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Ligand-Gated Ion Channel Subunit Partnerships: $GABA_A$ Receptor α_6 Subunit Gene Inactivation Inhibits δ Subunit Expression

A. Jones,[1a] E. R. Korpi,[2a] R. M. McKernan,[3a] R. Pelz,[4a], Z. Nusser,[5] R. Mäkelä,[2] J. R. Mellor,[1] S. Pollard,[6] S. Bahn,[1] F. A. Stephenson,[6] A. D. Randall,[1] W. Sieghart,[4] P. Somogyi,[5] A. J. H. Smith,[1] and W. Wisden[1]

[1]Medical Research Council Laboratory of Molecular Biology, Medical Research Council Centre, Cambridge CB2 2QH, United Kingdom, [2]Department of Pharmacology and Clinical Pharmacology, University of Turku, Fin-20014 Turku, Finland, Department of Alcohol Research, National Public Health Institute, Fin-00180 Helsinki, Finland, [3]Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Harlow, CM20 2QR, United Kingdom, [4]University Clinic for Psychiatry, Department of Biochemical Psychiatry, 1090 Vienna, Austria, [5]Medical Research Council Anatomical Neuropharmacology Unit, Oxford University Department of Pharmacology, Oxford, OX1 3TH, United Kingdom, and [6]School of Pharmacy, University of London, Brunswick Square, London, WC1N 1AX, United Kingdom

Cerebellar granule cells express six $GABA_A$ receptor subunits abundantly (α_1, α_6, β_2, β_3, γ_2, and δ) and assemble various pentameric receptor subtypes with unknown subunit compositions; however, the rules guiding receptor subunit assembly are unclear. Here, removal of intact α_6 protein from cerebellar granule cells allowed perturbations in other subunit levels to be studied. Exon 8 of the mouse α_6 subunit gene was disrupted by homologous recombination. In α_6 $-/-$ granule cells, the δ subunit was selectively degraded as seen by immunoprecipitation, immunocytochemistry, and immunoblot analysis with δ subunit-specific antibodies. The δ subunit mRNA was present at wild-type levels in the mutant granule cells, indicating a post-translational loss of the δ subunit. These results provide genetic evidence for a specific association between the α_6 and δ subunits. Because in α_6 $-/-$ neurons the remaining α_1, $\beta_{2/3}$, and γ_2 subunits cannot rescue the δ subunit, certain potential subunit combinations may not be found in wild-type cells.

Key words: $GABA_A$ receptor; α_6 subunit; granule cell; cerebellum; homologous recombination; gene targeting; transgenic mice; knockout mice; ligand-gated ion channel; subunit sorting; subunit assembly; internal ribosome entry site; dicistronic mRNA; muscimol; SR95531; Ro 15-4513; flunitrazepam

In vertebrate brains, $GABA_A$ receptors are the principal mediators of inhibitory synaptic transmission. They are agonist-gated anion channels formed of pentameric assemblies of subunits arranged around an aqueous pore (Seeburg et al., 1990; Sieghart, 1995; Stephenson, 1995; Tyndale et al., 1995; McKernan and Whiting, 1996). The subunit genes (α_{1-6}, β_{1-3}, γ_{1-3}, and δ) are differentially transcribed, and the polypeptides are assembled in many possible combinations depending on cell type (Persohn et al., 1992; Wisden et al., 1992; Fritschy and Möhler, 1995). There are several important unresolved issues. Why is this receptor heterogeneity needed for synaptic function? What is the subunit composition of native subtypes of receptor? Are there rules guiding which subunits assemble with each other?

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Correspondence should be addressed to W. Wisden, Medical Research Council Laboratory of Molecular Biology, Medical Research Council Centre, Hills Road, Cambridge, CB2 2QH, UK.

[a]These authors made equally important contributions to different aspects of this work.

The cerebellum is an excellent brain area for investigating these questions. Its clearly defined circuitry allows an almost complete account of which cerebellar cell types express which $GABA_A$ receptor subunit genes (Wisden et al., 1996). For example, cerebellar granule cells express six subunit genes abundantly (α_1, α_6, β_2, β_3, γ_2, and δ), and so they probably have several distinct $GABA_A$ receptor subtypes of unknown subunit stoichiometry. As for the canonical muscle nicotinic acetylcholine receptor subunits (Verrall and Hall, 1992; Green and Claudio, 1993; Kreienkamp et al., 1995), there is likely to be selective discrimination between $GABA_A$ subunits; however, assembling a neuronal receptor requires solving an extensive combinatorial element. To form a native receptor, subunits have to recognize and distinguish their neighbors. The assembly pathways used by granule cells to sort the six principal subunits into different receptor subtypes are not known. Granule cells receive a single GABAergic input from Golgi interneurons onto their distal dendrites. At this synapse, the $GABA_A$ receptor subtypes might be colocalized and intermingled (Nusser et al., 1995, 1996), and to date the α_1, α_6, $\beta_{2/3}$, and γ_2 subunits have been demonstrated to be present in the synaptic junction (Nusser et al., 1995, 1996; Somogyi et al., 1996).

Despite the comparative simplicity of the system, the receptor subunit composition of granule cell $GABA_A$ receptors is controversial. Current views accommodate $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$, $\alpha_1\alpha_6\beta_{2/3}\gamma_2$, $\alpha_1\alpha_6\beta_{2/3}\gamma_2\delta$, $\alpha_1\beta_{2/3}\gamma_2\delta$, and $\alpha_6\beta_{2/3}\delta$ combinations (Korpi and Lüddens, 1993; Mertens et al., 1993; Caruncho and Costa, 1994; Khan et al., 1994, 1996; Quirk et al., 1994; Caruncho et al., 1995; Korpi et al., 1995; Pollard et al., 1995). The evidence for such combinations is derived from antibody-based data and correlation

of pharmacological fingerprints of native binding sites, with binding profiles of subunits expressed in cell lines. Here we have targeted the α_6 subunit gene by homologous recombination techniques. Removal of α_6 protein from cerebellar granule cells allowed perturbations in other subunit levels to be studied and provided genetic evidence for a specific association between the α_6 and δ subunits. Our results begin to reveal the rules guiding receptor subunit assembly.

MATERIALS AND METHODS

Generation of mutant mice

The replacement vector for homologous recombination, designed for positive–negative selection (Mansour et al., 1988), was generated from a 6 kb mouse 129 strain α_6 subunit gene fragment (Jones et al., 1996), comprising part of exon 4 through to the middle of intron 8. This fragment was subcloned into pBluescript (Stratagene, La Jolla, CA) (see Fig. 1*A*). Into this plasmid, a *Bam*HI cassette (TAG_3IRESlacZpAMC1neopA) (Nehls et al., 1996) was inserted between the *Afl*II and *Nco*I sites located in exon 8, after the site ends were modified by adding *Bam*HI adaptors. This cassette, designed to report target gene expression and to provide a dominant marker to select for insertion into the gene, consisted of stop codons (TAG) in all three frames, followed by an internal ribosome entry site (IRES) linked to a lacZ reading frame and SV40 polyadenylation sequences. It also contained a neomycin resistance gene under independent transcriptional control (Nehls et al., 1996). The IRES-lacZ cassette was inserted with the lacZ coding sequence in the same transcriptional orientation as the α_6 gene coding sequences. For negative selection, a *Xho*I–*Sal*I fragment containing two MC1tk gene head-to-tail repeats (Smith et al., 1995) was placed in the targeting vector polylinker appending the longer homology arm (see Fig. 1*A*). The vector was linearized with *Sal*I and electroporated into 129 strain-derived embryonic stem (ES) cells (ES line "CCB," kindly supplied by Drs. W. Colledge and M. Evans, Wellcome/CRC, University of Cambridge).

Transfected ES cells were grown on $G418^r$ primary embryonic fibroblast feeder cells, in medium supplemented with leukemia inhibitory factor (Life Technologies, Paisley, UK) and selected in G418 (Life Technologies) and FIAU (Bristol-Myers, Hounslow, UK) (Mansour et al., 1988; Smith et al., 1995). Genomic DNA was isolated from individual colonies, digested with *Sph*I, Southern-blotted, and probed with an intron 8-derived *Sac*I–*Xba*I restriction fragment (see *PROBE A* in Fig. 1*A*). The addition of the neomycin gene creates an additional *Sph*I site in the α_6 gene locus, thus enabling the discrimination between wild-type (15 kb) and null (9 kb) alleles (see Fig. 1*A,B*). Confirmation of correct targeting events was established with restriction fragment probes from the lacZ and neo genes (marked on Fig. 1*A*) (data not shown) and probe B (the complete sequence of probe B, which comprises the promoter and 5′ untranslated region, is deposited in the EMBL database, accession number X97475; Jones et al., 1996) (see Fig. 1*A*). Additional diagnostic restriction digests of targeted genomic DNA used *Bam*HI (see Fig. 1*A*).

Male chimeras derived from targeted cells were mated with wild-type C57BL/6. Mutation germ-line transmission was determined by Southern blot analysis of agouti progeny tail DNA (see Fig. 1*B*). Heterozygotes were intercrossed to generate a homozygous α_6 −/− line.

β-Galactosidase staining

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed for 1 hr in 4% PFA, and then equilibrated at 4°C in PBS containing 30% sucrose. Sections (40 μM) were cut on a sliding microtome and incubated free-floating in 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) (Bonnerot and Nicolas, 1993). After X-Gal staining, some sections were counterstained with neutral red (Sigma, Poole, UK), allowing non-lacZ-expressing cells to be visualized. Alternatively, whole brains (see Fig. 3*C*) were immersed in the X-Gal staining reagent.

Staining of cultured granule cells. Cells on coverslips (see Granule Cell Culture and Electrophysiological Analysis) were washed in PBS and fixed in ice-cold 2% PFA/0.2% glutaraldehyde in PBS for 5 min. The coverslips were washed in PBS, incubated with X-Gal solution at 37°C overnight, and counterstained with neutral red.

