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Alterations in the expression of $GABA_A$ receptor subunits in cerebellar granule cells after the disruption of the α6 subunit gene

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Abstract

Any given subunit of the heteromultimeric type-A γ-aminobutyric acid (GABA) $GABA_A$ receptor may be present in several receptor subtypes expressed by individual neurons. Changes in the expression of a subunit may result in differential changes in the expression of other subunits depending on the subunit composition of the receptor subtype, leading to alterations in neuronal responsiveness to GABA. We used the targeted disruption of the α6 subunit gene to test for changes in the expression of other $GABA_A$ receptor subunits. Immunoprecipitation and ligand binding experiments indicated that $GABA_A$ receptors were reduced by ≈ 50% in the cerebellum of α6 –/– mice. Western blot experiments indicated that the α6 subunit protein completely disappeared from the cerebellum of α6 –/– mice, which resulted in the disappearance of the δ subunit from the plasma membrane of granule cells. The amount of β2, β3 and γ2 subunits was reduced by ≈ 50%, 20% and 40%, respectively, in the cerebella of α6 –/– mice. A comparison of the reduction in the level of α1, β2, β3, γ2, or δ-subunit-containing receptors in α6 –/– cerebellum with those observed after removal of α6-subunit-containing receptors from the cerebella of α6 +/+ mice by immuno-affinity chromatography demonstrated the presence of a significantly higher than expected proportion of receptors containing β3 subunits in α6 –/– mice. The receptors containing α1, β2, β3 and γ2 subunits were present in the plasma membrane of granule cells of α6 –/– mice at both synaptic and extrasynaptic sites, as shown by electron microscopic immunocytochemistry. Despite the changes, the α1 subunit content of Golgi-cell-to-granule-cell synapses in α6 –/– animals remained unaltered, as did the frequency of α1 immunopositive synapses in the glomeruli. Furthermore, no change was apparent in the expression of the α1, β2 and γ2 subunits in Purkinje cells and interneurons of the molecular layer.

These results demonstrate that in α6 –/– mice, the cerebellum expresses only half of the number of $GABA_A$ receptors present in wild-type animals. Since these animals have no gross motor deficits, synaptic integration in granule cells is apparently maintained by α1-subunit-containing receptors with an altered overall subunit composition, and/or by changes in the expression of other ligand and voltage gated channels.

Introduction

A widely used approach to study the functional role of a protein in the CNS is to generate animals in which the expression of the protein is selectively disrupted. Although temporally and spatially restricted genetic deletions have already been developed (Kuhn *et al*., 1995; Tsien *et al*., 1996; Jones *et al*., 1997), it is often a general assumption that the expression of only a single gene is altered, without any change in the amount or in the precise subcellular location of other gene products.

Inhibition in the brain is mainly mediated through type-A γ-aminobutyric acid (GABA) $GABA_A$ receptors, which are ligand-gated anion channels formed of pentameric assemblies of subunits. Although, the possible permutations of the 16 subunits to form pentameric channels are hundreds of thousands, it is generally accepted that only a limited number of receptor subtypes exists in the CNS (Sieghart, 1995; Stephenson, 1995; McKernan & Whiting, 1996; Mohler *et al*., 1996; Barnard *et al*., 1998). Cerebellar granule cells provide an excellent opportunity to study how the disruption of a gene for a single $GABA_A$ receptor subunit influences the amount and the precise subcellular location of other subunits, as these cells express six $GABA_A$ receptor subunits abundantly (Laurie *et al*., 1992; Persohn *et al*., 1992; Wisden *et al*., 1996). These subunits form $GABA_A$ receptor subtypes with distinct subunit compositions and distinct kinetic and pharmacological properties (Puia *et al*., 1994; Saxena & Macdonald, 1994, 1996; Kaneda *et al*., 1995; McKernan & Whiting, 1996; Tia *et al*., 1996b; Jechlinger *et al*., 1998). In addition, the microcircuit of the cerebellum is relatively simple; pre- and postsynaptic elements can easily be identified on ultrastructural grounds. For example, most granule cells receive GABAergic innerva-

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tion from Golgi cells and glutamatergic input from mossy fibre terminals only on their distal dendrites. Mossy fibre and Golgi cell terminals are easily distinguishable in electron microscopic preparations (Palay & Chan-Palay, 1974).

We have previously described the generation of a transgenic mouse line (Δα6lacZ; α6 –/–) with a disrupted gene encoding the α6 subunit of the $GABA_A$ receptor (Jones *et al*., 1997). In addition to the lack of the α6 subunit, we found that the δ subunit protein was greatly reduced in the cerebellum of α6 –/– animals. These results suggested that the α6 subunit protein is necessary for oligomerization and for surface expression of the δ subunit. In spite of the disappearance of the α6-subunit-containing receptors, no cerebellar-associated motor deficits have been observed in α6 –/– mice (Homanics *et al*., 1997; Jones *et al*., 1997; Korpi *et al*., 1999). Therefore in the present study we investigated whether there were compensatory changes in the expression of the remaining $GABA_A$ receptor subtypes and/or changes in their subcellular distribution. Quantitative immunoblot and immunoprecipitation analysis of $GABA_A$ receptor subunits together with light- and electron microscopic immunocytochemistry were applied in control and α6 –/– mouse cerebella with antibodies selective for $GABA_A$ receptor subunits (α1, α6, β2, β3, γ2 and δ) abundantly expressed in cerebellar granule cells.

Materials and methods

Preparation of animals and tissue for immunocytochemistry

Twelve adult female mice (C57/BL-6) and 12 adult female α6 –/– mice (strain 129/SvJXC57BL/6 J; Jones *et al*., 1997) were anaesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg i.p.) and perfused through the heart first with 0.9% saline for 1 min, then with fixatives containing either 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB; pH = 7.4); or 4% paraformaldehyde, 0.05% glutaraldehyde and ≈ 0.2% picric acid in PB for 7–20 min. After perfusion the brains were removed; blocks from the vermis of the cerebellum were then cut out and washed in several changes of PB.

Antibodies

The specificity of affinity-purified antibodies against the α1 (code P16: Zezula *et al*., 1991), α6 (P24: Nusser *et al*., 1996; R54XV: Thompson *et al*., 1992), β2/3 (bd17: Haring *et al*., 1985) and γ2 [γ2(1–29): Benke *et al*., 1996; γ2(319–366): Tretter *et al*., 1997; Jechlinger *et al*., 1998] subunits has been described earlier. For immunocytochemistry, the antibodies were used at the following final protein concentrations (μg/mL): P16, 1.3 and 6.3 for pre- and postembedding reactions, respectively; P24, 1.3 for pre-embedding reactions only; R54XV, 0.6 for pre-embedding reactions only; bd17, 40 and 160 for pre- and postembedding reactions, respectively; γ2(1–29), 1 for pre-embedding reactions only.

Rabbit polyclonal antibody (code δ(1–44)R5) was obtained from a different rabbit from that used previously (Jones *et al*., 1997). This antibody, as the previous one, was raised to maltose binding protein-δ-(1–44)-7His fusion protein and was purified by affinity chromatography on a column containing the corresponding glutathione S-transferase-δ(1–44)-7His fusion protein (Jechlinger *et al*., 1998). Antibody δ(1–44)R5 strongly reacted with a 51-kDa protein on Western blot and revealed a weak band at 119 kDa, but all immunolabelling was absent in δ –/– mice, demonstrating the specificity of the antibody and the immunostaining (Nusser *et al*., 1998b). Antibody δ(1–44)R5 was used at a final protein concentration of 1 μg/mL for pre-embedding reactions.

Antibodies β2(351–405)R23 and β3(345–408)R1 were found to be subunit-specific in immunoprecipitation experiments with recombinant $GABA_A$ receptors (Slany *et al*., 1995; Jechlinger *et al*., 1998). Antibodies β2R23 and β3R1 were used for pre-embedding reactions at final protein concentrations of 0.6–0.9 μg/mL and 1.3 μg/mL, respectively.

Antibody α6 (317–371) was used for Western blotting immunoprecipitation and immuno-affinity chromatography experiments. This antibody was generated against a maltose binding protein-α6-(317–371)-7His fusion protein and was purified by affinity chromatography on a column containing the corresponding glutathione S-transferase-α6-(317–371)-7His fusion protein. This antibody completely precipitated α6-subunit-containing receptors and may only have a very small crossreactivity with other $GABA_A$ receptor subunits as tested in immunoprecipitation experiments (see Table 2). Furthermore, it identified the same 56–57-kDa protein as the α6 P24 antibody, but yielded a higher signal-to-noise ratio in Western blots.

Controls

Selective labelling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies were either omitted or replaced by 5% normal serum of the species of the primary antibody. Using a monoclonal antibody to somatostatin (Vincent *et al*., 1985), or a polyclonal antibody to synapsin (Naito & Ueda, 1981), no plasma membrane labelling was observed with our postembedding immunogold method, demonstrating that the labelling observed on the plasma membrane is due to the antireceptor antibodies.

