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Gene Knockout of the α6 Subunit of the γ-Aminobutyric Acid Type A Receptor: Lack of Effect on Responses to Ethanol, Pentobarbital, and General Anesthetics

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SUMMARY

The α6 subunit of the γ-aminobutyric acid type A receptor ($GABA_A$-R) has been implicated in mediating the intoxicating effects of ethanol and the motor ataxic effects of general anesthetics. To test this hypothesis, we used gene targeting in embryonic stem cells to create mice lacking a functional α6 gene. Homozygous mice are viable and fertile and have grossly normal cerebellar cytoarchitecture. Northern blot and reverse transcriptase-polymerase chain reaction analyses demonstrated that the targeting event disrupted production of functional α6 mRNA. Autoradiography of histological sections of adult brains demonstrated that diazepam-insensitive binding of [^{3}H]Ro15–4513 to the cerebellar granule cell layer of wild-type mice was completely absent in homozygous mice. Cerebellar $GABA_A$-R density was unchanged in the mutant mice; however, the apparent affinity for muscimol was markedly reduced. Sleep time response to injection of ethanol after pretreatment with vehicle or Ro15–4513 did not differ between genotypes. Sleep time response to injection of pentobarbital and loss of righting reflex and response to tail clamp stimulus in mice anesthetized with volatile anesthetics also did not differ between genotypes. Thus, the α6 subunit of the $GABA_A$-R is not required for normal development, viability, and fertility and does not seem to be a critical or unique component of the neuronal pathway mediating the hypnotic effect of ethanol and its antagonism by Ro15–4513 in mice. Similarly, the α6 subunit does not seem to be involved in the behavioral responses to general anesthetics or pentobarbital.

The molecular mechanisms that mediate the effects of ethanol and volatile general anesthetics have remained enigmatic despite evidence that some of the effects of these distinct classes of drugs affect the brain through overlapping pathways. These drugs may primarily target specific proteins rather than interacting nonspecifically with cellular lipids (1). Several lines of evidence implicate the $GABA_A$-R complex (a ligand-gated chloride channel that contains binding sites for numerous drugs, including the clinically useful anxiolytic benzodiazepines) in mediation of at least some of the effects of these drugs (for reviews, see Refs. 2 and 3). For example, animals selected for behavioral hypersensitivity to ethanol show cross-sensitivity not only to anesthetics[1] but also to drugs such as picrotoxin, which are known to interrupt the GABA pathway by blocking the $GABA_A$-R (4). In addition, $GABA_A$-R-specific drugs, such as picrotoxin and bicuculline, are also able to modify some ethanol-induced behaviors, such as motor impairment and withdrawal severity (5).

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Activation of $GABA_A$-Rs results in an inward flow of chloride ions and consequently in neuronal hyperpolarization. These receptors are pentameric complexes of several different subunit polypeptides (α1–6, β1–3, γ1–3, δ). Several subunit mRNAs, including the α6 (6), exist in multiple splice variants. The functional significance of this plethora of subunits and splice variants and the role of

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[1] L. L. Firestone, E. R. Korpi, L. Niemi, P. H. Rosenberg, G. E. Homanics, and J. J. Quinlan, unpublished observations.

ABBREVIATIONS: GABA, γ-aminobutyric acid; $GABA_A$-R, γ-aminobutyric acid type A receptor; ES, embryonic stem; PGKNeo, neomycin phosphotransferase; DI, diazepam insensitive; LORR, loss of righting reflex; bp, base-pair(s); kb, kilobase(s); G3PDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase or transcription; PCR, polymerase chain reaction.

each in mediating the effects of ethanol and volatile anesthetics are currently unknown.

Various data suggest that the α6 subunit is involved in mediating at least some of the behavioral effects of ethanol and general anesthetics. The alcohol antagonist Ro15–4513 (7) binds with high affinity to many GABA$_A$-R isoforms; however, its binding to α6-containing receptors is atypical in that it is insensitive to displacement by diazepam and other agonists (8, 9). The alcohol-nontolerant rat line is hypersensitive to motor impairment by ethanol and benzodiazepines (4), and a point mutation in the α6 subunit gene cosegregates with the alcohol-sensitive phenotype (10). These mutant rats lack high affinity, DI Ro15–4513 binding to cerebellar α6-containing GABA$_A$-Rs (10) and are significantly more sensitive to the ataxic effects of volatile anesthetics (11) and pentobarbital (4) than are control rats. In addition, the volatile anesthetic halothane acts as a partial agonist at some GABA$_A$-Rs that contain the α6 subunit (12). Finally, chronic treatment of rodents with ethanol selectively increases (13–15) or decreases (16) expression of the α6 subunit in cerebellum.

Consistent with the ligand-binding data, α6-containing receptors are functionally insensitive to potentiation by diazepam, which is in contrast to receptors containing the α1 subunit (8, 17). Furthermore, when α6 is expressed in both stably transfected cells and *Xenopus laevis* oocytes, the classic benzodiazepine antagonists Ro15–1788 and Ro15–4513 both act as agonists (18). Barbiturates, which are known to modulate the GABA$_A$-R, differentially affect α6-containing receptors, such that 100 μM pentobarbital elicits a large direct permeability response in the absence of agonist (GABA) that is not noted when α1 is present instead (18, 19).

Among the GABA$_A$-R gene family, the temporospatial pattern of α6 gene expression is the most highly restricted. In rodents, α6 mRNA is first detectable at ~1 week of postnatal life, and maximum expression is reached at ~3 weeks and maintained throughout adulthood (20–22). Anatomically, α6 expression is limited to postmigratory granule cells of the cerebellum and cochlear nuclei (18, 20–22). Interestingly, this tissue-specific pattern of expression has been conserved throughout evolution from fish to human (23). The functional importance of α6-containing GABA$_A$-Rs is also suggested by the fact that ~40–60% of cerebellar GABA$_A$-Rs contain this subunit (9, 24).