Antibodies

α_1-specific antibodies. α_1(1–9), an N-terminal-specific antibody (Zezula and Sieghart, 1991); α_1-N, affinity-purified, raised against rat N terminus residues 1–14 (S. Pollard and F. A. Stephenson, unpublished data). α_1(328–382)/α_1L was prepared as described (Mossier et al., 1994). Rabbits were immunized with an MBP-α_1(328–382)-7His fusion protein, and the antibodies were purified with a GST-α_1(328–382)-7His fusion protein. This antibody precipitates $GABA_A$ receptors and is selective for the α_1 subunit (R. Pelz and W. Sieghart, unpublished data).

α_6-specific antibodies. α_6-N (Batch R54XV), affinity-purified polyclonal, was raised to bovine α_6 subunit N-terminal residues 1–16 (Thompson et al., 1992); α_6(429–434) batch P24, affinity-purified rabbit polyclonal antibody, was raised to rat α_6 subunit residues 429–434 (Tögel et al., 1994); α_6-C, affinity-purified rabbit polyclonal, was directed against the C-terminus sequence CSKDTMEVSSTVE (S. Pollard and F. A. Stephenson, unpublished data).

δ-specific antibodies. δ(318–400), rabbit polyclonal was raised against the rat cytoplasmic loop sequence between TM3 and TM4 (Quirk et al., 1995); δ(1–44) (rabbit R7) polyclonal was prepared by immunizing with an MBP-δ(1–44)-7His fusion protein and purifying by affinity chromatography, as described (Mossier et al., 1994; R. Pelz and W. Sieghart, unpublished data). This antibody is specific for the δ subunit and does not precipitate $\alpha1\beta3\gamma2$ receptors (R. Pelz and W. Sieghart, unpublished data).

Immunocytochemistry

Five α_6 −/− and five +/+ mice were transcardially perfused with 4% PFA, 0.05% glutaraldehyde, and ~0.2% picric acid for 7–17 min. After perfusion the brains were washed in 0.1 M phosphate buffer. Preembedding immunocytochemistry was carried out on 70-μm-thick vibratome sections (Somogyi et al., 1989). Floating sections were incubated in 20% normal goat serum (NGS) diluted in Tris-buffered saline (TBS), pH 7.4, for 1 hr. The purified antibodies were diluted in TBS containing 1% NGS. After they were washed, the sections were incubated for 2 hr in biotinylated goat anti-rabbit IgG (diluted 1:50 in 1% NGS containing TBS), followed by incubation in avidin–biotinylated horseradish peroxidase complex (diluted 1:100; Vector Laboratories, Peterborough, UK) for 90 min. Peroxidase enzyme reaction was with 3,3′-diaminobenzidine tetrahydrochloride as chromogen and H_2O_2 as oxidant. In some cases, Triton X-100 (0.1–0.3%) was added to the TBS throughout the experiment. The antibody concentrations used for immunocytochemistry were δ(1–44)R7, 0.7–2.2 μg/ml; α_6-N, 1.5–3.0 μg/ml.

For controls, selective labeling could not be detected when the primary antibodies were either omitted or replaced by 5% normal rabbit serum. No immunoreactivity was obtained when the antibodies were preincubated with the appropriate peptides used for immunization (Nusser et al., 1996).

Ligand autoradiography

The procedures were slightly modified from Olsen et al. (1990) and Wong et al. (1996). Cryostat sections (14 μm) from frozen nonfixed adult mouse brains were preincubated in 50 mM Tris-HCl, pH 7.4, and 120 mM NaCl for 15 min at 0°C, except for the GABA site assays when 0.31 M Tris-citrate solution, pH 7.1, was used. Incubations with ligands used fresh buffers of composition identical to those used for preincubation. For the benzodiazepine (BZ) site, [^{3}H]Ro 15-4513 (5 nM, Du Pont de Nemours, NEN Division, Dreieich, Germany) was used with and without 100 μM diazepam (Orion, Espoo, Finland) for a 60 min incubation at 0°C, followed by three 30 sec washes, a dip in distilled water, and rapid drying. The same conditions and washes were used for the GABA site, with [^{3}H]muscimol (20 nM, Amersham, Buckinghamshire, UK) and [^{3}H]SR 95531 (20 nM, Du Pont), except that the incubation time was 30 min. The sections were washed three times for 15 sec in 10 mM Tris-HCl, pH 7.4, followed by dipping in distilled water and air drying. Sections were exposed to Hyperfilm-^{3}H (Amersham) for 1–6 weeks. The images were produced by scanning the films. The nonspecific binding components to BZ and GABA sites were defined in the presence of 10 μM Ro 15-1788 (Hoffmann-La Roche, Basel, Switzerland) and 100 μM GABA, respectively.

Radioligand binding

Radioligand binding on membranes prepared from individual mouse cerebella was as described previously (Quirk et al., 1994). Membranes prepared from each animal were used for saturation binding with [^{3}H]Ro 15-1788 (0.1–17.0 nM), [^{3}H]zolpidem (1–30 nM), and [^{3}H]Ro 15-4513 (0.8–60.0 nM) displaced with Ro 15-4513 (10 μM) to define the total number of BZ binding sites, or with flunitrazepam (1 μM) to define binding to diazepam-sensitive sites only. Saturation binding with [^{3}H]muscimol (2–45 nM) used 1 mM GABA to determine nonspecific

levels. All assays used eight concentrations of ligand, with total and nonspecific binding measured in duplicate with 30–80 μg of protein/assay tube. B_{max} and K_d values were determined by nonlinear least-squares fit of the saturation curves using the data analysis software RS1 (Bolt, Beranek and Newman, Cambridge MA).

Immunoprecipitation analysis

Immunoprecipitation of $GABA_A$ receptors solubilized from individual mouse cerebella used antibody δ(318–400) bound to protein A-Sepharose as described previously (Quirk et al., 1994, 1995). [^{3}H]muscimol binding to the solubilized receptor was measured after gel filtration through Sephadex G-25 to remove any remaining endogenous GABA.

PAGE and immunoblotting

Membranes from individual +/+ and −/− cerebella were prepared, and equal amounts of protein per slot were subjected to SDS-PAGE in 10% polyacrylamide gels and immunoblotted. For the α_1(1–14), α_6(1–16), and α_6-C antibodies, the ECL Western blotting system (Amersham) was used for detection (Pollard et al., 1995). ECL blots were quantitated by normalizing with an anti-neuron specific enolase (NSE) antibody (Sigma) and then probing with α_1(1–14) (S. Pollard and F. A. Stephenson, unpublished data). Multiple exposures were taken for both anti-NSE and α_1(1–14) immunoreactivity and quantitated using a Molecular Dynamics Personal Quantitator. For the δ(1–44)R7 and α_1(328–382) antibodies, membranes were incubated with digoxygenin-labeled antibodies and were then treated with anti-digoxygenin-alkaline phosphatase Fab fragments (Boehringer Mannheim, Mannheim, Germany). Proteins were detected by fluorescence using the CSPD substrate (Tropix). Blots were quantitated by densitometry of Kodak X-Omat S films with the DocuGel 2000i gel documentation system using the RFLPscan software (MWG-biotech).

Granule cell culture and electrophysiological analysis

Cell culture. Cerebellar granule cells, attached to matrigel-coated coverslips, were cultured from postnatal day 5 (P5) mice as described for rat cells (Randall and Tsien, 1995). Minimal essential medium was supplemented with glucose (5 mg/ml), transferrin (100 μg/ml), insulin (5 mg/ml), glutamine (0.3 mg/ml), and 10% fetal calf serum. After 2 d, the cells were fed with media that was supplemented further with 4 μM cytosine arabinoside, and they were then fed every 5 d by a 50% replacement of the culture media. Electrophysiological measurements were made after 14–17 d *in vitro*.

Electrophysiology

Recordings were from single, visually identified, granule cells using both outside-out patches and whole cells pulled away from the underlying cell-attachment substrate. No differences were observed between data from patches and whole cells, and results from both data sets were therefore pooled. A piezoelectrically driven theta tube-based application system delivered 120 msec pulses of GABA. Concentration jumps from control to agonist and vice versa took place within ~1 msec. Five 120 msec 20 μM GABA pulses were applied at 0.1 Hz before and during the application of 1 μM flunitrazepam (Sigma). Recovery from the actions of flunitrazepam were studied with 20 additional GABA applications. Data were filtered at 2 kHz and sampled directly to computer at 10 kHz under control of the pClamp software suite. Because of the presence of some application-to-application variability in the current peak amplitude generated by GABA, an arbitrary threshold was set, with a 15% increase in the GABA response considered to be a potentiation above the baseline variability.

In situ *hybridization*

In situ hybridization with ^{35}S-labeled oligonucleotide probes was as described (Wisden and Morris, 1994). The oligonucleotide sequences used were α_4: 5′-TTCTGGACAGAAACCATCTTCGCCACATGCCATACTTTAAGCCTGT-3′ (EMBL accession number L08493) and δ: 5′-AGCAGCTGAGAGGGAGAAAAGGACGATGGCGTTCCTCACATCCAT-3′ (EMBL accession number M60596)

Behavioral observations

The animals (n = 23 for both +/+ and −/− lines, from two generations) were observed in their normal activities. Open field explorative activity was determined, under artificial lighting, in a round area (diameter 83 cm) divided into 19 segments. The mice were in the area for the first time. Their behavior was recorded for 5 min with a video recorder, and the behavioral parameters (number of segment crossings with all four feet and number of rearings) were scored blindly afterward. The number of fecal boli was counted before the area was cleaned for the next animal. The ability of the mice to learn to climb up onto a thin horizontal wire while initially hanging from their forepaws was tested in three trials during 1 d. Their ability to learn to stay on an accelerating rotating rod (Rotamex, Columbus Instruments, Columbus, OH) for 180 sec was tested during daily sessions. The initial session was 3 min on a nonmoving rod. On subsequent sessions, the mice were placed on the stationary rod, and the rotation speed was then set at 5 rpm and increased to 15 rpm over a 180 sec interval.