Pre-embedding immunohistochemistry

Normal goat serum (NGS) was used in 50 mM Tris-HCl containing 0.9% NaCl (TBS; pH = 7.4) as the blocking solution for 1 h. The sections were then incubated in the solution of the primary antibodies made up in TBS containing 1% NGS and 0.05% Triton X-100 overnight. After washing, the sections were incubated in either biotinylated goat anti-rabbit IgG or biotinylated goat antimouse IgG (diluted 1 : 50 in TBS containing 1% NGS; Vector Lab. Peterborough, UK) for 2 h. Then the sections were 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. After that, the sections were routinely processed for light- or electron microscopic examination.

Freeze substitution and embedding in Lowicryl resin

The same procedure was used as described earlier (Baude *et al*., 1993; Nusser *et al*., 1995a). Briefly, after perfusion, blocks of tissue were washed in PB; this was followed by Vibratome sectioning (500 μm thickness) and washing with PB overnight. The sections were placed into 1 M sucrose solution in PB for 2 h for cryoprotection before they were slammed (Reichert MM80 E) to a polished copper block cooled with liquid N_2. The sections were then transferred to a freeze-substitution apparatus (Leica CS Auto) for dehydration in methanol at –80 °C and embedding in Lowicryl HM 20 (Chemische Werke Lowi GMBH, Germany) at –50 °C.

Postembedding immunocytochemistry

Postembedding immunocytochemistry on slam-frozen, freeze-substituted and Lowicryl-embedded tissue was carried out on ≈ 80-nm ultrathin sections of the vermis as described earlier (Nusser *et al*., 1995a, b). The sections were incubated on drops of blocking solution (TBS containing 20% NGS) for 30 min, followed by an incubation

on drops of primary antibodies (made up in TBS containing 2% or 5% NGS) overnight at room temperature. After incubation in primary antibody, the sections were washed and transferred to drops of goat antimouse or goat antirabbit IgG coupled to 10 nm gold particles (diluted 1 : 100 in TBS containing 2% or 5% NGS, Nanoprobes Inc) for 2 h. Following several washes, the sections were contrasted with saturated aqueous uranyl acetate followed by lead citrate.

Quantification of immunopositive synapses and immunoparticles for the α1 subunit of the $GABA_A$ receptor

A similar method was used as described earlier (Nusser *et al*., 1995b). Briefly, four to five glomeruli were chosen from each animal on the basis of a clear demarcation by granule cell bodies, capillaries or the grid bars in the well-preserved strip of the ultrathin sections. The images of glomeruli were recorded on a CCD camera at a primary magnification of 4600 for subsequent area measurement at a magnification of 10 000 using NIH Image 1.59 software (Bethesda, USA). The border of glomeruli were delineated by granule cell and glial cell bodies and myelinated axons which were not included in the measured area, but the area covered by mossy fibre terminals was included. The glomeruli were visually scanned in the electron microscope at a magnification of 70 000, and every synapse between a Golgi cell terminal and a granule cell dendrite which was immunopositive for the α1 subunit was recorded at 75 000 × final magnification. A synapse was considered immunopositive if it contained at least two immunoparticles over the membrane specialization. Special care was taken to exclude particles associated with the extrasynaptic plasma membrane. For comparisons, the frequency of immunopositive synapses was calculated for a glomerular area of 100 μm^2. The immunoparticles were counted at a magnification of 75 000 in the anatomically defined synaptic junction. If the lateral displacement of a gold particle was more than 25 nm from the membrane, that particle was not considered to be specifically associated with the synapse.

Quantitative immunoblot analysis

Membranes from three α6 +/+ and three α6 –/– cerebella were prepared, and equal amounts (15–20 μg) of protein per slot were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis in the same 10% polyacrylamide gel (Jechlinger *et al*., 1998). Proteins were blotted to poly vinylidene difluoride membranes and detected by subunit-specific antibodies. Secondary antibodies $(F(ab')_2$ fragments of goat anti rabbit IgG, coupled to alkaline phosphatase, Jackson Immunoresearch Labs., Inc., Wesburg, NY, USA) were visualized by the reaction of alkaline phosphatase with CSPD (Tropix, Bedford, MA, USA) and the chemiluminescent signal was quantified by densitometry of Kodak X-omat S films with the DocuGel 2000i gel documentation system using the RFLP scan software (MWG-Biotech, Ebersburg, Germany). Different exposures of the same polyvinylidene difluoride membrane were used in order to ensure that the measured signal was in the linear range of the X-ray film.

Quantification of $GABA_A$ receptors by immunoprecipitation

$GABA_A$ receptors were solubilized from cerebellar membranes of α6 +/+ or α6 –/– mice using a deoxycholate buffer (0.5% deoxycholate, 0.05% phosphatidylcholine, 10 mM Tris-chloride, pH 8.5, 150 mM NaCl, 500 μM benzamidine, 200 μg/mL bacitracin and 300 μM phenylmethylsulphonylfluoride) as described previously (Jechlinger *et al*., 1998). For immunoprecipitation, 300 μL of the clear deoxycholate membrane extract were mixed with 30 μL of antibody solution (20 μg antibody), and the mixture was incubated under gentle shaking at 4 °C overnight. Then 50 μL of immunoprecipitin (Life Technologies, Inc., Gaithersburg, MD, USA) plus 150 μL of an IP (immunoprecipitation)-low buffer (0.2% Triton X-100, 50 mM Tris-chloride, pH 8.3, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 500 μM benzamidine, 200 μg/mL bacitracin, and 300 μM phenylmethanesulphonylfluoride: PMSF) additionally 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 IP-high buffer (0.5% Triton X-100, 50 mM Tris-chloride, pH 8.3, 600 mM NaCl, 1 mM EDTA, 500 μM benzamidine, 200 μg/mL bacitracin, and 300 μM PMSF) and once with 500 μL IP-low buffer.

Diazepam-sensitive or -insensitive [^{3}H]Ro 15–4513 binding sites were determined as described previously (Jechlinger *et al*., 1998). For [^{3}H]muscimol binding assays the precipitated receptors were suspended in 1 mL of a solution containing 0.1% Triton X-100, 50 mM Tris-citrate buffer, pH 7.1, and 20 nM [^{3}H]muscimol (17.1 Ci/mmol, DuPont NEN) in the absence or presence of 10 μM GABA, and were incubated for 60 min at 4 °C (Jechlinger *et al*., 1998). The suspensions were then filtered through Whatman GF/B filters and the filters were washed twice with 3.5 mL of a 50-mM Tris-citrate buffer (pH 7.1).

Total [^{3}H]muscimol binding sites were determined after precipitating all $GABA_A$ receptors present in the extract with an antibody mixture containing 8 μg β1(350–404), plus 8 μg β2(351–407), plus 10 μg β3(1–13) antibody, using the same assay as described above (Jechlinger *et al*., 1998).

The relative precipitation efficiency of the individual antibodies was determined using recombinant $GABA_A$ receptors containing the respective subunit by measuring [^{3}H]muscimol binding sites precipitated by the antibody as a percentage of the total [^{3}H]muscimol binding sites present in the extract. The factor obtained was then used to correct for 100% precipitation of the antibody. Data obtained after this correction were identical with data produced by measuring the percentage reduction in total [^{3}H]muscimol binding sites after complete elimination of receptors containing the respective subunit by affinity chromatography (Jechlinger *et al*., 1998).

Affinity chromatography of cerebellar $GABA_A$ receptors

Deoxycholate extracts from wild-type mouse cerebella were cycled three times through an affinity column containing immobilized α6(317–371) antibodies. In the efflux of this column α6-subunit-containing receptors were no longer detectable by immunoblotting. Binding of [^{3}H]muscimol was determined in the original extract and in the column efflux after immunoprecipitation with antibodies to one of the following subunits: α1, β2, β3, γ2 or δ. The percentage reduction in immunoprecipitated binding from the column efflux corresponds to the percentage of the respective subunit that is associated with α6 subunits in wild-type cerebella.