To assess the physiological role of the α6 subunit of the GABA$_A$-R, we used gene targeting in mouse ES cells to create mice with a disrupted α6 subunit gene. These mutant mice lacking functional α6 protein, and thus cerebellar DI GABA$_A$-Rs, were used to investigate the role of this subunit in development, pharmacology of GABA$_A$-Rs, and whole-animal responses to ethanol, pentobarbital, and the volatile anesthetics halothane and enflurane.

Materials and Methods

Generation of mutant mice. A targeting vector containing Strain 129-derived mouse genomic DNA was constructed as illustrated in Fig. 1A. The selectable marker PGKNeo was blunt-end ligated into the *Nco*I site in exon 8. This construct contains two copies of the negative selection marker herpes simplex virus thymidine kinase from pMC1TK (25), flanking 2.8 kb of 5′ homology and 3.7 kb of 3′ homology.

Approximately 2–4 $\times$ 10^7 R1 (26) ES cells/ml were electroporated

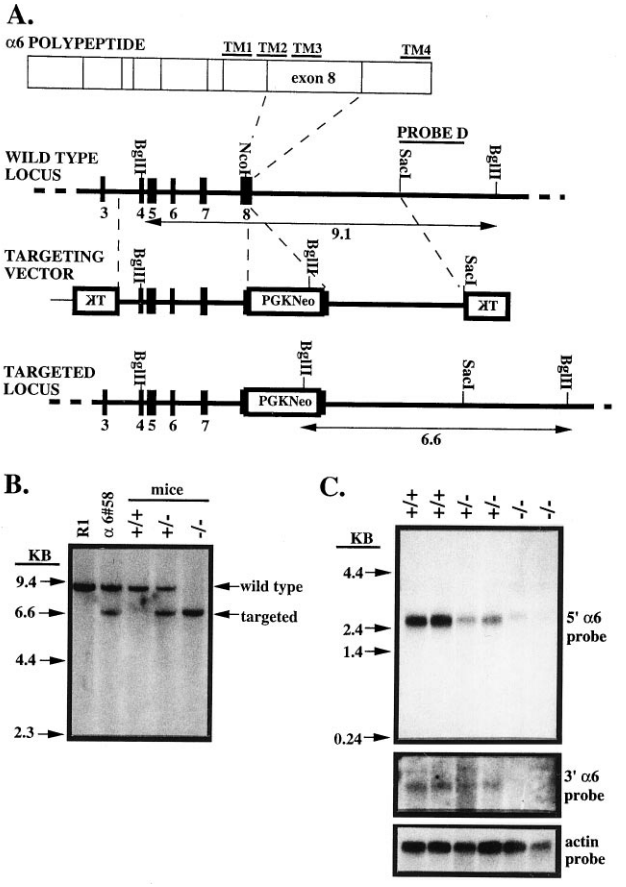

Fig. 1. Targeted gene disruption and molecular characterization. A, Gene targeting. The α6 polypeptide is illustrated with exon-exon boundaries. *TM1–4*, putative transmembrane domains. *Numbered filled boxes*, exons in genomic DNA. *Arrows*, 9.1-kb *Bgl*II fragment of the wild-type locus altered to 6.6 kb in the targeted locus. *TK*, reverse orientation of thymidine kinase cassette. B, Southern blot analysis of *Bgl*II-digested DNA from parental ES cells (R1), a targeted ES cell clone (α6#58), and mice that illustrate all three genotypes. C, Northern blot analysis of polyadenylated cerebellar RNA. mRNAs that hybridized with the 3′ α6 probe were the same size as those that hybridized with the 5′ α6 probe.

in a 0.5-cm cuvette in the presence of 5 nM linearized targeting vector at 250 V, 50 μF, 500-V capacitance, and 350-Ω resistance using an Electro Cell Manipulator 600 electroporation unit (BTX, San Diego, CA). Beginning at 24 hr after electroporation, ES cells were subjected to positive/negative selection (25) with geneticin (G418, 250 μg/ml; Life Technologies, Gaithersburg, MD) and ganciclovir (2 μM; gift of Syntex, Palo Alto, CA). Individual doubly resistant clones were expanded, an aliquot was frozen, and an aliquot was used to prepare genomic DNA. At all times, ES cells were maintained on feeder layers of mitotically inactivated mouse embryonic fibroblasts in medium that contained leukemia inhibitory factor (500 units/ml, ESGRO; Life Technologies).

ES cell clones were screened for targeting by genomic Southern hybridization analysis after digestion of DNA with *Bgl*II. Blots were hybridized with a genomic fragment that is external to the targeting construct (Fig. 1A, *PROBE D*). Correctly targeted ES cells clones were injected into C57BL/6J blastocysts to produce chimeric mice. Male chimeras were mated with C57BL/6J female mice. Heterozygous offspring were intercrossed to produce wild-type, heterozygous, and homozygous mice. All mice were genotyped as described above for ES cells. The mice used for these studies were derived from the α6#58 cell line, and the genetic background of all mice was Strain 129 X C57BL/6J F_2 or F_3. These mice have been given the strain

designation of *Gabrα6*^{tm1Geh} and can be obtained from the Induced Mutant Resource (Jackson Laboratories, Bar Harbor, ME).

Northern blot analysis. Polyadenylated RNA was isolated from cerebella of adult mice using the Microfast Track Kit (InVitrogen, San Diego, CA). Approximately 2.5 μg of polyadenylated RNA was electrophoresed, blotted to Hybond-N (Amersham, Arlington Heights, IL), and hybridized as previously described (27). The 5′ α6 probe is an RT-PCR product corresponding to nucleotides 250–963 of the α6 cDNA (28). The 3′ α6 probe is a genomic DNA restriction fragment that includes exon 9. Human β-actin (Clontech Laboratories, Palo Alto, CA) was used as a probe to assess the integrity of RNA and as a control for the amount of RNA loaded.