RESULTS

Creation of a mouse line with no $GABA_A$ receptor α_6 subunit protein: mapping α_6 expression with a dicistronic RNA encoding lacZ

Homologous recombination in embryonic stem cells was used to create a 129/Sv × C57BL/6 mouse line in which the α_6 subunit gene was disrupted at exon 8 (Fig. 1*A,B*) (see Materials and Methods). The mutation, located just after the TM2 (channel lining) region, consisted of an insertion of stop codons in all three reading frames, an IRES linked to a β-galactosidase (lacZ) open reading frame and SV40 polyadenylation site, and finally a neomycin resistance gene expressed from its own promoter (Mountford et al., 1994; Nehls et al., 1996) (Fig. 1*A*). Translation of the α_6 subunit mRNA from the mutant allele should terminate just after the TM2 region, resulting in a 300 amino acid protein designated α_6M2. The stop codon-IRES insertion generates a dicistronic mRNA in which β-galactosidase protein translation is then linked to an α_6 gene expression, i.e., lacZ expression is under α_6 transcriptional regulatory element control.

Homozygous mutant mice had no overt defects and could breed normally (see Behavioral Characterization of α_6 −/− Mice). By the criteria of Nissl staining, the size, folding of folia, and histological appearance of the cerebellum in α_6 −/− animals were completely normal. Immunocytochemistry with the α_6 N-terminal-specific antibody α_6-N (Thompson et al., 1992), however, demonstrated a total loss of α_6-specific immunoreactivity from the cerebellar granule cell layer of −/− mice (Fig. 2*A,B*). Using both N- and C-terminal α_6 subunit-specific antibodies (Thompson et al., 1992; S. Pollard and F. A. Stephenson, unpublished data), a complete loss of the 57 kDa immunoreactive α_6 band was also seen on Western blots of cerebellar protein extracts isolated from −/− animals (Fig. 2*C*). Identical results were found with an additional C-terminal anti-peptide antibody α_6(429–434) (R. Pelz and W. Sieghart, data not shown). Long exposure times of blots probed with the N-terminal antibody failed to show any α_6-specific degradation products in the −/− samples (data not shown), indicating that α_6M2 is not a stable entity. In a γ_2 subunit gene knockout study, which similarly used an exon 8 disruption, a truncated γ_2 form was also not detectable (Günther et al., 1995).

We mapped α_6 gene expression in adult −/− animals using β-galactosidase staining (Fig. 3). An intense blue coloration was seen in the cerebellar granule cell layer (Fig. 3*A,B*). The reaction product started to appear within the first 5 min of incubating the sections at room temperature in X-Gal and was fully developed within 30 min, thus demonstrating the extremely high level of α_6 locus expression. There were numerous small blue cells in the cerebellar molecular layer. These are probably ectopic granule cells (Fig. 3*D*) (cf. Thompson et al., 1992; Gao and Fritschy, 1995; Gutiérrez et al., 1996). Strong, blue coloration was also seen along the molecular layer outer edge, probably corresponding to β-galactosidase enzyme transported into granule cell axons, the parallel fibers. As expected, there were many blue granule cells in the dorsal cochlear nuclei (Fig. 3*E*) (Varecka et al., 1994). With

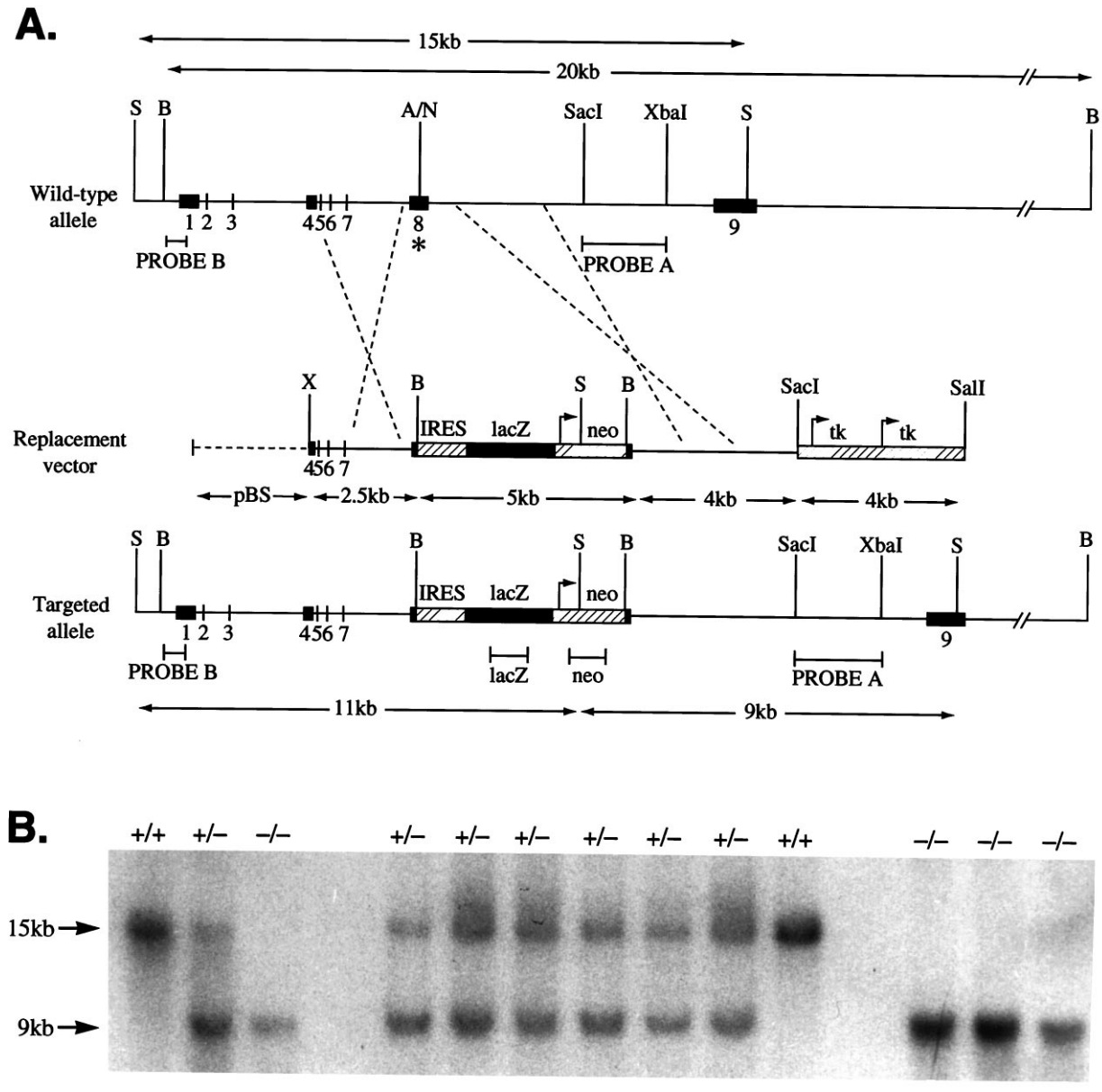

Figure 1. $GABA_A$ receptor α_6 subunit gene disruption by homologous recombination. *A*, Wild-type α_6 gene, targeting (replacement) vector, and the disrupted α_6 gene structures. *Numbers* indicate exons. On the *Replacement vector*, the *broken line* indicates pBluescript (*pBS*) sequences. On the wild-type allele, the *asterisk* marks exon 8 where the *IRES lacZ/neo* cassette was inserted. Only relevant restriction sites are shown. *A*, *Afl* II; *B*, *Bam*HI; *N*, *Nco*I; *S*, *Sph*I; *X*, *Xho*I. *Arrows* mark the *neo* and *tk* gene promoter sites and direction of transcription. The *lacZ* coding sequence orientation is the same as the α_6 gene, thus permitting its translation from the *IRES* sequence (*striped box*) to be initiated on the mRNA derived from the α_6 promoter. Expected restriction fragment lengths diagnostic for homologous recombination and the probes used to detect these are marked by *double-headed arrows* and *horizontal bars*, respectively. *B*, Confirmation of α_6 mutant allele germline transmission. Biopsy tail DNA samples were digested with *Sph*I, electrophoresed, and Southern-blotted. The membrane was probed with *PROBE A* (3′ flanking). Wild-type (+/+) individuals give a 15 kb band, the homozygous null (−/−) animals give a 9 kb band, and heterozygotes (+/−) give both bands.

little exception, the rest of the brain showed no detectable staining (Fig. 3*A*). Unexpectedly, however, many cells in the inferior colliculi dorsal regions stained blue (Fig. 3*C*), and there were other minor cell populations in the substantia nigra and thalamus (geniculate nuclei) with faint but consistent blue staining (data not shown). These populations of stained cells were not seen in wild-type animals, and thus were not attributable to endogenous β-galactosidase-like activity.

α_6 subunit expression has not been noted previously in the inferior colliculi, substantia nigra, or thalamus by *in situ* hybridization or immunoreactivity, although a rat α_6 gene proximal promoter fragment consistently drives lacZ expression in the inferior colliculi of transgenic mice (Jones et al., 1996). The reasons for the lack of detection in previous studies could include low α_6 mRNA and protein levels. Alternatively, α_6 may be part of presynaptic receptors transported to distant axon terminals outside the inferior colliculi and so may escape detection in the inferior colliculi nucleus itself. The long half-life of β-galactosidase in mammalian tissue contributes to the extreme sensitivity of the lacZ reporter method. Over time, low levels of transcription from the α_6-lacZ hybrid gene will lead to accumulating amounts of β-galactosidase. These results make