Results

Immunoreactivity for the α6 subunit

The distribution of immunoreactivity for the α6 subunit in mouse CNS was identical to that in rat brain (Thompson *et al*., 1992; Gao & Fritschy, 1995; Nusser *et al*., 1996). Granule cells in the cerebellum (Figs 1 and 2) and in the dorsal cochlear nuclei of control mice showed intense staining with either an N- (R54XV-) or a C-terminal (P24) antibody. The staining of granule cells completely disappeared in all α6 –/– animals (n = 11) with both antibodies (Figs 1 and 2; see Jones *et al*., 1997). In agreement with the immunocytochemical

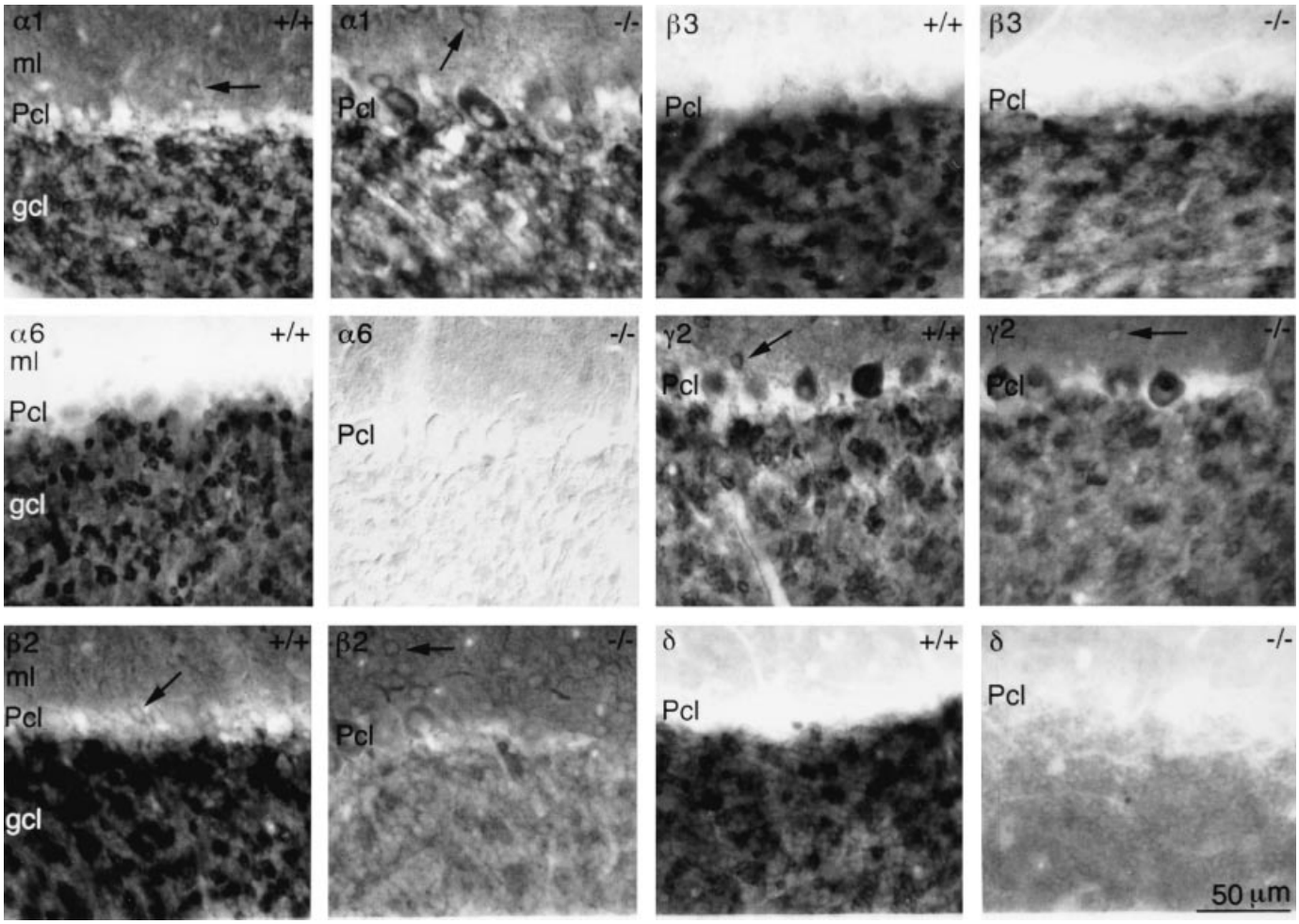


FIG. 1. Light microscopic demonstration of changes in the expression of $GABA_A$ receptor subunits in the cerebella of α6 –/– mice. Pre-embedding immunoperoxidase reactions with antibodies against the α1, α6, β2, β3, γ2 and δ subunits. Immunoreactivity for only the α1, β2 and γ2 subunits is present in the molecular layer (ml), originating from the staining of Purkinje cells and interneurons (arrows). The staining intensity of the molecular layer remains unchanged in α6 –/– mice. Immunoreactivity for the α6 subunit completely disappears in the granule cell layer (gcl) of α6 –/– mice. There is a great reduction in the immunoreactivity for the β2 and δ subunits and a moderate decrease in the staining for the α1, β3 and γ2 subunits in the granule cell layer of α6 –/– mice. Immunopositive glomeruli are present for the α1, β2, β3 and γ2 subunits, but not for the δ subunit. (α6 reactivity in α6 –/– mouse: differential interference contrast image; Pcl: Purkinje cell layer; all micrographs at same magnification.)

results, the 57-kDa protein on immunoblots was absent in cerebella of α6 –/– mice (Fig. 3).

It has recently been reported (Gutierrez *et al*., 1996) that the α6 subunit of the $GABA_A$ receptor is not exclusively present in granule cells of the cerebellum and the cochlear nuclei (Laurie *et al*., 1992; Persohn *et al*., 1992; Varecka *et al*., 1994; Nusser *et al*., 1996), but it is also found in the superior colliculi, olfactory bulb, retina and spinal cord. Gutierrez *et al*. (1996) detected immunoreactivity for the α6 subunit with an antibody against the N-terminal part (1–13) of the subunit. Using another antibody against the N-terminal sequence (1–15) of the α6 subunit (R54XV, Thompson *et al*., 1992), we obtained a similar staining of the olfactory bulb to that reported by Gutierrez *et al*. (1996). However, the labelling of the olfactory bulb could not be detected with the C-terminal antibody. In addition, immunostaining of the olfactory bulb with our N-terminal antibody was identical in wild-type and in α6 –/– mice. We could not detect any specific immunostaining of the superior colliculi using either of the antibodies in control or in α6 –/– mice. These results suggest that the staining of the olfactory bulb and the superior colliculi is due to crossreactivity of the N-terminal antibodies with an unknown protein(s), rather than representing α6 subunits. Immunoreactivity for the α6 subunit was not tested in the spinal cord or the retina in this study.

Immunoreactivity for the δ subunit in control and α6 –/– mice

The great reduction of immunoreactivity for the δ subunit in the granule cell layer of α6 –/– cerebella, as described in Jones *et al*. (1997), was confirmed using a δ-subunit-selective antibody from a different rabbit (Fig. 1; $n = 6$ pairs of animals). Immunostaining of the glomeruli could not be detected in α6 –/– mice at the light microscopic level (Fig. 2), suggesting that the remaining δ subunits in granule cells are not present in the dendritic plasma membrane. The remaining immunoreactivity for the δ subunit in α6 –/– cerebella could not be assigned to subcellular compartments using electron microscopy because of its very low level of expression. Quantitative immunoblot analysis of cerebellar membranes demonstrated ≈ 77% reduction of the δ subunit in α6 –/– mice (Fig. 3 and Table 1).