Pharmacological characterization. DI [^{3}H]Ro15–4513 binding to sagittal sections of adult mouse brain was determined as previously described (10). DI [^{3}H]Ro15–4513 binding to cerebellar homogenates was determined using a filtration assay similar to previous reports (29, 30). Membrane suspensions of ~100 μg of protein/ml of Krebs-Tris buffer were incubated to equilibrium with 25 nM [^{3}H]Ro15–4513 with or without diazepam (final concentration, 200 μM). Nonspecific binding was determined in the presence of flumazenil (10 μM).

[^{3}H]Ro15–4513 and [^{3}H]muscimol binding to mouse cerebellar membranes were determined using an ultracentrifugation binding assay (31). Membrane suspensions containing ~250 μg of protein/ml of Tris buffer were incubated to equilibrium (20 min, room temperature) with concentrations of [^{3}H]Ro15–4513 (30.4 Ci/mmol, New England Nuclear Research Products, Boston, MA) or [^{3}H]muscimol (19.1 Ci/mmol, New England Nuclear) approaching saturation with and without unlabeled flumazenil (10 μM) and muscimol (0.1 mM), respectively. Nonspecific binding was calculated by linear regression of free versus nonspecific binding data. Specific binding was calculated by subtracting nonspecific binding from total binding. Specific binding data were then fit to a logistic function of the form: specific binding = [maximal binding][(free slope)/(K_d slope + free slope)] using iterative nonlinear least-squares routines, yielding parameters such as the apparent binding affinity (K_d), maximal binding sites, and slope of the binding curve and their standard deviations (32). These parameters were compared using the Z statistic, a method that makes the fewest assumptions about the distribution of the data (33). The ratio of the difference between groups to the variance of that difference was referred to a standard normal distribution, with $p < 0.05$ considered significant.

RT-PCR. RNA from individual wild-type ($n = 5$) and homozygous ($n = 7$) cerebella were isolated to determine the relative patterns of expression of selected $GABA_A$-R subunit mRNAs. Total RNA was isolated from the dissected cerebella using Tri-Reagent (Sigma Chemical, St. Louis, MO) and aliquots (0.5 μg) were reverse-transcribed with Moloney murine leukemia virus RT (200 units) using random hexamers as described by the manufacturer. Parallel control reactions did not contain RT. The reaction (volume of 20 μl) included 1 mM deoxynucleotide triphosphate, 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and RNasin (30 units). RT was carried out first at room temperature for 10 min and then at 37° for 60 min, heat-denatured at 98° for 5 min, and quick chilled on ice. Eighty microliters of a solution containing 0.5 mM primer pairs (Table 1), 50 mM Tris·HCl. pH 9.0, 2.0 mM $MgCl_2$, 15 mM KCl, and DNA polymerase (2.5 units) was added to the resultant cDNA for the PCR. For quantification, [^{32}P]dCTP (1–2 μCi/reaction) was added to the PCR mixture. The mixture was amplified for 28–32 cycles (depending on the abundance of each subunit), in which each amplification cycle consisted of a denaturation step (95°, 30 sec), an annealing step (60°, 45 sec), and an elongation step (72°, 45 sec). After completion of PCR cycles, the PCR products were incubated at 72° for final extension for 15 min. Aliquots of the PCR products (20 μl) were loaded onto 1.8% agarose gels stained with ethidium bromide.

For semiquantitative RT-PCR, the amount of [^{32}P]dCTP incorporated into each band was determined using a scintillation counter. Preliminary experiments were performed for each primer pair to ensure that the extent of [^{32}P]dCTP incorporated into each band was an exponential function of the number of amplification cycles and to determine the range of linearity of the amplification curves. For each sample, we also analyzed the mRNA content of G3PDH, which was used to correct for variations in the amounts of RNA from individual cerebella. The cDNA corresponding to G3PDH was amplified as described above and the amount of the incorporated [^{32}P]dCTP after different numbers of amplification cycles was assessed by scintillation counting. The counts obtained from the amplifications of each subunit-specific primer pair were normalized for each cerebellar sample based on the amount of amplification product obtained using the G3PDH primers.

The mRNA content corresponding to each $GABA_A$-R subunit from each cerebellum was examined at least three times. Data from homozygous or wild-type cerebellar RNA for each individual subunit were pooled, and mean counts incorporated were calculated and compared between the two groups using analysis of variance and Student's t test.

Histology. Wild-type and homozygous mice were perfused with 4% paraformaldehyde, and brain sections were stained with toluidine blue or with antineurofilament (RT97). Neurofilament staining was detected with fluorescein isothiocyanate-conjugated secondary antibodies.

Sleep time assay. *In vivo* drug administration was by intraperitoneal injection. Ethanol was diluted in 0.9% saline (20% w/v) and administered at 0.02 ml/g of body weight. Ro15–4513 (Research Biochemicals, Natick, MA) was dissolved in a drop of Tween 80, diluted in saline (1.0 mg/ml), and sonicated. Ro15–4513 or vehicle

TABLE 1
RT-PCR primers

Primer	Sequence (5′ to 3′)	Position	GenBank no.
Mouse α6 sense	TTCTAGCCTCCTCCAGTATGATTTG	816–840	X51986
PGKNeo antisense	GCTACCGGTGGATGTGGAATGTGT	544–521	M18735
Mouse α6 sense	TTCTAGCCTCCTCCAGTATGATTTG	816–840	X51986
Mouse α6 antisense	TACTCAACAGTACTGCTCACTTCC	1571–1548	X51986
Rat G3PDH sense	AAGTTCAACGGCACAGTCAAGGCT	228–251	X02231
Rat G3PDH antisense	GGTGCAGGATGCATTGCTGACAAT	524–501	X02231
Rat α1 sense	AGCTATACCCCTAACTTAGCCAGG	1174–1197	L08490
Rat α1 antisense	AGAAAGCGATTCTCAGTGCAGAGG	1478–1455	L08490
Mouse α3 sense	CAACATAGTGGGAACCACCTATCC	1362–1385	M8656
Mouse α3 antisense	ATACAGTGCTCTGGAAGCTGTGCT	1691–1668	M8656
Rat β2 sense	AAGATGCGCCTGGATGTCAACAAG	1130–1153	X15467
Rat β2 antisense	TTTTGTGCCACATGTCGTTCCAGG	1350–1327	X15467
Rat γ2 sense	GAGCAACCGGAAACCAAGCAAGGA	1121–1144	L08497
Rat γ2 antisense	AATGCGAATGTGTATCCTCCCGTG	1355–1332	L08497
Mouse δ sense	ACGGGCTTCAGCTATCAAAGCTCT	1006–1029	S42882
Mouse δ antisense	CAGTAGATGATGTTGACCGCTGCA	1431–1408	S42882