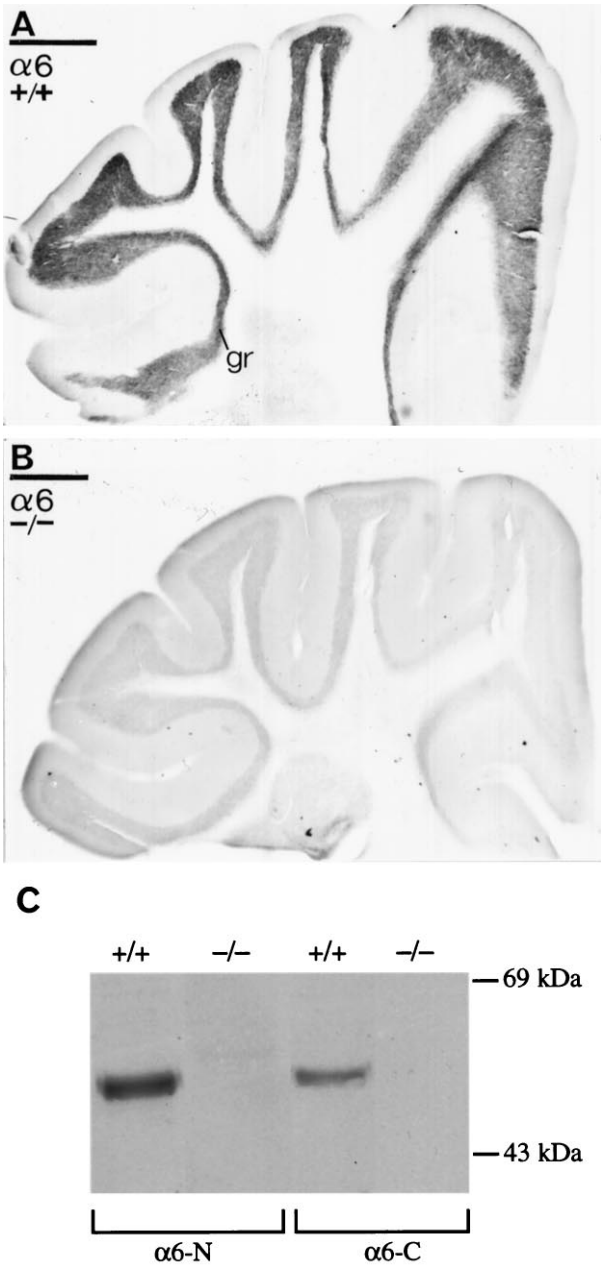

Figure 2. Immunodetection of the α_6 subunit of the $GABA_A$ receptor in α_6 +/+ (*A, C*) or α_6 −/− (*B, C*) cerebella as visualized with either light microscopic immunoperoxidase reactions (*A, B*) or immunoblotting (*C*). *A, B*, An intense immunoreactivity for the α_6 subunits in the granule cell layer (*gr*) disappeared in α_6 −/− mice (*B*). The sections and one immunoblot were reacted with the same N-terminal domain-specific antibody. Scale bars: *A, B*, 500 μm. *C*, The 57 kDa α_6 protein is absent in α_6 −/− cerebella, as shown with either α_6-N, an N-terminal-specific antibody, or α_6-C, a C-terminal-specific antibody to the α_6 subunit.

clear the usefulness of tracking gene expression using dicistronic-based reporters (Mountford et al., 1994; Nehls et al., 1996).

Pharmacological characterization of α_6 −/− cerebellar granule cells: BZ sensitivity

The α_6 protein absence was established further by pharmacological analysis. $GABA_A$ receptors containing the α_6 subunit are insensitive to most types of BZs, such as diazepam or flunitrazepam (Lüddens et al., 1990; Hadingham et al., 1996). Ro 15-4513, however, is a BZ that binds to all subtypes of $GABA_A$ receptor with $\alpha_x\beta\gamma_2$ subunit combinations, including those containing the α_6 subunit (Lüddens et al., 1990; Sieghart, 1995). Thus, a diagnostic assay for α_6 in cerebellar granule cells is the high level of [^{3}H]Ro 15-4513 binding on granule cell membranes that is insensitive to full BZ agonists such as diazepam (Sieghart et al., 1987; Malminiemi and Korpi, 1989; Lüddens et al., 1990; Turner et al., 1991). Normally, more than half of the [^{3}H]Ro 15-4513 binding in the granule cell layer is diazepam insensitive (DIS) but can be displaced by micromolar concentrations of the BZ antagonist flumazenil (also known as Ro 15-1788). This profile is thought to be attributable to the abundant expression of $\alpha_6\beta_{2/3}\gamma_2$ receptors on granule cells (Lüddens et al., 1990; Korpi and Lüddens, 1993; Korpi et al., 1993; for review, see Wisden et al., 1996). In the α_6 −/− animals, DIS binding over the cerebellar granule cell layer is completely absent (Fig. 4*A*), whereas in wild-type brains [^{3}H]Ro 15-4513 still binds over the granule cell layer even in the presence of 100 μM diazepam (Fig. 4*A*). From binding studies using isolated cerebellar membranes, the contribution that the α_6 subunit makes to the number of total cerebellar Ro 15-4513 binding sites was estimated to be ~40% (see Evaluation of α_1 Subunit Levels; also see Table 1).

The α_6 subunit has a closely related homolog, the α_4 subunit, which is expressed in certain forebrain areas such as the thalamus (Wisden et al., 1991, 1992). The recombinant α_4 subunit in an $\alpha_4\beta_x\gamma_2$ configuration displays a pharmacological profile identical to that of $\alpha_6\beta_x\gamma_2$ receptors, and α_4 mRNA is found at low levels in cerebellar granule cells of adult rats (Wisden et al., 1991; Laurie et al., 1992). Thus we looked to see whether there had been a compensatory change in α_4 expression in the cerebellum of α_6 −/− mice (Fig. 4*D*); however, consistent with the absence of DIS binding in α_6 −/− animals, there was no upregulation of α_4 mRNA in −/− cerebella (Fig. 4*D*).

The BZ sensitivity of $GABA_A$ receptors in α_6 −/− cerebellar granule cells was investigated directly using electrophysiology on cultured granule cells isolated from P5 animals. After 14–17 d *in vitro*, we tested the effects of BZ agonist flunitrazepam (1 μM) coapplication on the current amplitude generated by 20 μM GABA. Results of a typical culture are shown in Figure 5. Examination of wild-type cells revealed a heterogeneous response: flunitrazepam-induced potentiation of the GABA response took place in approximately half (20 of 36) of the cells tested (Fig. 5*C*, *top row*). In those cells with flunitrazepam-potentiated GABA responses, the average potentiation was 58 ± 7%. In contrast, in age-matched cultures derived from α_6 −/− cerebella, 16 of 18 cells tested had flunitrazepam-induced potentiations of their GABA responses. The average potentiation was 62 ± 7% (Fig. 5*C*).

To examine a possible reason for the heterogeneity of the $GABA_A$ receptor response, the extent of gene expression from the α_6 locus in cultured α_6 −/−granule cells was analyzed with β-galactosidase histochemistry. At 3 weeks in culture, numerous cells strongly stained dark blue after incubation with X-Gal (Fig. 5*A,B*); however, there were many adjacent "granule-like" cells that either contained just a few blue particles or were completely unstained (Fig. 5*B*). This applied both to isolated cells and to cells in large clusters. There was no obvious correlation between the location of cells (isolated or in clusters) and lacZ expression. This mosaic of blue cells is evidence that at least in culture, not all granule cells or granule-like cells express the α_6 gene (cf. Santi et al., 1994), and may explain the heterogeneous nature of the BZ potentiation seen in our cultures.

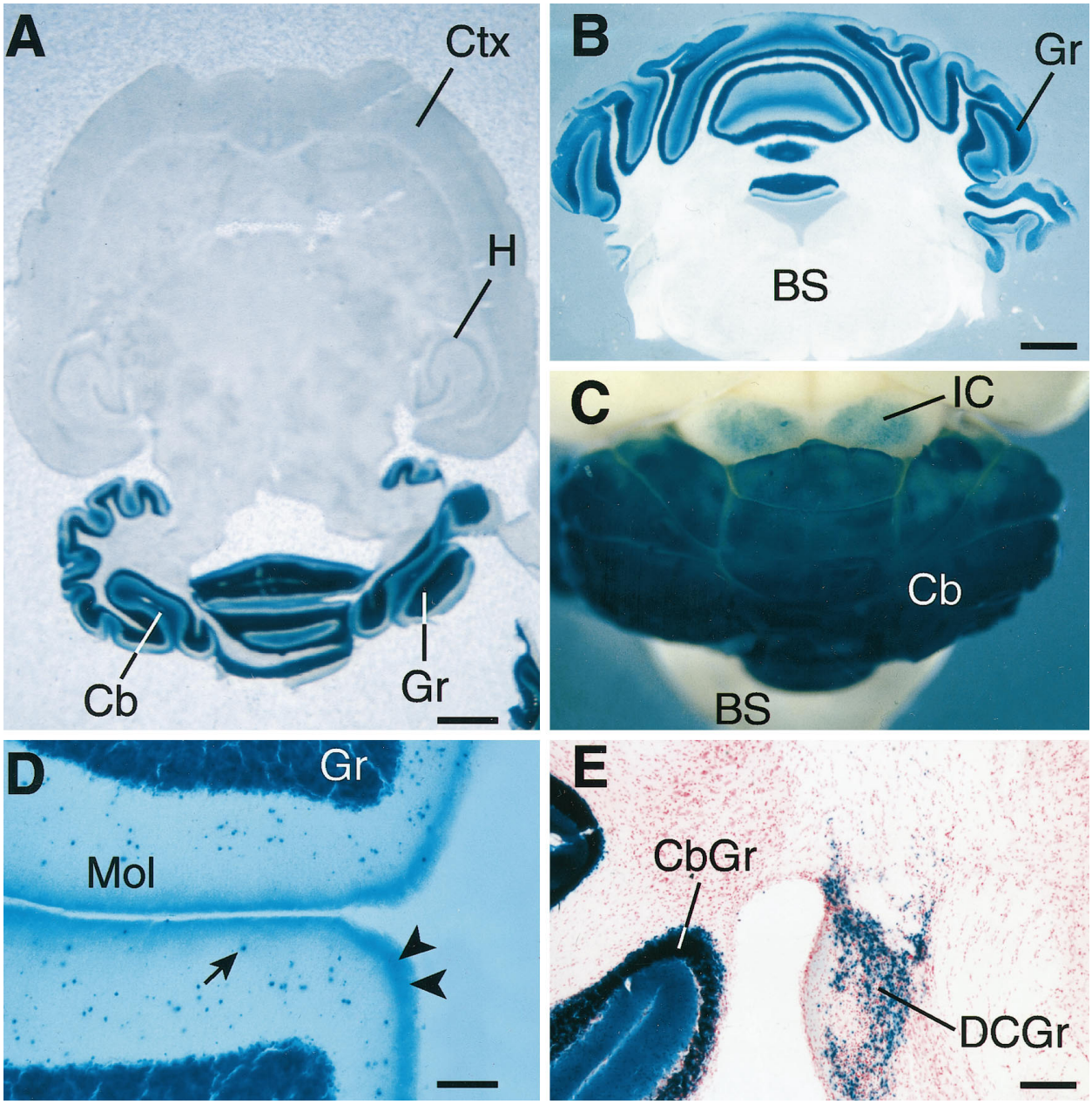

Figure 3. LacZ expression driven from the α_6 gene locus in α_6 −/− adult mouse brains illustrated in horizontal (*A*), coronal (*B*), and whole-mount views (*C*); *blue coloration* indicates lacZ activity. *A* and *B* show the confined expression of the α_6 gene to the cerebellar granule cell layer; *C* shows the expression in the dorsal regions of the inferior colliculi; *D* shows higher-power view of α_6 gene expression in the molecular layer of the cerebellum. The *arrow* indicates an example of the numerous lacZ positive cells in the molecular layer. These are probably nonmigrated granule cells. The *arrowheads* mark putative parallel fiber staining; *E*, α_6 gene expression in the dorsal cochlear nucleus granule cells. *BS*, Brainstem; *Cb*, cerebellum; *CbGr*, cerebellar granule cell layer; *Ctx*, neocortex; *Gr*, cerebellar granule cells; *DCGr*, dorsal cochlear nucleus granule cells; *H*, hippocampus; *IC*, inferior colliculi; *Mol*, cerebellar molecular layer. Scale bars: *A*, 1.3 mm; *B*, *C*, 1 mm; *D*, 150 μm; *E*, 300 μm.