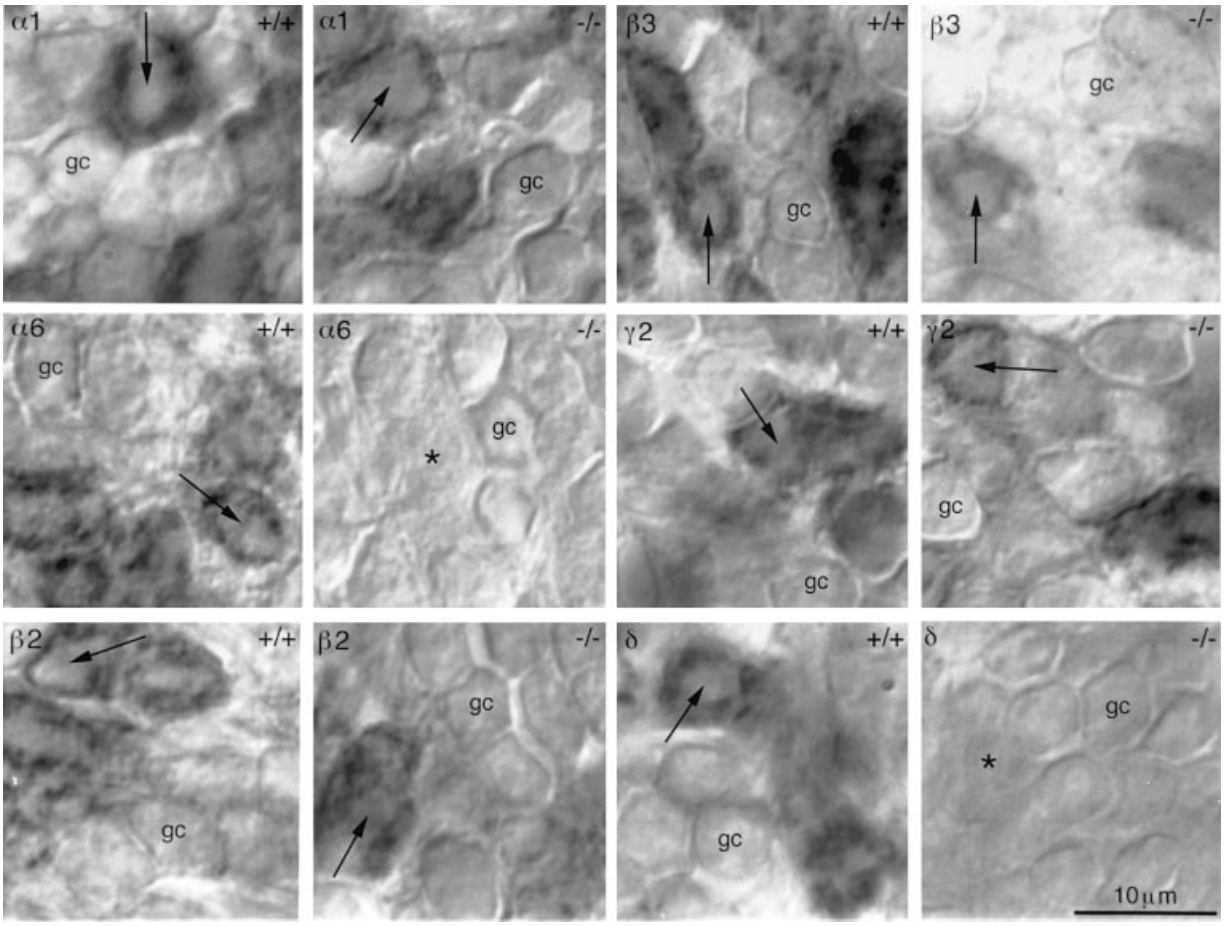


FIG. 2. High-power light microscopic images of control (+/+) and α6 –/– mice cerebellar granule cell layer immunoreacted for $GABA_A$ receptor subunits. In control animals, immunoreactivity for all subunits is mainly localized to glomeruli (e.g. arrows); the granule cell bodies (gc) were only weakly outlined by the reaction end-product. Glomeruli appear as dark rings of the immunopositive granule cell dendrites surrounding a pale centre (arrows) representing the immunonegative mossy fibre terminals. Although, the immunostaining for the α1, β2, β3 and γ2 subunits is reduced in the granule cell layer of α6 –/– mice, immunopositive glomeruli have a similar appearance to that observed in control mice. This result indicates that the remaining immunoreactive receptors may have a similar distribution to that in control mice. Every glomerulus is immunonegative (asterisks) for the α6 and δ subunits, suggesting that the remaining immunoreactive δ subunits may not be present on the surface of granule cell dendrites. (Differential interference contrast images; all micrographs at same magnification.)

Immunoreactive β2, β3 and γ2 subunits are reduced in granule cells of α6 –/– mice

In the cerebellar cortex of control mice, immunostaining for the β2 subunit was most intense in the granule cell layer (Fig. 1), demonstrating a high level of expression in granule cells. Strong reactivity was confined to the glomeruli, whereas granule cell bodies were only weakly outlined by the reaction end-product. The glomeruli appeared as dark rings with a pale centre, representing the strongly reactive granule cell dendrites at the periphery and the immunonegative mossy fibre terminals at the centre (Fig. 2). Purkinje cells and interneurons of the molecular layer were also immunopositive (Fig. 1). There was a great reduction in the staining intensity in the granule cell layer of α6 –/– mice (Fig. 1; $n = 4$ pairs of animals), although the intensity of immunostaining for the β2 subunit in the molecular layer was approximately the same in control and α6 –/– animals. Quantitative immunoblot analyses revealed that in α6 –/– cerebella the expression of β2 subunits was reduced to 47% of the wild-type level (Fig. 3, Table 1). In spite of the large reduction in the β2 subunit, immunopositive glomeruli could be detected in the granule cell layer of α6 –/– mice (Fig. 2), indicating that the remaining β2 subunits were present on the surface of granule cells.

Immunostaining for the β3 subunit was restricted to the granule cell layer (Fig. 1) in the cerebellar cortex of control mice. At high magnification, the staining of the granule cell layer resembled that obtained for the β2 subunit. Strongly immunopositive glomeruli, which appeared as dark rings with a pale centre, dominated over the weakly reactive granule cell bodies (Fig. 2). In α6 –/– mice, a reduction in the staining of the granule cell layer was apparent. The remaining immunopositive glomeruli had a similar appearance to that observed in control mice (Fig. 2; $n = 3$ pairs of animals), but were weaker. On average, ≈ 21% of the β3 subunit protein disappeared in α6 –/– cerebella (Fig. 3, Table 1).

To confirm that the remaining β subunits in α6 –/– mice were indeed in the plasma membrane of granule cells, the β2/3 subunits were localized with an electron microscopic immunogold procedure. Gold particles for the β2/3 subunits were enriched in Golgi-cell-to-granule-cell synapses, and were also present at a lower density on the extrasynaptic somatic and dendritic membranes of control mice (Fig. 4), similar to that described in rat cerebellum (Nusser *et al.*, 1995b). In α6 –/– mice, a similar pattern of labelling was observed. Some Golgi synapses contained several gold particles (Fig. 4), whereas others had either zero or only a few particles, indicating a variability

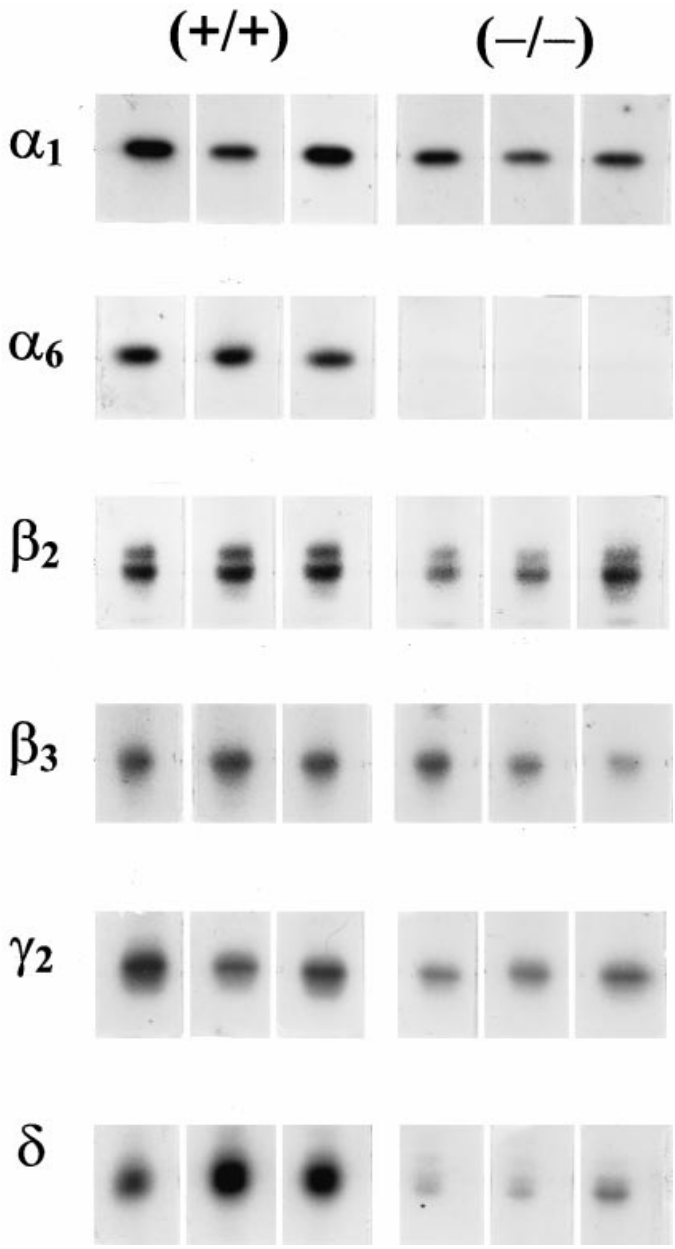


FIG. 3. Immunoblot analysis of the α1, α6, β2, β3, γ2 and δ subunits in wild-types (+/+) and α6 –/– mice cerebellar membranes. The immunoreactive band for the α6 subunit completely disappears in α6 –/– cerebella. There is a large reduction in the intensity of immunoreactive bands for the β2 and δ subunits in α6 –/– cerebella compared with the control. A more moderate decrease in the intensity of the bands for the α1, β3 and γ2 subunits is detected in α6 –/– mice. The results of the quantification of these immunoblots are included in Table 1.

in the receptor content of individual synapses, as also described for cerebellar stellate cells (Nusser *et al*., 1997). Immunoparticles were also detected on the extrasynaptic membranes. These results demonstrate that most of the β subunits in α6 –/– granule cells are in the plasma membrane, as predicted from the staining pattern of the glomeruli revealed by light microscopy. Immunoparticles for the β2/3 subunits could not be detected in the glutamatergic mossy fibre to granule cell synapses of α6 –/– mice (Nusser *et al*., 1998b).