(Tween 80 in saline) was administered at 0.01 ml/g of body weight 15 min before injection with ethanol. Pentobarbital was dissolved in saline (3.1 mg/ml) and injected at 0.01 ml/g of body weight. Sleep time (i.e., duration of the LORR) in response to ethanol (with and without pretreatment with Ro15–4513), and pentobarbital was assayed as previously described (34). Mice that were 57–88 and 84–119 days old were used for the ethanol and pentobarbital assays, respectively. All assays were performed by an investigator who was unaware of the individual genotypes of the mice being tested. For the ethanol sleep time assay, the effects of genotype and pretreatment were analyzed by analysis of variance and Scheffé's *post hoc* analysis (Stat-View; Abacus Concepts, Berkeley, CA). The effect of genotype on pentobarbital-induced sleep time was analyzed by *t* test.

LORR. For each LORR determination, six to eight unrestrained mice were placed in individual wire-mesh cages mounted on a carousel in a sealed Plexiglas chamber maintained at 33–35°. Soda lime scattered on the chamber floor maintained the ambient CO_2 tension at <0.05 atmosphere. Controlled amounts of each volatile agent [halothane (clinical grade, Ayerst, New York, NY); enflurane (Anaquest, Madison, WI)] diluted with oxygen were delivered from a copper kettle or a specific vaporizer and monitored on-line either by mass spectroscopy (Ohmeda 6000) or with a piezoelectric analyzer (Siemens 120). After equilibration for 15 min at each anesthetic concentration, mice were assessed for LORR by an observer who was unaware of the genotype being tested, while the carousel was rotated five complete turns at 4 rpm. Scoring was quantal: mice that passively rolled over twice were scored as positive for the LORR. The chamber was then evacuated, and the mice were allowed to recover in air for 10 min before the next determination. This process was repeated until all general anesthetic concentrations (usually five) were tested. Rectal temperature was measured after each assay to ensure normothermia.

Dose-response data for each cohort of mice were fit to sigmoid curves using the iterative nonlinear least-squares method described by Waud (32). The ED_{50} values and estimates of the standard errors were derived from these curves. Slope is defined as the steepness of the anesthetic concentration-response curve and is a parameter generated by the nonlinear least-squares fitting routine. After unblinding, data from groups (wild-type and homozygous) were pooled and refit to sigmoid functions. Statistical comparisons between groups were performed as described above using the *Z* statistic (33).

Tail-clamp/withdrawal assay. Briefly, equilibration of mice with anesthetic agents in a heated, enclosed chamber and the monitoring of chamber temperature, F_{IO_2} (inspired oxygen concentration), and anesthetic concentrations were similar to procedures used for the LORR assay. After equilibration at a given anesthetic concentration, a tail-clamp stimulus was applied. If the mouse exhibited motor activity (typically of the limbs), the concentration was considered to be one that permitted a positive response. The mice were allowed to recover in an oxygen-rich atmosphere for ≥20 min before being equilibrated with the next anesthetic concentration and retested. ED_{50} values for fractional group response were calculated as for the LORR assay.

Results

Creation and molecular characterization of mutant mice. Of 64 ES cell clones analyzed for gene targeting, 17 displayed predicted restriction fragment length polymorphisms indicative of correct gene targeting at the α6 locus. As indicated in Fig. 1, A and B, probe D (which is external to the targeting construct) hybridized only to the 9.1-kb *Bgl*II fragment from the wild-type allele in the parental ES cell line (R1). In correctly targeted clones (e.g., α6#58), this probe also hybridized to a 6.6-kb fragment from the targeted allele. Targeting was further confirmed with several other restriction digests and by hybridization with a PGKNeo probe and with a probe from the 5′ arm of the targeting construct (data not shown). Two correctly targeted clones (α6#55, and α6#58) were transferred through the mouse germline. Intercrossing of heterozygous F_1 mice (within each clone) produced wild-type, heterozygous, and homozygous mice (see Fig. 1B) at the expected frequencies. All mice were morphologically and behaviorally indistinguishable, and no overt signs of ataxia or motor incoordination were apparent.

To determine the effects of gene targeting on expression of the α6 subunit gene, we conducted Northern blot analysis. Hybridization of cerebellar polyadenylated RNA with an α6 specific probe that hybridizes 3′ of the PGKNeo insertion site showed the absence of the ~2.70-kb α6 mRNA in homozygous mice (Fig. 1C). Rehybridization of the same blot with a probe that is 5′ of the PGKNeo insertion site showed the presence of a low-abundance ~2.75-kb message in homozygous cerebella (Fig. 1C). The size of this message, which originated from the targeted α6 allele, indicated that it likely represented a chimeric message that originated from the α6 promoter and terminated at the polyadenylation site of PGKNeo.