Selective δ subunit protein loss from cerebellar granule cells of α_6 −/− mice

A key and controversial question for cerebellar granule cell GABA$_A$ receptors has been which subunits co-assemble *in vivo* (Wisden et al., 1996). To examine one aspect of this, we immunoprecipitated deoxycholate-solubilized cerebellar GABA$_A$ receptors from α_6 −/− mice with a δ-specific polyclonal antiserum, δ(318–400), raised against the putative intracellular loop domain between TM3 and TM4 (Quirk et al., 1994, 1995). In wild-type and α_6 +/− cerebella, the δ(318–400) antiserum precipitated the same number of muscimol binding sites (Fig. 6*A*). By this assay, the δ protein was also present in both pure wild-type 129/Sv and pure C57BL/6 cerebella (data not shown). In contrast, immunoprecipitation of δ-containing receptors from α_6 −/− cerebella was greatly reduced (Fig. 6*A*). These data were extended by Western blot analysis of membrane protein samples isolated from individual +/+ and α_6 −/− cerebella. With use of a δ-subunit-specific antibody, δ(1–44)R7, raised against the N terminus (R. Pelz and W. Sieghart, unpublished data), α_6 −/− samples showed a dramatic reduction in the 53 ± 1 kDa δ subunit band intensity to 25 ± 8% of +/+ levels (Fig. 6*B*). The residual δ protein in the α6 −/− tissue had the same molecular weight as that in wild-type tissue (Fig. 6*B*).

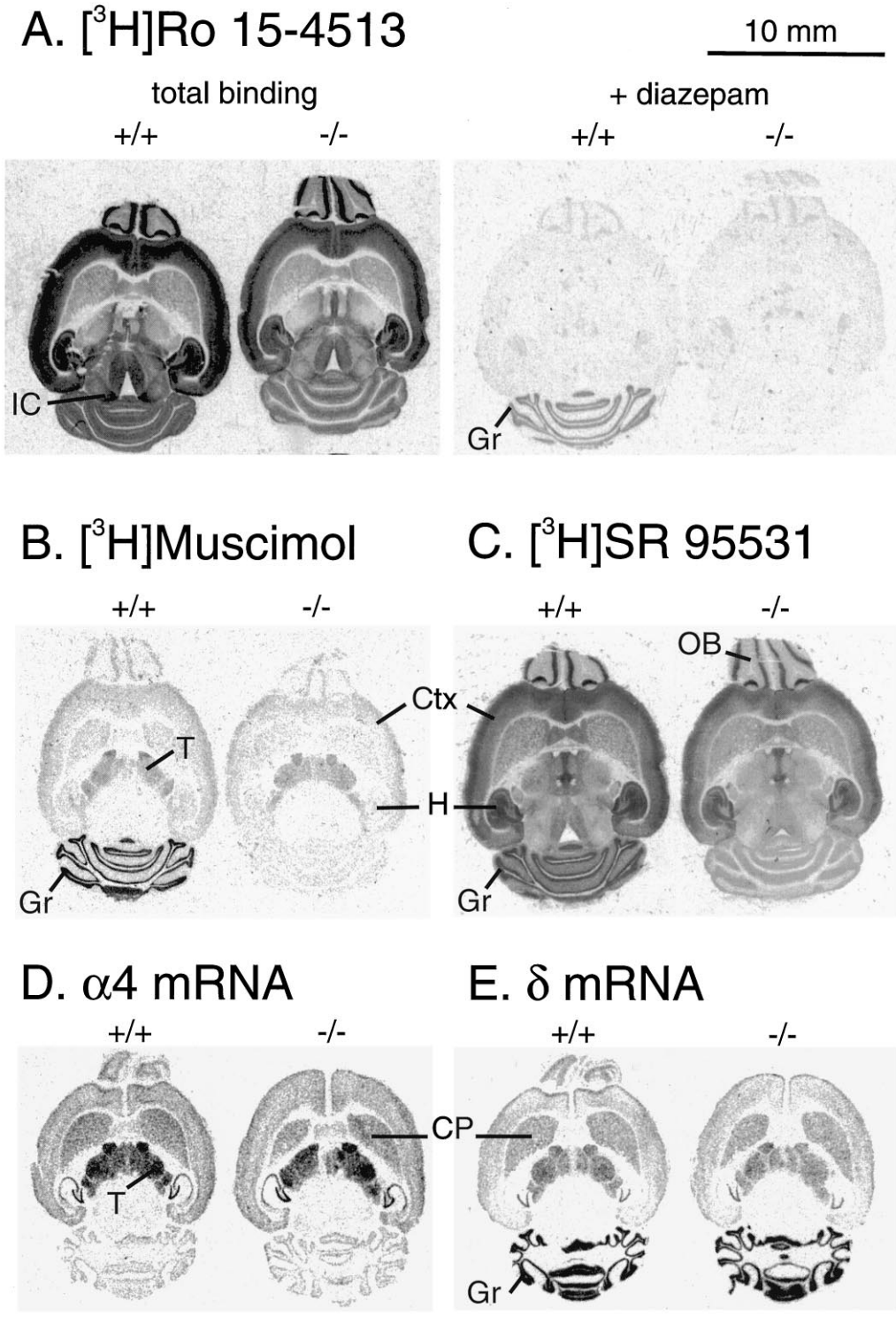

Figure 4. Autoradiographic analysis of $GABA_A$ receptor binding sites in wild-type (+/+) and α_6 −/− mice. *A*, Benzodiazepine sites labeled by 5 nM [^{3}H]R0 15-4513 showing total and diazepam-insensitive binding in the presence of 100 μM diazepam. *B*, $GABA_A$ receptor sites labeled by 20 nM [^{3}H]muscimol, showing total binding. The nonspecific signal in the presence of 100 μM GABA was at the film background level. *C*, $GABA_A$ receptor sites labeled by 20 nM [^{3}H]SR 95531, showing total binding. The nonspecific binding signal in the presence of 100 μM GABA was similar in wild-type and −/− brains (data not shown). Similar distinct pharmacological profiles were observed between the wild-type and α_6 −/− brains in each of seven pairs of adult mice studied. *D, E, In situ* hybridization x-ray film autoradiographs of adult mouse brains hybridized with α_4 (*D*) and δ-specific (*E*) ^{35}S-labeled oligonucleotide probes. Wild-type (+/+) brains are on the *left*; α_6 −/− brains are on the *right*. No differences can be seen in subunit mRNA levels between +/+ and −/− brains. Note also the very similar pattern of α_4 and δ gene expression in the forebrain/thalamus regions, and the correlation with the distribution of [^{3}H]muscimol (*B*). *Cbgr*, Cerebellar granule cells; *CP*, caudate-putamen; *Ctx*, cerebral cortex; *Gr*, cerebellar granule cell layer; *H*, hippocampus; *IC*, inferior colliculus; *OB*, olfactory bulb; *T*, thalamus.

Table 1. Determination of Bz binding in α_6 −/− mice

	+/+		−/−	
Ligand	K_d	B_{max}	K_d	B_{max}
Ro 15-1788	0.91 ± 0.16	1222 ± 144	0.84 ± 0.17	1021 ± 141
Ro 15-4513 total sites	9.4 ± 2.0	2106 ± 203	6.3 ± 1.5*	1159 ± 107**
DS	5.9 ± 0.8	1199 ± 114	5.7 ± 0.5	1088 ± 174
Zolpidem	17.9 ± 4	1155 ± 152	19.6 ± 4.8	993 ± 161
Muscimol	6.3 ± 0.8	2568 ± 326	6.2 ± 1.7	661 ± 234

Data shown are the mean ± SEM of cerebellar membranes prepared independently from six animals. Saturation analysis used eight concentrations of ligand in duplicate for each animal. B_{max} (fmol/mg protein) and K_d (nM) values were determined by nonlinear least-squares fit of the saturation curves using the data analysis software RS1. *Significantly different from +/+ ($p < 0.05$); **significantly different from +/+ ($p < 0.005$). DS, Diazepam sensitive.