The distribution of immunoreactive γ2 subunits in the cerebellum of control mice was similar to that in the rat (Gutierrez *et al*., 1994; Fritschy & Mohler, 1995; Somogyi *et al*., 1996). Purkinje cells and interneurons of the molecular layer showed prominent labelling for the γ2 subunit, which appeared unchanged in α6 –/– mice (Fig. 1). However, the staining of the granule cell layer was reduced in α6 –/– mice when compared with control animals (Fig. 1; $n = 4$ pairs of mice). Immunoblot analysis revealed a 41% reduction of the γ2 subunit protein in α6 –/– cerebella (Fig. 3, Table 1). Similar to the β2 and β3 subunits, but unlike the δ subunit, strongly immunopositive glomeruli were detectable in the granule cell layer of α6 –/– mice (Fig. 2), suggesting that the γ2 subunit is also present in the plasma membrane of granule cells.

The α1 subunit is not significantly up-regulated in GABAergic Golgi synapses of α6 –/– granule cells

Distribution of immunostaining for the α1 subunit was similar in the cerebellar cortex of control mice to that observed in the rat, cat and monkey (Somogyi *et al*., 1989, 1996; Zimprich *et al*., 1991; Fritschy *et al*., 1992; Fritschy & Mohler, 1995). In the molecular layer, cell bodies of interneurons and the dendrites of Purkinje cells and interneurons were immunostained. In the granule cell layer, strongly positive glomeruli dominated over weakly stained granule cell bodies (Figs 1 and 2). In two out of four α6 –/– mice immunohistochemically tested for the α1 subunit, a reduction in the immunostaining of the granule cell layer was observed (Fig. 1), in the other two animals, no obvious change in the staining was detected. Quantitative Western blot analysis performed on membrane extracts from α6 +/+ and α6 –/– mice revealed a significant reduction of 27% in the 51-kDa immunoreactive band representing the α1 subunit (Fig. 3, Table 1).

Although the amount of α1 subunit slightly decreased in the whole cerebella of α6 –/– mice, the number of α1-subunit-containing receptors could have increased in Golgi-cell-to-granule-cell synapses, compensating for the loss of α6-subunit-containing synaptic receptors. Such a reorganization of extrasynaptic and synaptic receptors could have accounted for the fact that no obvious motor deficits were observed in α6 –/– mice (Homanics *et al*., 1997; Jones *et al*., 1997; Korpi *et al*., 1999). We have tested the above hypothesis by quantitative immunogold localization of the α1 subunit in Golgi-cell-to-granule-cell synapses. Immunogold labelling for the α1 subunit in cerebellar granule cells of control mice was similar to that in the rat and cat (Nusser *et al*., 1995b, 1996). Gold particles were enriched in synaptic junctions made by Golgi cell terminals and granule cell dendrites and were also present at a lower density on the extrasynaptic somatic and dendritic membranes (Fig. 5). The number of immunoparticles per immunopositive Golgi synapse was not significantly higher in α6 –/– than in control mice ($n = 3$ pairs of animals, Fig. 6).

A potential mechanism maintaining inhibitory function with synapses that lost the α6-subunit-containing receptors is an increase in the number of synapses equipped only with α1-subunit-containing receptors on granule cells. However, the frequency of α1 subunit immunopositive synapses in the glomeruli of control and α6 –/– animals was not different (Fig. 6), assuming that the distribution of the size of synaptic areas was unaltered in α6 –/– mice. The lack of change in immunopositive synapses demonstrates the absence of up-regulation in the synaptic pool of the α1-subunit-containing receptors. Thus, the ≈ 26% reduction of the α1 subunit protein very probably results in a lower density of the extrasynaptic α1 subunits, provided most of the reduction took place on granule cells.

Overall reduction of the $GABA_A$ receptors in α6 –/– cerebellum

In order to estimate the number of $GABA_A$ receptors present in mouse cerebellum, $GABA_A$ receptors were extracted from cerebella

TABLE 1. Quantification of subunit proteins in cerebellar membranes by Western blot analysis

Antibodies	(+/+)	(*n*)	(–/–)	(*n*)	Reduction (%)	*P*-value
α1	100.0 ± 5.8	(17)	73.5 ± 6.7	(17)	26.5	< 0.01
β2	100.0 ± 7.6	(12)	46.6 ± 5.8	(12)	53.4	< 0.01
β3	100.0 ± 7.1	(15)	78.8 ± 6.2	(15)	21.2	< 0.05
γ2	100.0 ± 8.4	(9)	58.9 ± 7.0	(9)	41.1	< 0.01
δ	100.0 ± 10.5	(6)	22.7 ± 4.3	(6)	77.3	< 0.01

Equal amounts of cerebellar membrane proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subunit levels were compared in α6 +/+ and α6 –/– membranes by Western blot analysis. Results are expressed as percentages of the mean subunit level found in α6 +/+ membranes ± SEM (*n* = number of experiments). For statistical comparisons unpaired Student's *t*-test was used.

TABLE 2. Quantification of $GABA_A$ receptor subtypes by immunoprecipitation with subunit-specific antibodies and [^{3}H]muscimol binding

	α6 +/+ mice		α6 –/– mice			α6 +/+ mice	
Antibodies to subunit	Percentage of total receptors	(*n*)	Reduction (%)	(*n*)	*P*-value	Reduction by α6 column (%)	(*n*)
β1 + β2 + β3	100.0 ± 4.8	(18)	49.4 ± 3.7	(18)	< 0.0001	55.7 ± 1.2	(3)
α1	74.8 ± 6.0	(3)	26.0 ± 6.7	(3)	= 0.05	32.3 ± 2.5	(6)
α6	55.7 ± 1.2	(3)	98.2 ± 0.5	(3)	< 0.0001	–	
β2	90.0 ± 4.2	(3)	53.6 ± 3.3	(3)	< 0.01	51.3 ± 4.8	(3)
β3	36.4 ± 1.6	(12)	28.9 ± 9.8	(12)	< 0.05	62.9 ± 4.3	(6)*
γ2	68.3 ± 5.6	(3)	36.7 ± 1.4	(3)	< 0.01	45.0 ± 4.2	(3)
δ	26.7 ± 1.5	(3)	89.6 ± 4.0	(3)	< 0.01	98.7 ± 0.8	(3)

$GABA_A$ receptors were extracted from α6 +/+ and α6 –/– cerebella and then precipitated by subunit-specific antibodies. Receptor subtypes containing the respective subunit were quantified using [^{3}H]muscimol binding. In the second column, the proportion of receptors containing a given subunit were expressed as a percentage of the total receptors obtained after precipitation with a mixture of three antibodies directed against each of the three β subunits (100% value). Data were calculated after normalization for precipitation efficacy. The percentage reduction in these receptors in α6 –/– animals (fourth column) was calculated from a direct comparison of immunoprecipitation data from α6 +/+ and α6 –/– cerebellar extracts used in the same individual experiments. In all cases, there was significantly less receptor in the cerebellum of α6 –/– animals compared with wild-type animals. In the seventh column, the reduction of receptors containing the respective subunits was determined after removal of α6-subunit-containing receptors from cerebellar extracts of α6 +/+ mice by immuno-affinity chromatography. A comparison of the fourth and seventh columns indicates that the difference between the two sets of animals was significant (*$P < 0.05$) only for the β3 subunit content. Results are expressed as means ± SEM. For statistical comparisons, unpaired Student's *t*-test was used.

of control and α6 –/– mice. After solubilization, 68% of the [^{3}H]Ro 15–4513 or [^{3}H]muscimol binding sites present in the membranes could be recovered in the extract as determined by immunoprecipitation with a combination of β1, β2 and β3 antibodies (Jechlinger *et al*., 1998). This corresponds to 93% of the binding sites identified in the extract and in the 100 000 ***g*** membrane pellet after extraction. Since there was no significant difference in the efficiency of solubilization by detergent between [^{3}H]muscimol binding sites and diazepam-sensitive or -insensitive [^{3}H]Ro 15–4513 binding sites, it can be concluded that the extracted receptors were representative of the entire functional $GABA_A$ receptor population.