To test this possibility, RT-PCR analysis was conducted using primers that were designed to specifically amplify a chimeric transcript that originates in α6 and terminates in PGKNeo. As shown in Fig. 2A, this primer set specifically produced a 431-bp product from homozygous cerebellar mRNA but failed to produce a product from wild-type mRNA. To further characterize the result of the targeting event on α6 mRNA production, we conducted RT-PCR using primers specific for α6 that flank the PGKNeo insertion site. As

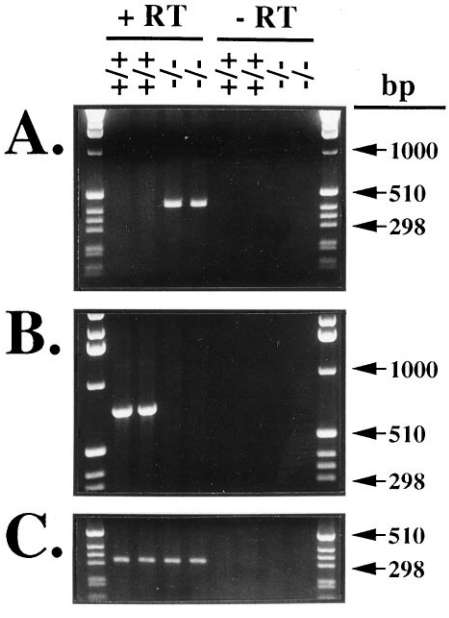

Fig. 2. RT-PCR analysis of transcripts produced from the GABA$_A$-R α6 locus in wild-type (+/+) or homozygous (−/−) cerebella. Two representative samples of each genotype are shown. These samples were amplified in the presence (+*RT*) or absence (−*RT*) of RT. A, RT-PCR analysis using primers that selectively amplify a chimeric transcript that originates in α6 and terminates in PGKNeo. This transcript is present only in mRNA from homozygous mice. B, RT-PCR analysis using primers specific for α6 that bind to sites that flank the PGKNeo insertion site. This wild-type transcript is readily detectable in wild-type mice but is completely absent in homozygous mice. C, Control RT-PCR analysis using primers specific for G3PDH.

shown in Fig. 2B, this primer set readily amplifies a 755-bp product from wild-type cerebellar mRNA but fails to produce a product from homozygous mRNA. This indicates that no wild-type message is produced in homozygous mice. We also used a primer pair specific for PGKNeo and α6 sequences that are downstream of the PGKNeo insertion site to screen for read-through chimeric mRNA that originates in the PGKNeo cassette and terminates in α6 sequences. In some homozygous cerebellar mRNA samples, this primer set produced a faint band on ethidium-stained gels (data not shown). The ability of G3PDH to amplify a product from all mRNAs (Fig. 2C) verified the integrity of all mRNA preparations.

Thus, the wild-type and the modified α6 genes produced distinctly different transcripts. In homozygous cerebella, no wild-type α6 transcripts are produced; only chimeric mRNAs between α6 and PGKNeo are detectable. Based on the predicted sequence of the low-abundance chimeric transcript that originates in α6 and terminates in PGKNeo, if it were translated, it would be expected to produce a truncated protein that contains amino acids 1–293 of α6 (28) fused to four amino acids from PGKNeo promoter sequences. Such a protein should be nonfunctional because it would lack the carboxyl-terminal 150 amino acids of α6 that include a portion of transmembrane 2 and all of transmembrane domains 3 and 4, as well as the putative intracellular loop. The other low-abundance chimeric transcript, which originates in PGKNeo and terminates in α6, would be expected to produce only protein from the PGKNeo coding sequence because of the presence of in-frame stop codons.

Pharmacological changes in mice lacking α6. Both wild-type and homozygous mouse brain possess a large quantity of binding sites for [^{3}H]Ro15–4513 (Fig. 3A). In wild-type mouse brain, this binding is displaceable from all $GABA_A$-Rs by diazepam except for the receptors that include the α6 subunit (Fig. 3B); this DI [^{3}H]Ro15–4513 binding is limited almost exclusively to the cerebellar granule cell layer. In marked contrast to the DI [^{3}H]Ro15–4513 binding observed in wild-type mouse brain, diazepam completely displaced all [^{3}H]Ro15–4513 binding in homozygous brain. Heterozygous brain possessed DI [^{3}H]Ro15–4513 binding that was intermediate between that of wild-type and homozygous brain. This autoradiographic analysis was confirmed with an *in vitro* binding assay that used cerebellar homogenates (Table 2A).

Specific binding of both the GABA ligand [^{3}H]muscimol and the benzodiazepine ligand [^{3}H]Ro15–4513 to mouse cerebellum was saturable. The slopes of the concentration-specific binding curves were statistically indistinguishable for both ligands in wild-type and homozygous animals (Table 2, B and C), allowing valid comparisons of their apparent affinities. Absence of the α6 subunit was associated with a marked decrease in the affinity of cerebellar $GABA_A$-Rs for [^{3}H]muscimol [Table 2B, wild-type values were similar to previously published values (35–37)]. However, homozygous mice did not differ from wild-type in the maximal number of GABA binding sites (Table 2B). [^{3}H]Ro15–4513 binding also showed a similar maximal receptor number in the wild-type and homozygous tissue (Table 2C); however, unlike that obtained with [^{3}H]muscimol, the apparent affinity constant for [^{3}H]Ro15–4513 did not differ between genotypes and was similar to previously published values (30, 38). Together, the [^{3}H]Ro15–4513 and [^{3}H]muscimol binding data suggest the possibility that another $GABA_A$-R subunit or subunits substituted for the missing α6 subunit to maintain the normal number of $GABA_A$-Rs. Furthermore, the substituted subunit or subunits must assemble to form a $GABA_A$-R isoform that exhibits a lower affinity for [^{3}H]muscimol and a comparable affinity for [^{3}H]Ro15–4513.

Semiquantitative RT-PCR. To identify a $GABA_A$ R subunit that might be up-regulated and could account for the observed pharmacology, we examined the abundance of several subunit mRNAs by semiquantitative RT-PCR analysis. As shown in Fig. 4, the amounts of the α1, α3, β2, γ2, and δ mRNAs did not statistically differ between wild-type and homozygous cerebella.

Normal cerebellar histology in mice lacking α6. Toluidine blue staining in homozygous cerebellum (Fig. 5B) revealed a granule cell layer and a molecular layer that were similar in width and cell density to wild-type cerebellum (Fig.