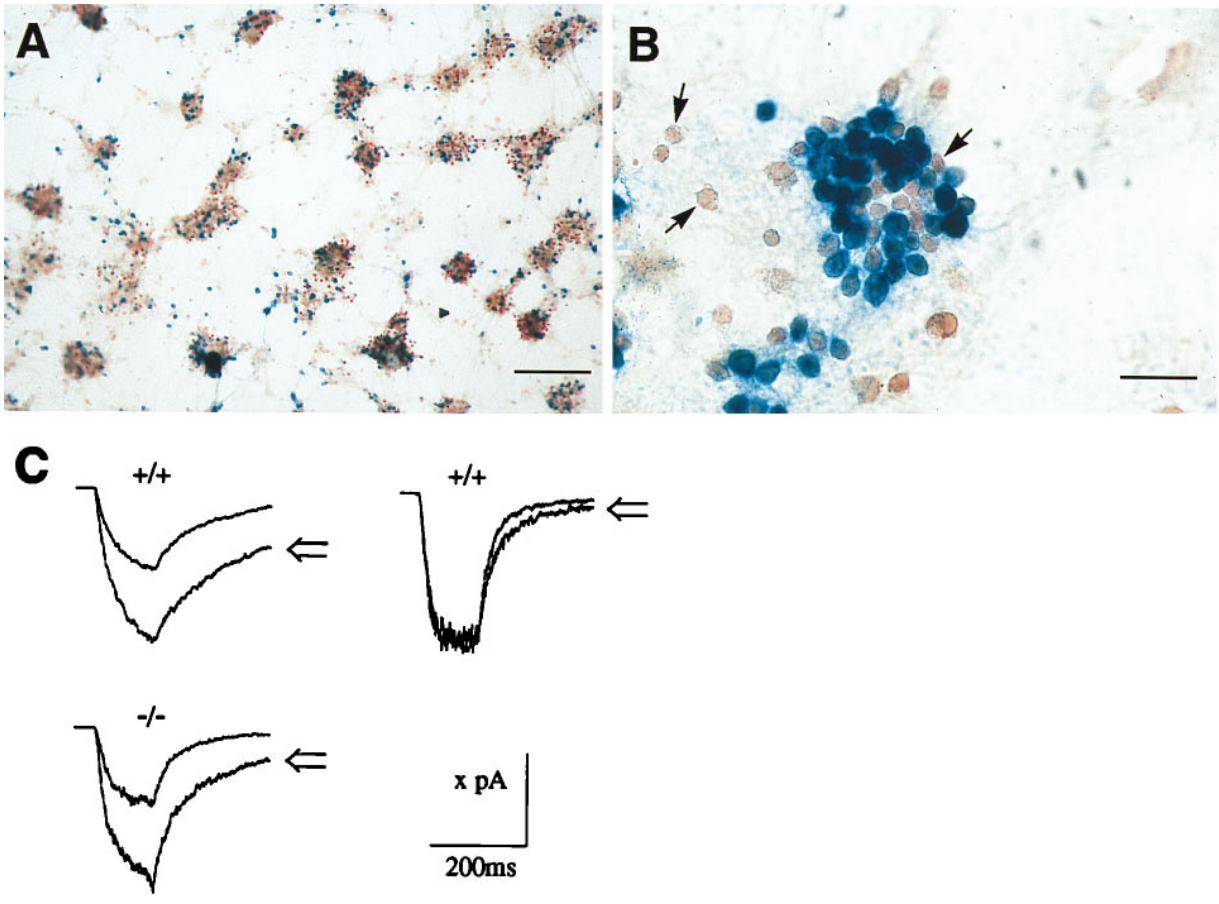

Figure 5. Electrophysiological characterization of $GABA_A$ receptors in cerebellar granule cells from wild-type and α_6 −/− cells. Photomicrographs in *A* and *B* show typical examples of lacZ-expressing cerebellar granule cells, isolated from P5 α_6 −/− mouse cerebella, and cultured for 3 weeks. *A* is a low-magnification view, showing the mosaic of *blue* (lacZ-positive) *cells* scattered throughout the culture. Both isolated and clustered *blue cells* can be seen. Within any given cluster, not all the cells are *blue* and therefore are not expressing the α_6 gene. The cells have been counterstained with neutral red. All electrophysiological recordings were from isolated cells. *Arrows* in *B* show examples of non-lacZ-expressing cells. Scale bars: *A*, 200 μm; *B*, 30 μm. *C*, Example responses to 120 msec applications of 20 μM GABA alone, and 20 μM GABA with 1 μM flunitrazepam (*open arrows*). The *top row* shows an example of wild-type cells (+/+) with $GABA_A$ receptors that responded to flunitrazepam (*left trace*) or were insensitive to flunitrazepam (*right trace*). The *bottom row* shows a typical $GABA_A$ response from an α_6 −/− cell and the associated flunitrazepam potentiation. From *left to right* and *top to bottom*, the value *x* on the scale bar corresponds to 200, 230, and 170 pA, respectively. The traces were averages of three to five consecutive records.

Immunocytochemistry with the δ(1–44)R7 antibody clearly supported the Western blot and immunoprecipitation data (Fig. 7). Light microscopic immunocytochemistry with this antibody revealed a very intense cerebellar granule cell layer staining in wild-type animals (Fig. 7*A*), similar to that reported earlier using a different δ-specific antibody (Benke et al., 1991; Gao and Fritschy, 1995). The immunoreactivity originated mainly from staining of the glomeruli, and granule cell bodies were only weakly outlined (Fig. 7*C*). The glomeruli appeared as dark rings of labeled granule cell dendrites surrounding pale centers representing the unstained mossy fiber terminals (Fig. 7*C*). In contrast to the wild-type animals, in α_6 −/− mice the granule cell layer immunostaining for the δ subunit was virtually absent (Fig. 7*B*). In particular, no immunoreactivity could be detected in the glomer-

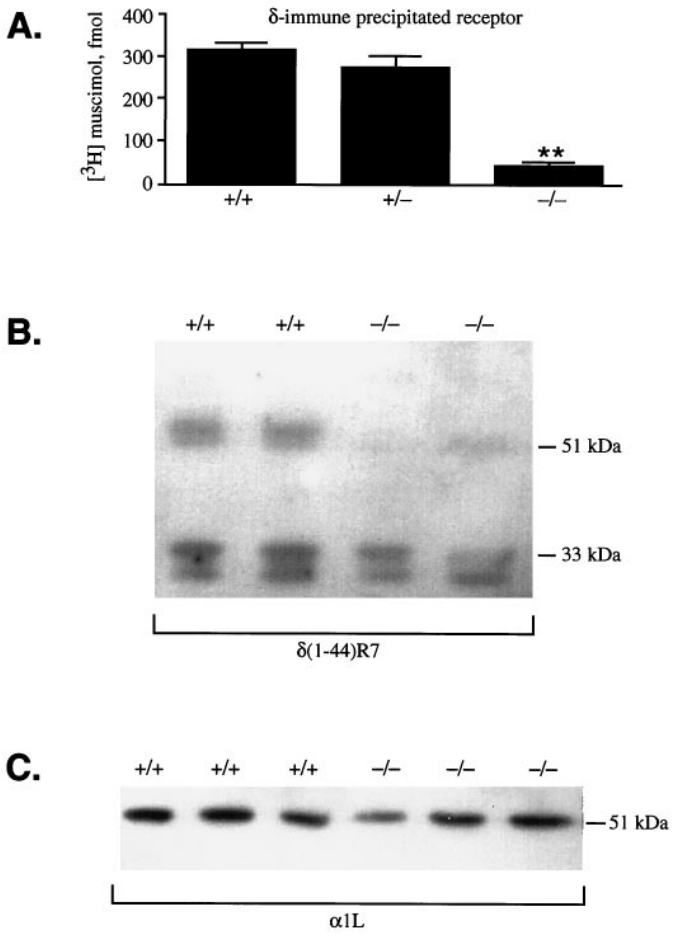

Figure 6. Immunoprecipitation and immunoblot analysis of $GABA_A$ receptor δ subunit levels in wild-type and α_6 −/− cerebella. *A*, After cerebellar $GABA_A$ receptors were solubilized in Triton X-100/deoxycholate, the number of [^{3}H]muscimol binding sites immunoprecipitated by the δ(318–400) antiserum from +/+, +/−, and −/− cerebella was determined (n = 10–14). *B*, Immunoblot analysis: the marked δ subunit reduction in α_6 −/− cerebella detected with the δ(1–44)R7 antiserum. The identity of the low molecular weight doublet (33 and 31 kDa) seen in all samples is unknown. *C*, A 51 kDa α_1 immunoreactive band is present in both −/− and +/+ cerebellar samples as detected with the α1(328–382)/α1L antibody.

uli (Fig. 7*D*), suggesting that α_6 −/− granule cell dendrites contain either no δ subunit protein or an undetectably low level. Electron microscopic examination of the immunoreactivity for the δ subunit in the granule cell layer further confirmed the lack of δ subunit immunoreaction in α_6 −/− granule cells (not shown). Therefore, the residual δ subunit immunoreactivity seen on Western blots may represent a level of protein undetectable by immunocytochemistry under our conditions, or it could come from cell types other than granule cells, because whole cerebella were used to prepare the protein extracts.

The δ subunit loss occurs post-translationally

One possibility to explain the loss of δ protein from α_6 −/− granule cells is through a change in regulation at the mRNA level; however, the δ subunit mRNA level in the cerebellar granule cells was at normal levels when examined by *in situ* hybridization (Fig. 4*E*). High levels of δ mRNA were seen in both wild-type and −/− granule cells. δ mRNA expression was also examined in both pure 129/Sv and C57BL/6 strain wild-type animals and found not to differ (not shown). This result suggests that the loss of δ subunit from the α_6 −/− cerebellar granule cells occurs post-translationally.

[^{3}H]Muscimol and [^{3}H]SR95531, two ligands that mark out α_6-and δ-containing $GABA_A$ receptors

[^{3}H]Muscimol and [^{3}H]SR95531 are ligands that highlight restricted GABA molecule conformations (Sieghart, 1995). In particular, [^{3}H]muscimol is the classic $GABA_A$ ligand and has been used extensively for mapping $GABA_A$ receptors in the brain (Palacios et al., 1980; Olsen et al., 1990). We used [^{3}H]muscimol and [^{3}H]SR95531 to autoradiographically probe the remaining $GABA_A$ receptors in the δ subunit-deficient/α_6 −/− cerebellar granule cell layer. A clear cut, but completely unanticipated, pharmacological feature was revealed: the selective and extensive loss of high-affinity [^{3}H]muscimol (Fig. 4*B*) and [^{3}H]SR95531 (Fig. 4*C*) binding from the granule cells. Binding over the cerebellar molecular layer with these ligands remained unchanged, as did the levels of binding in the forebrain, e.g., normal levels of [^{3}H]muscimol binding remain over the thalamus of −/− animals (Fig. 4*B*). The decrease in [^{3}H]muscimol binding seen by autoradiography in α_6 −/− individuals was further quantified by studying [^{3}H]muscimol binding to membranes from whole cerebella (Table 1). The level of high-affinity [^{3}H]muscimol sites was reduced to ~25% of that found in control animals (Table 1). No significant reduction in [^{3}H]muscimol binding was seen in +/− animals (data not shown). Saturation analysis revealed no change in the observed K_d values for [^{3}H]muscimol in −/− animals (K_d is ~6 nM; Table 1). The residual binding is likely to come from sites within the molecular layer, the granule cell layer, and the deep cerebellar nuclei, all of which contain an $\alpha_1\beta_{2/3}\gamma_2$ component. Under autoradiographic conditions, however, [^{3}H]muscimol does not highlight these α1-containing receptors in the cerebellum. Rather, [^{3}H]muscimol and [^{3}H]SR95531 seem to selectively highlight α_6δ-containing receptors.