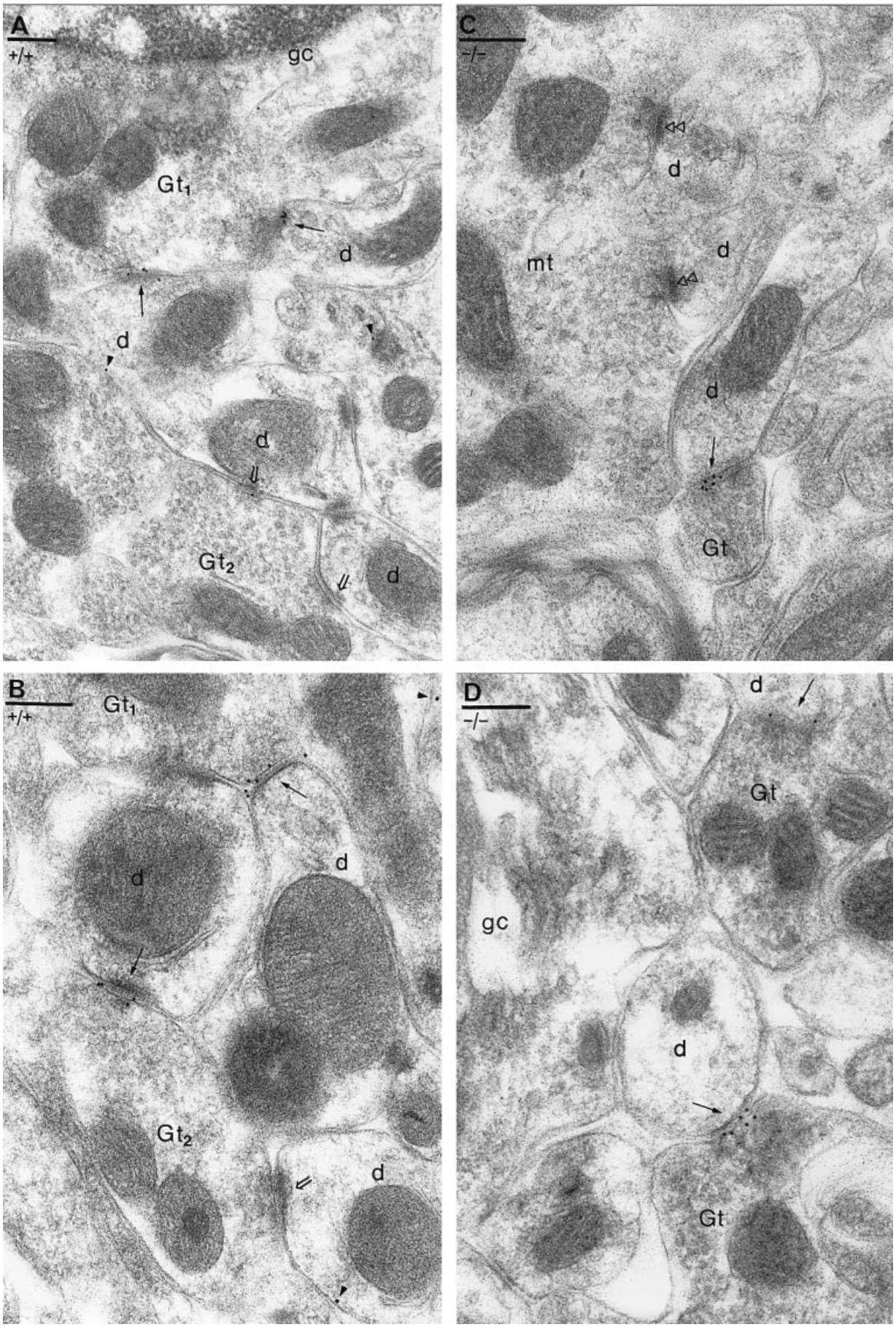
Quantitative immunoprecipitation of $GABA_A$ receptors in cerebellar extracts using subunit-specific antibodies revealed that 75, 56, 90, 36, 68 and 27% of all $GABA_A$ receptors in the extract from control cerebella contained α1, α6, β2, β3, γ2 and δ subunits, respectively (Table 2). The proportional change in the amount of different subunits in α6 –/– cerebella as determined with immunoprecipitation was very similar to that obtained with the immunoblotting experiments (cf. Tables 1 and 2). Interestingly, the number of $GABA_A$ receptors precipitated by the mixture of β1, β2 and β3 antibodies in extracts from α6 –/– cerebella (range 0.9–1.7 pmol/mg protein) was only 51% of that precipitated by these antibodies in control cerebella (range 1.9–3.4 pmol/mg protein), showing a 49% decrease in the total cerebellar $GABA_A$ receptors in α6 –/– mice.

Comparison of receptor subunit composition in α6 –/– and wild-type cerebella

In order to compare the amount of different subunits in α6 –/– cerebellum with that expected to be present in wild-type cerebellum in the absence of α6-subunit-containing receptors, deoxycholate extracts from the cerebellum of α6 +/+ mice were passed through an immuno-affinity chromatography column containing immobilized antibodies to the α6 subunit (residues 317–371). In the efflux of this column α6-subunit-containing receptors were no longer detectable by immunoblotting. Receptors containing α1, β2, β3, γ2 and δ subunits were quantified in the original extract and in the efflux of the column by immunoprecipitation with subunit-specific antibodies and [^{3}H]muscimol binding. The difference between the two values represents the percentage of subunits associated with α6 subunits in wild-type cerebellum. The data indicated that α1, β2, γ2 or δ subunits were reduced in the efflux to an extent similar to that observed in α6 –/– cerebella. The reduction of β3 subunits by the α6 subunit immuno-affinity column, however, was significantly larger than that observed in α6 –/– cerebella (Table 2).

Discussion

The restricted expression of the α6 subunit of the $GABA_A$ receptor to granule cells of the cerebellum and the cochlear nuclei (Laurie *et al*., 1992; Turner *et al*., 1993; Varecka *et al*., 1994; Nusser *et al*., 1996) suggested that the disruption of the α6 subunit gene may result in dysfunctions of cerebellum-associated behaviours such as movement co-ordination, motor learning and maintenance of orientation and balance. However, no such cerebellar-associated motor deficits were observed in α6 –/– mice (Homanics *et al*., 1997; Jones *et al*., 1997; Korpi *et al*., 1999), indicating that $GABA_A$ receptors in α6 –/– cerebellar granule cells could satisfy the tested functional requirements. There are at least four possibilities regarding changes in the quality and the quantity of $GABA_A$ receptors in granule cells of α6 –/– mice. Firstly, the expression of new $GABA_A$ receptor subunit genes (subunits without


A
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a significant expression in control granule cells, e.g. α2, α3, α4 or α5) may be turned on to compensate for the loss of the α6 subunit. However, quantitative immunoblot analysis revealed that the expression of the α2 and α4 subunits was not elevated in the cerebellum of α6 –/– mice (data not shown). Secondly, receptors that do not contain the α6 subunit ($\alpha_1\beta_{2/3}\gamma_2$, $\alpha_1\beta_{2/3}\delta$; Jechlinger *et al*., 1998) may be upregulated in GABAergic Golgi synapses, resulting in a qualitative, but not necessarily a quantitative change in the receptor content of these synapses. Thirdly, the loss of α6-subunit-containing receptors may not be compensated by expression of other receptors, resulting in a large overall reduction in the amount of $GABA_A$ receptors in the cerebellum of α6 –/– animals. Fourthly, the subunit composition of the remaining receptors in the cerebellum may change resulting in functional compensation for the loss of α6-subunit-containing receptors.

