined with the fact that in cerebellum α_6 subunits are exclusively expressed in granule cells (where they are colocalized with δ subunits in wild-type mice) and with the observation suggesting an increased formation of $\alpha_6\beta\gamma_2$ receptors in δ−/− mice, this indicates that most of the changes in receptor composition observed in mice with a targeted disruption of the δ subunit were induced in granule cells.

Although a significant increase in the expression of α_1 and β_3 subunits was observed in Western blot studies, no apparent change in the expression of these subunits was observed in immunocytochemical studies in δ−/− cerebella. Because changes in α_1 and β_3 subunit expression were small and varied in different animals, they might have escaped detection in immunocytochemical studies. Because there was no change in the number of DS [^{3}H]Ro15-4513 binding sites in the cerebellum of δ−/− mice, and because the extent of coassembly of α_1 and β_3 subunits with γ_2 receptors was comparable in extracts from δ+/+ and δ−/− cerebella, the increased abundance of α_1 and β_3 subunits did not result in an increased formation of $\alpha_1\beta_3\gamma_2$ receptors. These subunits, therefore, either formed $\alpha_1\beta_3$ receptors that contributed to the number of [^{3}H]muscimol but not of [^{3}H]Ro15-4513 binding sites (32) or represented unassembled α_1 and β_3 subunits. The first possibility seems to be more likely, because specific [^{3}H]muscimol binding in cerebellar membranes or membrane extracts was slightly, although not significantly, increased in δ−/− mice.

Molecular Mechanism Causing the Changes in GABA$_A$ Receptor Subunit Composition in δ−/− Cerebellum—Immunoaffinity purification on an anti-α_6 subunit column showed that δ subunits almost exclusively are coassembled with α_6 subunits. The corresponding GABA$_A$ receptors are composed of $\alpha_6\beta\delta$ and $\alpha_1\alpha_6\beta\delta$ subunits in the rat (13). Because there was no change in the expression of α_6 subunits in the cerebellum of δ−/− mice, $\alpha_6\beta\delta$ and $\alpha_1\alpha_6\beta\delta$ receptors presumably were converted into

$\alpha_6\beta$, $\alpha_1\alpha_6\beta$, and $\alpha_6\beta\gamma_2$ receptors.

Studies on $GABA_A$ receptor assembly in non-neuronal cells have indicated that α and β subunits form subunit tetramers and pentamers, whereas α, β, and γ subunits exclusively form pentamers (20). The rate of formation of $\alpha\beta$ pentamers, therefore, presumably is significantly slower than that of $\alpha\beta\gamma$ (and possibly also of $\alpha\beta\delta$) pentamers (33, 34). Once formed, however, $\alpha\beta$ pentamers are transported to the cell surface (34) and form functional receptors (35). It is, therefore, possible that most of the $\alpha\beta$ subunit combinations that do not assemble with γ_2 subunits in the cerebellum of δ−/− mice represented completely assembled and functional receptors. However, the presence of functional $\alpha\beta$ receptor on the surface of these cells needs to be directly demonstrated.

The relatively stable $\alpha\beta$ tetramers, however, presumably also act as a trap for γ subunits. Any γ subunit newly synthesized at sites where preformed $\alpha\beta$ tetramers are located may preferentially combine with the tetramers to complete the assembly of receptors composed of $\alpha\beta\gamma$ subunits (33). Such a mechanism may explain the presence of $\alpha_6\beta\gamma_2$, $\alpha_1\alpha_6\beta\gamma_2$, $\alpha_6\beta$, and $\alpha_1\alpha_6\beta$ receptors in the cerebellum of δ−/− mice.

Comparison with Previous Studies on Targeted Disruption of GABA$_A$ Receptor Subunits—Previous studies have indicated that the targeted disruption of γ_2, β_3, or α_6 subunits did not induce a compensatory up-regulation of the transcription of other $GABA_A$-receptor subunit genes in mutant mouse brain (16, 36–38). But these studies also indicated that the availability of α and β subunits was essential for receptor formation. Thus, upon disruption of the α_6 or β_3 subunit expression, the extent of reduction in the number of the remaining receptors indicated a complete loss of receptors containing the respective subunit and a corresponding reduction in the number of the remaining $GABA_A$ receptors (39).

Upon disruption of the γ_2 subunit expression, however, neither the number of $GABA_A$ receptors nor the protein level of the subunits α_1, α_2, α_3, $\beta_{2/3}$, were reduced, indicating the predominant formation of receptors composed of $\alpha\beta$ subunits (36). In addition, the protein level of γ_1 and γ_3 subunits remained unaltered in γ_2−/− mice as shown by Western blotting. This may indicate that the supply of γ_1 and γ_3 subunits is not linked to the availability of $\alpha\beta$ subunits, because receptors containing these subunits either are not synthesized in the same cells or the same subcellular compartments as those containing γ_2 subunits. The extent of expression of δ subunits was not investigated in this study.

The likely presence of receptors composed of $\alpha\beta$ subunits without additional subunit classes in the cerebellum of δ−/− mice is consistent with the expression of these receptors in γ_2−/− mice (36). The additional up-regulation of $\alpha_6\beta\gamma_2$ receptors in δ−/− mice can be explained if the level of γ_2 subunit expression is linked to the function of the granule cells. The latter mechanism might be unique for γ_2 subunits, which are the most abundant $GABA_A$ receptor subunits in the brain and seem to be responsible for receptor anchoring (40, 41). Alternatively, it is possible that in wild-type mice γ_2 and δ subunits compete for α_6 and β subunits during assembly of receptors and that subunits unable to find assembly partners are degraded (34). A lack of δ subunits in δ−/− mice might then reduce degradation of the surplus γ_2 subunits by increasing their chances to find assembly partners, thus leading to an increased formation of $\alpha_6\beta\gamma_2$ receptors. Future studies will have to decide which of these possibilities most likely occur.

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