Evaluation of α_1 subunit levels in α_6 −/− and δ-deficient cerebella

The α_1 protein is expected to account for the majority of the remaining α subunits in the cerebellum of α_6 −/− mice (Sieghart, 1995; McKernan and Whiting, 1996; Wisden et al., 1996). To examine whether there was any concomitant change in the α_1-receptor population in the α_6 −/− cerebella, the portion of α_1 subunits complexed with the $\beta_{2/3}$ and γ_2 subunits was measured using three different ligands targeting the BZ binding site. These assays are not likely to measure any α_1 subunits that are complexed with the δ but not the γ_2 subunits, e.g., $\alpha_1\beta_{2/3}\delta$, because the GABA responses of these complexes cannot be modulated by BZs (Saxena and Macdonald, 1994).

Full saturation analysis was carried out on cerebellar membranes to determine the K_d and B_{max} values for [^{3}H]Ro 15-1788 binding, diazepam-sensitive [^{3}H]Ro 15-4513 binding, and [^{3}H]zolpidem binding (Table 1). All three ligands identified approximately the same number of binding sites in the cerebellar membranes (~990–1160 fmol/mg protein). There was no statistically significant difference between the number of binding sites for any ligand between the α_6 +/+ and δ-deficient/α_6 null groups, although the trend was always toward a reduced number of sites in the −/− animals. Total [^{3}H]Ro 15-4513 binding sites (both diazepam-sensitive and -insensitive components, with the nonspecific binding being defined in the presence of 10 μM Ro 15-4513), however, were decreased by 44% in the −/− cerebella (Table 1), with a minor change in the affinity K_d constant. This figure is in

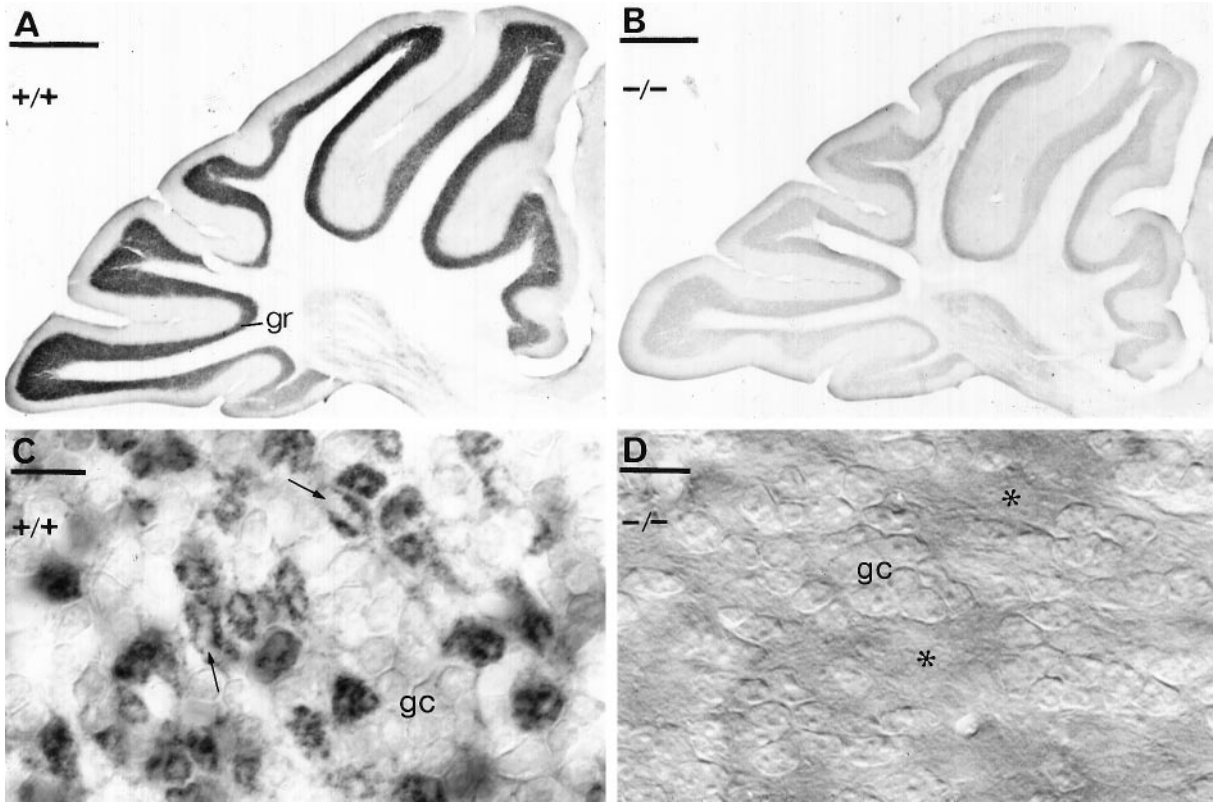

Figure 7. Immunodetection of the δ subunit of the $GABA_A$ receptor in α_6 +/+ (*A*, *C*) or α_6 −/− (*B, D*) cerebella, using a polyclonal antibody δ R7 and immunoperoxidase reaction. The granule cell layer showed intense immunoreactivity in α_6 +/+ animals but almost no staining was observed in the α_6 −/− mouse. *C*, At higher magnification, it is evident that the δ subunit is localized mainly in the glomeruli, granule cell bodies (*gc*) being only weakly outlined. The glomeruli appear as dark rings of granule cell dendrites surrounding a pale center (*arrows*) representing the unstained mossy fiber terminal. *D*, In the α_6 −/− mice, both the granule cell bodies (*gc*) and the glomeruli (*asterisks*) are immunonegative for the δ subunit. *C* and *D* were photographed using DIC optics. Scale bars: *A, B*, 500 μm; *C, D*, 10 μm.

line with the 30–40% contribution that the $\alpha_6\beta_{2/3}\gamma_2$ component has been reported to make to the total Ro 15-4513 binding sites in the cerebellum (Sieghart et al., 1987; Turner et al., 1991; Korpi et al., 1993; Quirk et al., 1994).

These binding results suggest that the amount of total α_1 protein complexed with $\beta_{2/3}$ and γ_2 in the cerebellum is essentially unchanged between δ-deficient/α_6 null and wild-type animals. Furthermore, immunocytochemistry with a polyclonal α_1-specific antibody (P16) showed no overt change in granule cell layer immunostaining at the light microscopic level in α_6 −/− cerebella compared with wild-type tissue (data not shown); however, this method may not pick out small changes in subunit levels. In fact, immunoblotting with α_1-specific antibodies did show a downward trend in α_1 protein levels between δ-deficient/α_6 null and +/+ cerebellar samples (Fig. 6*C*). In −/− animals, a small reduction with high variability was seen in the α_1 51 kDa band intensity, as determined by densitometric measurements. This was observed independently with three different α_1-specific antibodies: α_1(1–14) (S. Pollard and F. A. Stephenson, unpublished data), α_1(1–9) (Zezula and Sieghart, 1991), and α_1(328–382) (R. Pelz and W. Sieghart, unpublished data) (see Materials and Methods).

Behavioral characterization of α_6 null/δ-deficient mice

The total loss of α_6 from the cerebellum and the associated severe reduction of δ subunit levels might be expected to have consequences for nervous system function in the mutant mice. The cerebellum integrates sensory input needed for maintaining balance and orientation, has a prominent role in the refinement of motor action, and may also participate in motor memory storage (Raymond et al., 1996). We looked for evidence of cerebellar-associated motor deficits in the −/− mice. In terms of simple observable behavior, mutant mice seem indistinguishable from wild-type littermates. The adult α_6 null/δ-deficient mice are active and agile, whether they be in the cage or roaming freely, and exhibit spontaneous activity, such as walking upside down on the ceiling of their cages. In an open field test, mutant mice showed as much exploratory activity as individuals with normal levels of α_6 and δ proteins (Table 2). Mutant as well as wild-type mice learned the horizontal wire task (data not shown; see Materials and Methods). Both the wild-type and α_6 null/δ-deficient mice reached the rotating rod test learning criterion (Table 2). With these tests, we found no evidence to suggest any form of ataxia associated with cerebellar dysfunction. Additionally, a detailed behavioral analysis on an independently generated α_6 −/− mouse line, where the same exon was disrupted by insertion of a neo gene (exon 8, *Nco*I site) in a 129xC57BL/6 background, showed no abnormalities in motor behaviors (Gregg E. Homanics, Department of Anesthesiology, University of Pittsburgh, personal communication).