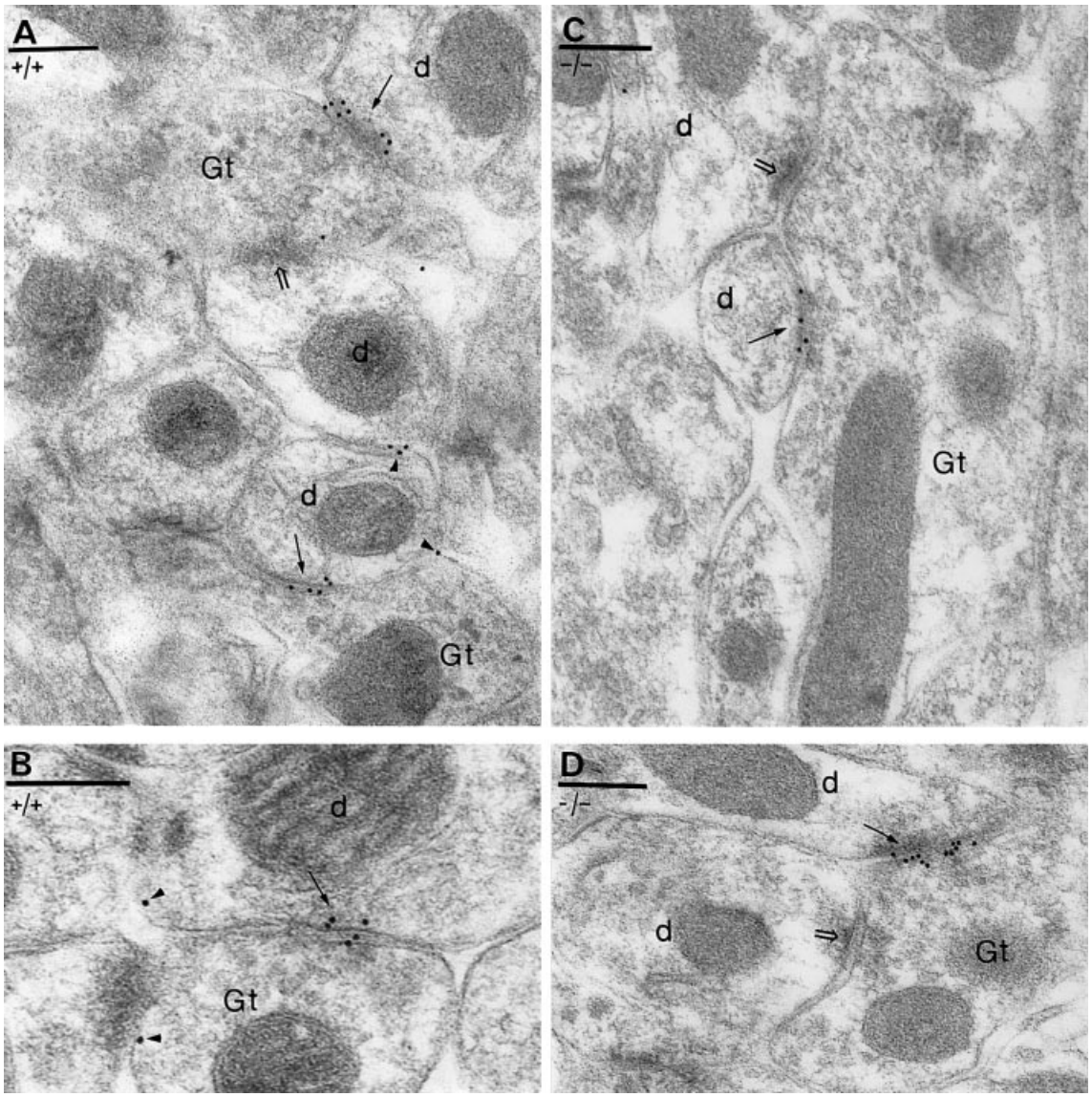

FIG. 5. Electron microscopic demonstration of immunoreactivity for the α1 subunit in the granule cell layer of control (A and B; +/+) and α6 –/– mice (C and D; –/–). Postembedding immunogold (10 nm particles) reactions on Lowicryl resin-embedded tissue. Gold particles are concentrated in some synapses (arrows) made by Golgi cell terminals (Gt) with granule cell dendrites (d). Immunonegative Golgi synapses (open arrows) are also present in both control (A) and α6 –/– (C and D) mice. Extrasynaptic receptors can also be seen (arrowheads). The number of particles per synapse is variable, with a similar range in both control and α6 –/– animals (also see Fig. 6). Scale bars, 0.2 μm.

FIG. 4. Electron micrographs showing immunoreactivity for the β2/3 subunits in cerebellar glomeruli of control (A and B; +/+) and α6 –/– mice (C and D; –/–). Postembedding reactions on Lowicryl resin-embedded tissue with 10 nm gold particles. Although, there is a great reduction of the β subunit content of granule cells, the remaining β subunits are present on the surface of granule cells at both synaptic and extrasynaptic sites. In control animals (A and B), symmetrical synapses (arrows) between Golgi cell terminals (Gt) and granule cell dendrites (d) show an enrichment of gold particles. The number of immunoparticles per synapse is variable, some of the synapses are immunonegative (open arrows by Gt_2), some others contain a large number of particles (arrows by Gt_1). Some extrasynaptic particles are also present (arrowheads). In α6 –/– mice (C and D), an enrichment of gold particles can be seen in synapses (arrows) made by Golgi cell terminals with granule cell dendrites. These synapses contain a similar number of particles to that observed in control mice. Asymmetrical synapses (double open triangles) made by a mossy fibre terminal (mt) with granule cell dendrites are immunonegative. Scale bars: 0.2 μm.

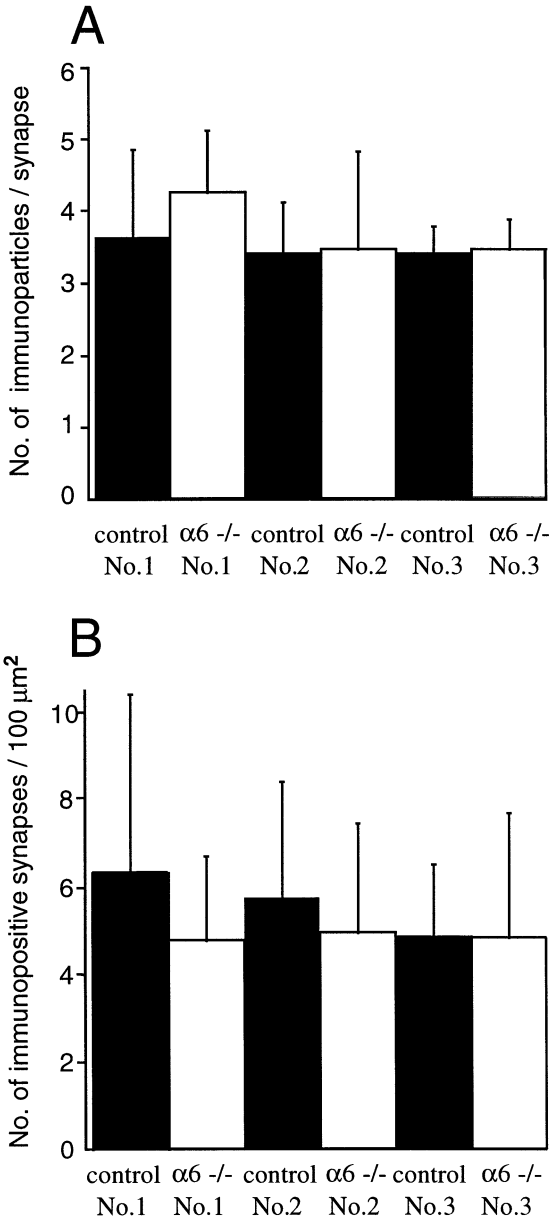

FIG. 6. Comparison of the α1 subunit content and frequency of Golgi synapses in control and α6 –/– mice. A. The number of immunoparticles per immunopositive (at least two particles) Golgi synapses is compared in three pairs of animals. Immunoreactivity of synapses is not significantly different ($P > 0.2$, Mann–Whitney *U*-test) between control (No.1: 3.7 ± 1.2 (mean ± SD), $n = 21$; No.2: 3.4 ± 0.7, $n = 27$; No.3: 3.4 ± 0.3, $n = 17$) and α6 –/– mice (No.1: 4.3 ± 0.9, $n = 18$; No.2: 3.5 ± 1.3, $n = 19$; No.3: 3.5 ± 0.4, $n = 18$). B. The frequency of immunopositive synapses is not significantly different ($P > 0.3$, Mann–Whitney *U*-test) between pairs of animals, or between the control (No.1: 6.4 ± 4.0, $n = 21$; No.2: 5.8 ± 2.6, $n = 27$; No.3: 4.8 ± 2.9, $n = 17$) and α6 –/– groups of mice (No.1: 4.8 ± 1.9, $n = 18$; No.2: 5.8 ± 2.6, $n = 19$; No.3: 4.8 ± 1.7, $n = 18$).

Overall reduction in the amount of $GABA_A$ receptors in α6 –/– cerebellum

All comparisons of immunoprecipitation data are based on the assumption that the subunit composition of receptors does not affect their solubilization differentially. We are not aware of any evidence contradicting this assumption for $GABA_A$ receptors. The determination of the abundance of $GABA_A$ receptor subtypes in mouse cerebellum in the present study shows that ≈ 55% of all $GABA_A$ receptors contain the α6 subunit. A slightly smaller proportion was found in the rat cerebellum (Jechlinger *et al*., 1998). The finding that $GABA_A$ receptors in α6 –/– mice were reduced by 49% compared with control mice, indicates that there is no significant compensation for the loss of α6-subunit-containing receptors by an up-regulation of other $GABA_A$ receptors in the cerebellum. Light microscopic immunocytochemistry supported the conclusion of an overall reduction in $GABA_A$ receptors in granule cells. However, a functional up-regulation of receptors through the redistribution of receptor pools in the plasma membrane, for example by increasing synaptic receptor numbers as demonstrated for hippocampal granule cells (Nusser *et al*., 1998a), could take place even at reduced overall receptor level. The testing of the synaptic receptor pool by quantitative immunogold localization at the electron microscopic level showed no significant up-regulation in the frequency of immunopositive Golgi cell synapses or in their α1 subunit content. These results, in agreement with previous findings (Jones *et al*., 1997; Makela *et al*., 1997), demonstrate that after the targeted disruption of the α6 subunit gene, the number of $GABA_A$ receptor subtypes is greatly reduced, which results in a large decrease in the overall amount of $GABA_A$ receptors in granule cells.