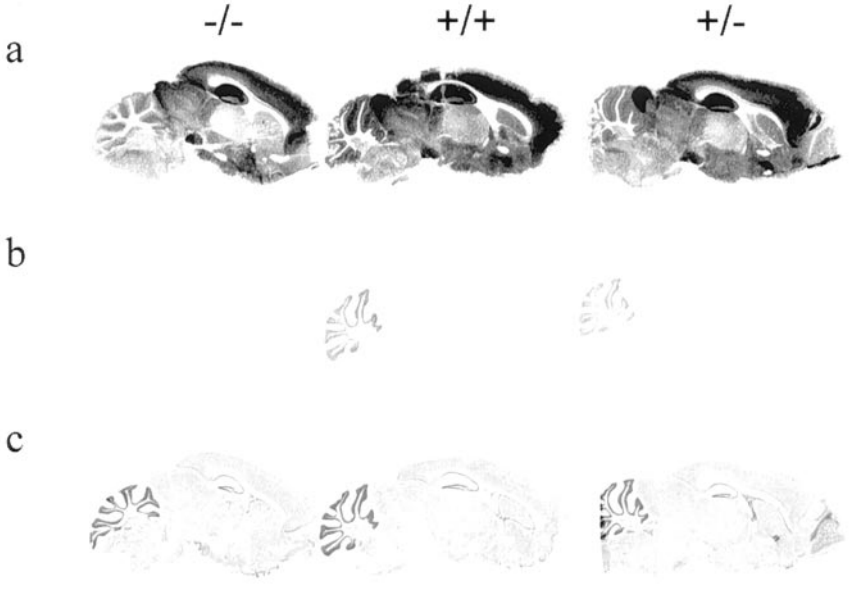

Fig. 3. Autoradiographic analysis of sagittal sections of adult wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mouse brain. A, Total binding of [^{3}H]Ro15–4513. B, DI binding of [^{3}H]Ro15–4513. C, Thionin staining.

TABLE 2
α6 Binding data

	Wild-type	Homozygous	*p*
A. Diazepam-insensitive [^{3}H]Ro15-4513 binding to cerebellum (*fmol/mg of protein*)[a]			
Specific binding in absence of diazepam	796 ± 38[b]	674 ± 61[b]	
Specific binding in presence of 200 μM diazepam	92 ± 11[c]	17 ± 8[d]	
B. [^{3}H]Muscimol binding to cerebellum[e]			
Apparent K_d (*nM*)	14.9 ± 2.0	66.3 ± 19.3	0.008
Maximal binding (*pmol/mg*)	4.3 ± 0.2	4.6 ± 0.6	0.81
Slope	1.0 ± 0.1	1.2 ± 0.2	0.37
C. [^{3}H]Ro15-4513 binding to cerebellum[f]			
Apparent K_d (*nM*)	12.4 ± 2.5	13.6 ± 5.9	0.90
Maximal binding (*pmol/mg*)	2.7 ± 0.2	2.4 ± 0.3	0.41
Slope	1.1 ± 0.2	0.9 ± 0.2	0.48

[a] Data are mean ± standard deviation of eight individual binding assays.
[b] $p = 0.09$, wild-type compared with homozygous.
[c] $p < 0.001$, wild-type compared with background nonspecific binding.
[d] $p = 0.30$, homozygous compared with background nonspecific binding.
[e] These data were pooled from four and five separate experiments for the wild-type and homozygous tissue, respectively. Two separate membrane preparations were used for each genotype. Each experiment consisted of 24 specific binding determinations.
[f] These data were pooled from six separate experiments using two separate membrane preparations for each genotype. Each experiment consisted of 24 specific binding determinations.

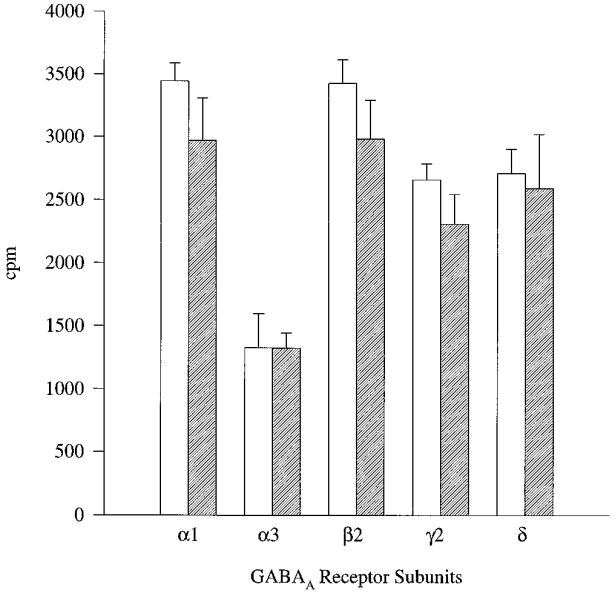

Fig. 4. Semiquantitative RT-PCR assay of cerebellar mRNAs for selected GABA$_A$-R subunits. The amounts of α1, α3, β2, γ2, and δ subunit mRNAs are expressed as [^{32}P]dCTP cpm incorporation into the PCR products. Data from wild-type (▨) and homozygous (□) mice demonstrate no significant difference in abundance of these subunits between mice of different genotypes.

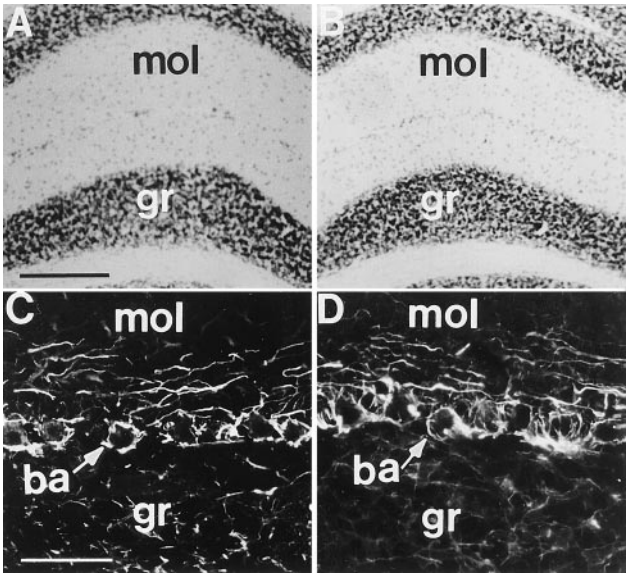

Fig. 5. Cerebellum of mice lacking the α6 subunit of the GABA$_A$-R exhibit normal cytoarchitecture. In cryostat sections of wild-type (A) and homozygous (B) cerebellum stained with toluidine blue, note the similar widths of granule cell layers and molecular layers in wild-type and homozygous cerebella. *Bar*, 50 μm. In cryostat sections of wild-type (C) and homozygous (D) brain stained for neurofilament, note the similar density of neurofilament-positive axons in the molecular layer and strongly stained basket cell neurons in both wild-type and homozygous cerebella. *Bar*, 200 μm. *gr*, granule cell layer; *mol*, molecular layer; *ba*, basket cell axons.