DISCUSSION

A mouse line lacking functional $GABA_A$ receptor α_6 subunit protein has been generated. Because of the restricted α_6 gene expression profile, this mutation was expected to principally affect the cerebellum. Furthermore, in the cerebellum, the granule cell δ subunit protein level was markedly reduced relative to wild-type levels. Thus

Table 2. Open field activity and rotating rod learning of wild-type and α_6 −/− mice

		Open field activity/5 min		
	n	Number of crossings	Number of fecal boli	Number of rearings
+/+	10	96 ± 5	3.3 ± 0.7	6.7 ± 1.5
−/−	10	84 ± 18	3.2 ± 0.5	5.2 ± 1.1

		Rotarod performance: seconds on the rod per 180 sec/(percent of animals staying for 180 sec)					
	n	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
+/+	15	114 ± 11 (20%)	147 ± 9 (40%)	160 ± 11 (67%)	179 ± 1 (87%)	179 ± 1 (87%)	180 ± 0 (100%)
−/−	14	144 ± 11 (43%)	148 ± 12 (57%)	167 ± 8 (79%)	176 ± 3 (86%)	176 ± 4 (93%)	180 ± 0 (100%)

Results are given as means ± SEM. No statistically significant differences ($p < 0.05$; Student's *t* test and repeated measures ANOVA). The Rotarod learning test was repeated with another batch of mice with similar results.

these mice effectively harbor a region-specific double subunit knockout, and the $GABA_A$ receptor complexity on granule cells is reduced to receptors largely containing just α_1, $\beta_{2/3}$, and γ_2 subunits. Two issues are discussed: the significance of multiple α subunits and defined assembly pathways for receptor subunits.

$GABA_A$ receptor α subunit heterogeneity

Surprisingly, in spite of a large loss of granule cell $GABA_A$ receptors, the α_6 null/δ-deficient mice are not grossly impaired in motor skills. This lack of phenotype under laboratory conditions was not anticipated from α_6 gene comparative studies. Both the conservation of peptide sequence in the N-terminal domain and a granule cell-specific expression pattern in the cerebellum of fish, birds, rodents, and humans imply that there has been a continual selection for the α_6 protein (Bahn et al., 1996; Hadingham et al., 1996).

In the α_6 null/δ-deficient mice, physiological changes in granule cell $GABA_A$ receptors are expected, but these have not obviously impaired cerebellar function. Removal of two of the six subunits from granule cells will still leave functional receptors with $\alpha_1\beta_{2/3}\gamma_2$ subunit combinations. Nevertheless, substitution of different α subunits in an $\alpha_x\beta_x\gamma_2$ complex may influence the inhibitory postsynaptic current kinetics (Gingrich et al., 1995; Tia et al., 1996). Synaptic transmission at GABAergic synapses is generated by millisecond pulses of 0.5–1 mM GABA (reviewed by Mody et al., 1994). Under these conditions, recombinant $\alpha_1\beta_{2/3}\gamma_2$ and $\alpha_6\beta_{2/3}\gamma_2$ receptors do behave differently, with the α_6-containing receptors having a slower deactivation rate (Tia et al., 1996). The physiological role of the δ subunit remains obscure (Shivers et al., 1989). During long applications of micromolar GABA, δ-subunits slow the acute macroscopic desensitization rate of recombinant $GABA_A$ receptors (Saxena and Macdonald, 1994); however, this property has not been studied using fast, brief GABA application.

Selective subunit partnerships

The α_6 −/− mouse has provided insight into $GABA_A$ receptor subunit assembly pathways in neurons. The α_6 protein derived from the targeted gene should terminate just after TM2. An analogous example has been studied for the mouse muscle nicotinic receptor δ subunit. When truncated just after TM2 (δM2) and co-expressed with wild-type nicotinic α, β, γ, and δ subunits in COS cells, δM2 interferes with receptor assembly (Verrall and Hall, 1992). Similarly, the truncated $GABA_A$ α_6 protein (α_6M2) may prevent $\alpha_6\delta$-containing receptors from reaching the granule cell surface. The association of α_6M2 and δ may inhibit mature receptor expression by forming specific complexes in the endoplasmic reticulum that are not permissive for further receptor assembly and/or trafficking. These will be retained and degraded (Verrall and Hall, 1992; Connolly et al., 1996). As for the nicotinic acetylcholine and glycine receptors (Verrall and Hall, 1992; Kuhse et al., 1993; Sumikawa and Nishizaki, 1994; Kreienkamp et al., 1995), the information needed for specific assembly of the $GABA_A$ receptor α_6 and δ proteins is likely to be in their N-terminal domains, because the N-terminal domain of α_6 is sufficient to block δ expression. Because they interact as an assembly intermediate, α_6 and δ probably occur adjacent to each other in the mature receptor subunit ring.

There are several other scenarios. The α_6M2 polypeptide could be degraded before pairing with the δ subunit. Because the δ subunit is not efficiently incorporated with other subunits, this might in turn be degraded. Alternatively, if no α_6 protein is present, the δ mRNA might be translated inefficiently, implying that α_6 protein levels feed back to regulate the translation of δ mRNA. Although this is an interesting possibility, there is no known mechanism.

Our results seem to confirm the antibody-based data suggesting that *in vivo*, δ predominantly assembles with α_6 and not α_1 (Caruncho and Costa, 1994; Quirk et al., 1994; Caruncho et al., 1995). Nevertheless, from the genetic results alone, an α_6 and δ interaction may be simply the first step allowing other subunits such as α_1 to subsequently join the complex. Thus, both $\alpha_1\alpha_6\beta\delta$ or even $\alpha_1\alpha_6\beta\gamma_2\delta$ might exist *in vivo* (Mertens et al., 1993; Pollard et al., 1995; R. Pelz and W. Sieghart, unpublished observations); however, because α_1, $\beta_{2/3}$, and γ_2 subunits cannot rescue the δ subunit in an α_6 −/− background, we predict that the $\alpha_1\beta_x\delta$ and $\alpha_1\beta_x\gamma_2\delta$ combinations will not be found to any great extent *in vivo*.

In recombinant systems [*Xenopus* oocytes, human embryonic kidney (HEK) 293 cells, and mouse L929 fibroblast cells], the situation is different. The δ subunit can assemble to form functional receptors with either α_1 or α_6 as $\alpha_1\beta_x\delta$, $\alpha_6\beta_x\delta$, and possibly $\alpha_1\beta_x\gamma_2\delta$ complexes, with the exact β subunit used having little influence (Saxena and Macdonald, 1994, 1996; Ducic et al., 1995; Krishek et al., 1996). Thus there may be unique architectural editing or chaperone mechanisms present in granule neurons that are not found in *Xenopus* oocytes or HEK cells. Alternatively, the subunits may differ slightly in affinity for each other. In a recombinant system, the large amounts of protein present may allow many combinations to assemble, even if they have nonoptimal association parameters. The results presented here demonstrate the importance of studying subunit assembly pathways in the brain.

[^{3}H]Muscimol as a selective autoradiographic probe for α_4, α_6, and δ subunit associations

It has been suggested that under autoradiographic binding conditions the $GABA_A$ site ligands [^{3}H]muscimol and [^{3}H]SR 95531 highlight a subpopulation of receptors in native membranes (Olsen et al., 1990). In a wide range of vertebrates, a hallmark of $GABA_A$ sites in the CNS is the high levels of [^{3}H]muscimol binding over the cerebellar granule cell layer (Palacios et al., 1980; Schmitz et al., 1988; Olsen et al., 1990; for review, see Wisden et al., 1996). A striking feature of our study was the almost total loss of high-affinity [^{3}H]muscimol and [^{3}H]SR95531 binding from the granule cell layer of α_6 null/δ-deficient cerebella (Fig. 4*B,C*), suggesting that the α_6 and/or δ subunits are responsible for these ligand profiles. From recombinant data, the $\alpha_6\beta_x\delta$ subunit combination is insensitive to BZs (Saxena and Macdonald, 1996) and sensitive to GABA (EC_{50} in the low micromolar range) but gives small currents (Saxena and Macdonald, 1994, 1996; Ducic et al., 1995). These properties would be consistent with the pharmacology of the cerebellar δ-containing receptors immunoprecipitated with a δ-specific antibody: high muscimol affinity and no BZ binding (Quirk et al., 1994).

Despite the absence of autoradiographic signal in −/− cerebella, muscimol is still an effective agonist of $GABA_A$ receptors on cultured α_6 −/− granule cells (J. Mellor and A. D. Randall, unpublished observations), although electrophysiological assays most likely use the low-affinity site. The remaining $\alpha_1\beta_{2/3}\gamma_2$ receptors in α_6 −/− granule cells should have a K_d for [^{3}H]muscimol of ~5 nM (Lüddens et al., 1990) and could be expected to bind [^{3}H]muscimol, but this is not the case under autoradiographic assay conditions. Similarly, in the inferior colliculi of wild-type and −/− animals, given the high concentration of $\alpha_1\beta_2\gamma_2$ receptors present (Persohn et al., 1992; Wisden et al., 1992; Fritschy and Möhler, 1995), it is difficult to explain the virtual absence of [^{3}H]muscimol binding sites in autoradiography (Fig. 4*B*). There may be some factor specifically associated with $\alpha_1\beta_{2/3}\gamma_2$ receptors on native membranes that prevents high-affinity [^{3}H]muscimol binding.

As pointed out previously (Shivers et al., 1989; Laurie et al., 1992), both δ subunit mRNA and protein closely parallel the distribution and abundance of [^{3}H] muscimol binding (Fig. 4*B,E*), e.g., highest in cerebellar granule cells, followed by the thalamus, and then caudate-putamen, neocortex, and dentate gyrus (Shivers et al., 1989; Olsen et al., 1990; Benke et al., 1991). In the forebrain, α_4 subunit mRNA also largely follows δ subunit distribution (compare Fig. 4, *B* and *D*; and see Wisden et al., 1992). The α_4 and α_6 subunits are closely related and form a subgroup set apart from the other α subunits (Seeburg et al., 1990; Ortells and Lunt, 1995; Tyndale et al., 1995). The total evidence suggests strongly that $\alpha_4\delta$ forms part of a $GABA_A$ receptor that is an $\alpha_6\delta$ combination homolog. A phylogenetic clock comparison calculated that α_4 and α_6 are the oldest α subunits and that the δ subunit is the oldest of all $GABA_A$ receptor subunit genes (Ortells and Lunt, 1995). Therefore, a selective interaction of α_4 and α_6 with δ might represent an early vertebrate $GABA_A$ receptor subtype.

In conclusion, we have provided evidence for a specific association between the α_6 and δ subunits in granule cell $GABA_A$ receptors. It seems likely that similar assembly rules exist for other brain heteromeric ligand-gated channels, e.g., the neuronal nicotinic acetylcholine receptor (Vernallis et al., 1993) and ionotropic glutamate receptors.

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