The reduction in the α1, β2, γ2 and δ subunits correlates well with the expected reduction from the complete loss of α6 receptors in α6 –/– mouse cerebellum

Quantitative immunoblot analysis showed a significant reduction of the α1, β2, β3, γ2 and δ subunits in cerebellar membranes of α6 –/– mice. The data for the α1 and δ subunits confirm previous results (Jones *et al*., 1997). In order to investigate a possible change in the composition of $GABA_A$ receptors in the cerebellum of α6 –/– mice, the reductions in different subunits were compared with that expected to remain after the complete removal of α6-subunit-containing receptors from wild-type cerebellum by α6 subunit-specific immunoaffinity chromatography.

The α1 subunit is present in ≈ 75% of all cerebellar $GABA_A$ receptors, as shown in this and previous studies (Khan *et al*., 1993, 1996; Pollard *et al*., 1995). A 32% reduction of α1 subunits observed in these experiments agrees reasonably with the 26% reduction of α1 subunits in α6 –/– cerebella, and supports the conclusion that there was no significant compensatory upregulation of receptors containing α1 subunits in the cerebellum of α6 –/– mice. The same holds true for β2- or γ2-subunit-containing receptors. After complete removal of α6-subunit-containing receptors from α6 +/+ cerebella by immuno-affinity chromatography, β2 or γ2-subunit-containing receptors were reduced by 51 and 45%, respectively. These values were not significantly different from the 54 or 37% reduction of β2 or γ2-subunit-containing receptors observed in α6 –/– cerebella.

These results are unexpected, and could be interpreted as indicating that the expression of more α1βxγ2 receptors instead of α6βxγ2 receptors was unnecessary for maintaining the function of cerebellar granule cells. Nevertheless, the results do not exclude the possibility that changes in granule cells were masked by different changes in other cerebellar cell types. However, the light microscopic immunocytochemical results did not reveal large changes in the immunoreactivity of cells outside the granule cell layer.

After complete removal of α6-subunit-containing receptors from α6 +/+ cerebella, δ-subunit-containing receptors were almost completely eliminated, indicating that the δ subunits are exclusively associated with α6 subunits in the cerebellum of the mouse (Jones *et al*., 1997). Immunoprecipitation experiments in α6 –/– cerebella indicated a reduction of the δ subunit by 90%. This percentage is close to that remaining after removal of α6-subunit-containing recep-

tors from α6 +/+ cerebellum. In Western blot experiments, however, δ subunits were reduced by only 77% in α6 –/– cerebella, confirming previous results (Jones *et al*., 1997). The remaining δ subunits may represent an incompletely assembled pool of subunits in intracellular compartments of α6 –/– cerebella.

The altered abundance of the β3 subunit in α6 –/– cerebellum suggests changes in receptor subunit composition

After complete precipitation of α6-subunit-containing receptors from α6 +/+ cerebella, β3-subunit-containing receptors were reduced by 63%, compared with only a 29% reduction of these receptors in α6 –/– cerebella. The unexpectedly high abundance of β3 subunits in α6 –/– mouse cerebellum may be the consequence of a selective sparing of the β3 subunit expression. The remaining β3 subunits may form receptors predominantly with the α1 and γ2 subunits in granule cells, but with an altered subunit composition.

Some of the $GABA_A$ receptor subunits (α1, β2, γ2 and δ) were reduced as expected, whereas the β3 subunit appears to be less affected in the cerebellum of α6 –/– mice. It is therefore possible that the composition of $GABA_A$ receptors in the cerebellum changed as a consequence of the missing α6-subunit-containing receptors, leading to functional compensation for the elimination of α6-subunit-containing receptors. The main difference between receptors containing the β2 or β3 subunits in a heterologous expression system is in their regulation by cAMP-dependent protein kinase (PKA). Phosphorylation of the β3 subunit led to an enhancement of the GABA-activated response, whereas the β2 subunit was not phosphorylated by PKA (McDonald *et al*., 1998). Therefore, the increased proportion of β3 subunits in α6 –/– cerebella would render a larger proportion of $GABA_A$ receptors more susceptible to PKA-mediated functional up-regulation.

Because the cerebellum contains several parallel and/or sequentially connected GABAergic neuronal types, and our biochemical analysis was carried out on whole cerebellar extracts, the sites of change in receptor composition have not been determined. The immunocytochemical results indicate no major changes in the abundance of receptors in the molecular cell layer, but the deep cerebellar nuclei have not been analysed. It is also possible that changes in voltage- and/or other ligand-gated ion channels, such as the *N*-methyl-D-aspartate (NMDA) receptor, compensate for this large reduction in the amount and complexity of $GABA_A$ receptors. Indeed, following the selective elimination of Golgi cells, which release GABA onto the granule cells, NMDA-receptor-mediated excitation is down-regulated, possibly as a compensatory adaptation for the lost inhibitory influence (Watanabe *et al*., 1998).

Reduction in the number of $GABA_A$ receptor subtypes in granule cells of α6 –/– mice

Although cerebellar granule cells receive GABAergic input only from a single cell type, the Golgi cell, two distinct types of $GABA_A$ receptor mediated inhibition (tonic and phasic) have recently been described in adult rats (Brickley *et al*., 1996; Wall & Usowicz, 1997; Rossi & Hamann, 1998). At least six $GABA_A$ receptor subtypes are expressed by granule cells (Caruncho & Costa, 1994; Khan *et al*., 1994, 1996; Quirk *et al*., 1994; Pollard *et al*., 1995; Wisden *et al*., 1996; Jechlinger *et al*., 1998) with dissimilar kinetic and pharmacological properties (Pritchett *et al*., 1989; Verdoorn *et al*., 1990; Angelotti & Macdonald, 1993; Puia *et al*., 1994; Saxena & Macdonald, 1994, 1996; Brickley *et al*., 1995, 1996; Kaneda *et al*., 1995; Tia *et al*., 1996a,b). We recently proposed (Nusser *et al*., 1998a) that a differential cell surface distribution of distinct $GABA_A$ receptor subtypes, together with their dissimilar functional properties, may underlie the different forms of inhibition observed in granule cells. Namely, since δ-subunit-containing receptors ($\alpha_6\beta_{2/3}\delta$ and $\alpha_6\alpha_1\beta_{2/3}\delta$) are only present extrasynaptically (Nusser *et al*., 1998b), have high affinity for GABA (Saxena & Macdonald, 1996) and do not desensitize upon prolonged presence of agonist (Saxena & Macdonald, 1994), they are well suited to mediate tonic inhibition which originates from the persistent activation of $GABA_A$ receptors (Brickley *et al*., 1996). In contrast, the synaptic γ2-subunit-containing receptors are more likely to underlie the phasic inhibition, because they have a much lower affinity for GABA and have a much faster desensitization rate than the δ-subunit-containing receptors (Saxena & Macdonald, 1994, 1996; Tia *et al*., 1996b). Although γ2-subunit-containing receptors are clearly concentrated in the synaptic junction, they are also present in the extrasynaptic membrane (Somogyi *et al*., 1996; Nusser *et al*., 1998b). In addition, the extrasynaptic receptors may also prolong the decay of inhibitory postsynaptic currents (IPSCs) due to their delayed activation by transmitter spilling over from synaptic junctions (Rossi *et al*., 1998). It is therefore possible that the lack of the α6 and δ subunits from the surface of α6 –/– granule cells results in a loss or great reduction of tonic inhibition, as well as a reduction in phasic inhibition in granule cells. Due to the loss of synaptic α6-subunit-containing receptors ($\alpha_6\beta_{2/3}\gamma_2$ and $\alpha_1\alpha_6\beta_{2/3}\gamma_2$) and the possible changes in the subunit composition of the remaining receptors, the amplitude and kinetics of inhibitory synaptic currents may also be altered. Although the roles of the two different forms of inhibition are not understood for cerebellar function, it is likely that tonic inhibition may regulate passive membrane properties of granule cells (e.g. membrane time constant and input resistance) to influence the time window for synaptic integration (Gabbiani *et al*., 1994; Hausser & Clark, 1997), whereas phasic inhibition may modify the output pattern of granule cells (Hausser & Clark, 1997). This hypothesis remains to be tested in α6 –/– animals.

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Abbreviations

GABA, γ-aminobutyric acid; $GABA_A$, *γ-aminobutyric acid type A (receptor); NGS, normal goat serum; NMDA, N*-methyl-D-aspartate ; PB, 0.1 M phosphate buffer; PKA, protein kinase A; TBS, 50 mM Tris-HCl containing 0.9% NaCl.

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