5A). Neurofilament staining was also indistinguishable between wild-type and homozygous cerebella (Fig. 5, C and D) and showed typical strong staining for basket cell neurons surrounding Purkinje cells and weaker staining in the molecular layer. Sections from homozygous and wild-type cerebella were also stained with antibodies directed against synaptophysin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and P84, an antigen associated with synaptic regions (39). Staining patterns of these synapse-associated antigens in both homozygous and wild-type sections appeared to be identical (data not shown). Thus, the general morphology and cytoarchitecture of the cerebellum of knockout mice seemed to be identical to those of wild-type control mice.

Normal behavioral responses. To investigate the importance of DI Ro15–4513 binding sites to whole-animal response to a variety of pharmacological agents, behavioral assays (sleep time, LORR, and tail-clamp/withdrawal) were used to compare wild-type mice with homozygous mice. Sleep time in response to ethanol did not differ between groups (Table 3). It has been suggested that the pathway by which Ro15–4513 antagonizes ethanol-induced sleep time involves DI sites (8, 40), although this has been debated (41). To directly test the involvement of DI sites, we compared the ability of Ro15–4513 to reduce ethanol-induced sleep time in wild-type and homozygous mice. As shown in Table 3, Ro15–4513 was equally effective in mice of each genotype. Thus, at this dose of Ro15–4513 (10 mg/kg), the reduction in sleep time in response to ethanol does not seem to be mediated by DI receptors.

TABLE 3
Sleep time assay
Duration of the LORR (i.e., sleep time) in response to ethanol (3.5 g/kg) was measured after pretreatment with vehicle or Ro15-4513 (10 mg/kg). Sleep time in response to pentobarbital (31 mg/kg) was measured without pretreatment. Values are mean ± standard error.

Genotype	Sleep time		
	Vehicle + ethanol	Ro15-4513 + ethanol	Pentobarbital
	min		
Wild-type	49.3 ± 5.3 (n = 15)	26.9 ± 3.5[a] (n = 14)	30.3 ± 3.2 (n = 24)
Homozygous	41.7 ± 5.9 (n = 15)	28.6 ± 4.4[a] (n = 15)	27.4 ± 2.3 (n = 23)

[a] Significant main effect of pretreatment ($p < 0.001$).

$GABA_A$-Rs containing the $\alpha 6$ subunit are electrophysiologically distinct from receptors containing other α subunits in their response to barbiturates, as measured in oocyte expression systems (19). Pentobarbital has a higher affinity and efficacy for $\alpha 6$-containing receptors. If these *in vitro* results are a true model of endogenous $GABA_A$-Rs in the brain and these receptors are physiologically important, one could predict that mice lacking the $\alpha 6$ subunit would be less sensitive to the sedative/hypnotic effects of pentobarbital. To test the physiological relevance of these *in vitro* observations directly on whole-animal responses, we measured sleep time in response to pentobarbital. As shown in Table 3, sleep time did not differ between the two genotypes. Thus, it appears that $\alpha 6$-containing $GABA_A$-Rs are not required to mediate the sedative/hypnotic effects of barbiturates in whole animals, at least as measured by this sleep time assay.

Similarly, for two halogenated volatile anesthetics from structurally distinct categories (i.e., halothane, an alkane, and enflurane, an ether), the EC_{50} values for LORR did not differ statistically between genotypes (Table 4; $p > 0.10$ for halothane, $p > 0.25$ for enflurane). In addition, no difference was found between genotypes in tail-clamp/withdrawal response to enflurane ($p > 0.15$).

Discussion

Gene targeting technology is a powerful technique for furthering understanding of the biological role of specific gene products in the nervous system. For example, mice that lack a functional $GABA_A$-R $\gamma 2$ subunit gene were used to establish the role of that subunit for benzodiazepine agonist action (42). Similarly, the reduced response of mice lacking the γ isoform of protein kinase C to alcohol support that protein kinase isozymes may be important mediators of some of the effects of ethanol (43). Because abundant evidence suggests that $GABA_A$-Rs are involved in mediating the effects of ethanol and general anesthetics, we used the same gene knockout approach to examine the role of specific subunits of the $GABA_A$-R. In the current study, mice were created that lacked a functional $\alpha 6$ subunit gene. Northern blot, RT-PCR, autoradiographic, and pharmacological analyses indicate that the modified $\alpha 6$ allele is a true null allele (i.e., no functional $\alpha 6$ protein is produced, which ultimately results in the absence of cerebellar DI $GABA_A$-Rs). The results presented indicate that $GABA_A$-Rs containing $\alpha 6$ subunit polypeptides are not required for normal brain development, viability, or fertility. In addition, DI $GABA_A$-Rs do not seem to be a critical or unique component of the neuronal pathway mediating the hypnotic effect of ethanol and its antagonism by Ro15–4513 in mice. Likewise, the sedative/hypnotic effects of volatile anesthetics and barbiturates do not require $\alpha 6$-containing receptors.

TABLE 4
Behavioral responses to volatile anesthetics

Genotype (n)	Anesthetic	ED_{50} (% Atmosphere)	Slope
LORR assay			
Wild-type (28)	Enflurane	1.09 ± 0.06	10.53 ± 1.75
Homozygous (28)	Enflurane	1.17 ± 0.06	9.15 ± 1.58
Wild-type (20)	Halothane	0.69 ± 0.04	8.48 ± 1.59
Homozygous (20)	Halothane	0.79 ± 0.04	11.66 ± 2.27
Tail-clamp/withdrawal response			
Wild-type (20)	Enflurane	1.58 ± 0.07	13.55 ± 2.68
Homozygous (20)	Enflurane	1.74 ± 0.09	10.45 ± 2.02

Values are mean ± standard error.

The conclusion that the ability of Ro15–4513 to reduce sleep time in response to ethanol is not mediated by DI receptors is in contrast to that of Harris *et al.* (40). These differing conclusions may be due to different treatment protocols. In the current study, we used pretreatment with a high dose of Ro15–4513, which may have masked the contribution of DI sites. In the study by Harris *et al.*, this same dose administered after injection with ethanol did not reduce ethanol-induced sleep time. Their conclusion that DI sites are involved in mediating the effects of Ro15–4513 on ethanol-induced sleep time were based on lower doses of Ro15–4513 that were effective at reducing sleep time. Additional studies are warranted in the gene knockout mice.

The absence of a dramatic alteration in behavioral response to the neuroactive compounds tested is somewhat surprising given the unique pharmacology that has been demonstrated *in vitro* for $\alpha 6$-containing $GABA_A$-Rs. The absence of a behavioral phenotype in the mutant mice could be due to the substitution of the $\alpha 6$ subunit by other $GABA_A$-R subunits. This type of compensatory mechanism is supported by the observation that $GABA_A$-Rs in cerebella of homozygous mice have a greatly reduced affinity for muscimol, despite being as abundant as those in wild-type cerebella. That is, the reduced affinity for the GABA agonist muscimol indicates a reduction in the numbers of $\alpha 6$-containing $GABA_A$-Rs (17, 18, 44), whereas the lack of a change in the maximal binding for muscimol indicates no change in total receptor numbers. This suggests that the absence of the $\alpha 6$ subunit is tolerated provided that the total numbers of inhibitory $GABA_A$-Rs are maintained. Acute knockdown of the $\alpha 6$ receptor subunit in cerebellar granule cells *in vitro* using antisense oligodeoxynucleotides results in an increase in the EC_{50} value for GABA-gated Cl^- currents, as measured with whole-cell voltage-clamp recordings (45). In that study, there was a marked reduction in the maximal current responses mediated by the antisense treatment consistent with a decrease in total numbers of $GABA_A$-Rs. Results of these acute subunit knockdown experiments contrast with the results presented here with respect to the homozygous $\alpha 6$ null mutants and suggest that reductions in receptor subunits and receptor numbers may be possible *in vitro* but that additional

levels of control may be operative if the inhibitory tone is insufficient to maintain neuronal homeostasis.

The molecular basis and the physiological significance of the reduced affinity for muscimol of the GABA$_A$-Rs in the α6 homozygous mutants have yet to be elucidated. We examined the level of expression of additional GABA$_A$-R subunit mRNAs (including the α1 subunit) in cerebella from wild-type and homozygous mice by using a semiquantitative RT-PCR based assay. No significant differences in mRNA content between wild-type and homozygous mouse cerebellum were detected using this approach. Although minor but physiologically important changes may not have been detected by this assay, the data indicate that increased expression of additional subunits does not likely contribute to the pharmacological results we report here. Alterations in GABA$_A$-R production in these mutant mice are of great interest because they may yield insight into the mechanisms regulating and coordinating production of the diverse array of GABA$_A$-R isoforms present in the mammalian brain. In rats, both the α1 and α6 subunit mRNAs increase during the second and third postnatal weeks (20). Although the α1 subunit is predominant early postnatally, the α6 subunit mRNA increases significantly during the same time frame. In the adult, mixed populations of receptors containing α1, α6, and α1/α6 combinations have been shown to coexist in cerebellar GABA$_A$-Rs (9, 46). These studies demonstrate that the mixed α1/α6 receptors show a pharmacology that is not dominated by either subunit but rather the pharmacology of these receptors shows properties of both subunits. This may be related to the lack of a difference with respect to the actions of volatile anesthetics and barbiturates in the homozygous mice reported in the current study. That is, 39% of cerebellar receptors contain α6 subunits, and of these, 41–45% also contain an α1 receptor subunit (9, 46). It may be that the action of volatile anesthetics and barbiturates at α1/α6 hybrid receptors is less evident than actions at GABA$_A$-Rs that contain only one type of α subunit *in vivo*.

Our data showing that the homozygous mice exhibit an increased K_d value for muscimol binding is consistent with the formation of receptors dominated by the α1 subunit, which has a lower affinity for GABA agonists than does the α6 subunit (17, 18, 44). Furthermore, the similar maximal binding for muscimol in both wild-type and homozygous mice is consistent with the hypothesis that normal levels of expression of the α1 subunit are sufficient to compensate for the α6 deficiency in terms of establishing the requisite numbers of GABA$_A$-Rs for normal cerebellar development. This also suggests that in wild-type mice, the α1 and α6 subunits compete during the process of assembly, with the total numbers of GABA$_A$-Rs determined by additional (or extrinsic) factors.

An alternative point to consider concerning the lack of a behavioral phenotype is that our rudimentary knowledge of the nervous system and our predictions about expected phenotypes are naive. It is also plausible that although pharmacological observations in isolated artificial systems *in vitro* are accurate predictors of other *in vitro* responses, they may be inadequate to reliably predict behavioral correlates in the context of intact animals. Thus, one of the goals of future research in this area should be to define the physiological role of each subunit of the GABA$_A$-R in whole-animal systems. Although this goal is unrealistic at present, mutant mice lacking GABA$_A$-R subunits (singly and in combination) represent the state of the art. Research with such mice will undoubtedly yield exciting insights into the contribution each subunit makes to the neuronal pathways that mediate the behavioral effects of ethanol, barbiturates, benzodiazepines, and volatile general anesthetics.

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