

# Mice devoid of $\gamma$ -aminobutyrate type A receptor $\beta 3$ subunit have epilepsy, cleft palate, and hypersensitive behavior

(gene targeting/benzodiazepine/Angelman syndrome/anesthesia)

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**ABSTRACT**  $\gamma$ -Aminobutyric acid type A receptors (GABA<sub>A</sub>-Rs) mediate the bulk of rapid inhibitory synaptic transmission in the central nervous system. The  $\beta 3$  subunit is an essential component of the GABA<sub>A</sub>-R in many brain regions, especially during development, and is implicated in several pathophysiologic processes. We examined mice harboring a  $\beta 3$  gene inactivated by gene targeting. GABA<sub>A</sub>-R density is approximately halved in brain of  $\beta 3$ -deficient mice, and GABA<sub>A</sub>-R function is severely impaired. Most  $\beta 3$ -deficient mice die as neonates; some neonatal mortality, but not all, is accompanied by cleft palate.  $\beta 3$ -deficient mice that survive are runted until weaning but achieve normal body size by adulthood, although with reduced life span. These mice are fertile but mothers fail to nurture offspring. Brain morphology is grossly normal, but a number of behaviors are abnormal, consistent with the widespread location of the  $\beta 3$  subunit. The mice are very hyperactive and hyperresponsive to human contact and other sensory stimuli, and often run continuously in tight circles. When held by the tail, they hold all paws in like a ball, which is frequently a sign of neurological impairment. They have difficulty swimming, walking on grids, and fall off platforms and rotarods, although they do not have a jerky gait.  $\beta 3$ -deficient mice display frequent myoclonus and occasional epileptic seizures, documented by electroencephalographic recording. Hyperactivity, lack of coordination, and seizures are consistent with reduced presynaptic inhibition in spinal cord and impaired inhibition in higher cortical centers and/or pleiotropic developmental defects.

$\gamma$ -Aminobutyric acid type A receptors (GABA<sub>A</sub>-Rs) mediate the bulk of rapid inhibitory synaptic transmission in the central nervous system. GABA<sub>A</sub>-Rs are heteromeric ligand-gated chloride channels that are encoded by at least 15 separate subunit genes and they form a number of binding sites for clinically important drugs such as benzodiazepines, barbiturates, and anesthetics (1, 2). The  $\beta 3$  subunit is a major constituent of the GABA<sub>A</sub>-R in selected regions, especially cerebral cortex, hippocampal formation, hypothalamus, cranial nerve ganglia, and spinal cord in adults, and even more widespread and abundant in prenatal and neonatal brain,

suggesting an important developmental role (3, 4). The  $\beta 3$  gene is clustered with two other GABA<sub>A</sub>-R subunits ( $\alpha 5$  and  $\gamma 3$ ) on mouse chromosome 7; deletion of these three genes and surrounding loci results in neonatal mortality, craniofacial abnormalities, and neurological signs (5–7). The syntenic region on human chromosome 15, including the cluster of three GABA<sub>A</sub>-R subunits, is the area associated with the human genetic disease Angelman syndrome (8–10). The gene for the GABA<sub>A</sub>-R  $\beta 3$  subunit has been proposed as a candidate for some of the phenotypic features of this syndrome (8, 11). To test directly the physiologic and developmental functions of the  $\beta 3$  subunit, we examined mice harboring a  $\beta 3$  gene inactivated by gene targeting.

## METHODS

**Production of Knockout Mice.** Strain 129 mouse genomic DNA was isolated from a P1 phage library (Genome Systems, St. Louis). Details of library screening and vector construction are available upon request. Following electroporation of R1 (12) embryonic stem (ES) cells, clones were screened for targeting by Southern blot analysis of *EcoRV*-digested genomic DNA. Blots were hybridized with a 3' probe that is external to the targeting construct. This probe is a 1.1-kb *BglII*–*BamHI* genomic subclone. Targeting was confirmed with several other restriction digests and by hybridization with a neomycin phosphotransferase probe and with a 5' arm probe (data not shown). Two correctly targeted ES cell clones ( $\beta 3\#347$ ,  $\beta 3\#372$ ) were transferred through the germ line. Results presented here are from the  $\beta 3\#347$  clone. A cursory analysis of  $\beta 3\#372$  yielded similar results. The genetic background of all mice was Strain 129  $\times$  C57BL/6J F<sub>2</sub> or F<sub>3</sub>. These mice have been given the strain designation *Gabrb3<sup>miGeh</sup>* and are available from the Induced Mutant Resource (The Jackson Laboratories). For Northern blot analysis, poly(A)<sup>+</sup> RNA was isolated from whole brain of neonatal mice using the Microfast Track kit (Invitrogen). The  $\beta 3$  exon 1–2 probe is a genomic DNA restriction fragment. The  $\beta 3$  exon 9 probe is a PCR product (10). Human  $\beta$ -actin (CLONTECH) was used as a

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Abbreviations: GABA,  $\gamma$ -aminobutyric acid; GABA<sub>A</sub>-R, GABA type A receptor; ES, embryonic stem cell; DRG, dorsal root ganglion; EEG, electroencephalogram.

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probe to assess the integrity of the RNA and as a control for the amount of RNA loaded.

**Analysis of Palate Morphology.** Neonate heads were collected and fixed on the day of birth. We detected a slight difference in the overall head sizes, with the  $\beta 3^{-/-}$  heads smaller than  $p^{cp}/p^{cp}$  heads. This difference is likely due to strain variation as  $\beta 3^{+/-}$  and  $p^{cp}/p^{6H}$  show a similar size difference. To minimize the measurements being biased by the size of individual heads, the ratio,  $W_{1/2}/L$  (width of cleft at the midsection divided by anterior-posterior length of cleft), was calculated.

**GABA<sub>A</sub>-R Pharmacology.** Binding of a prototypic GABA site agonist ( $[^3H]$ muscimol) and an imidazobenzodiazepine ( $[^3H]$ Ro15-4513) that recognizes all known central benzodiazepine sites (at six different concentrations, each in quadruplicate) to whole brain homogenates was measured using a centrifugation method (13, 14). Data were pooled from three to four separate experiments using two separate membrane preparations ( $\approx 40$  neonatal brains and four adult brains per membrane preparation, respectively) and fit to a logistic function using an iterative nonlinear least squares method, yielding binding parameters and their standard errors. Parameters were compared using the  $z$  statistic (15).

For autoradiography, horizontal and sagittal serial sections (20  $\mu$ m) from postnatal day 1 mice ( $n = 6$  for each genotype) were preincubated for 15 min at 0–4°C in 0.31 M Tris-citrate (pH 7.1) or 50 mM Tris-HCl (pH 7.4) supplemented with 120 mM NaCl for the GABA and benzodiazepine sites, respectively. The GABA sites were labeled by  $[^3H]$ muscimol (20 nM; Amersham) (16) during a 30-min incubation at 0–4°C. The benzodiazepine sites were labeled by  $[^3H]$ Ro15-4513 (10 nM; DuPont/NEN) (17) with and without zolpidem (Synthelabo Recherche, Bagneux, France) during a 60-min incubation at 0–4°C. The incubations were carried out in the respective preincubation buffers, and terminated by three washes, a dip in distilled water, and quick air-drying under a fan. The sections were apposed to Hyperfilm- $^3H$  (Amersham) for 6–12 weeks. Nonspecific binding to the GABA and benzodiazepine sites was determined in the presence of 100  $\mu$ M GABA (Sigma) or 10  $\mu$ M flumazenil (Ro15-1788; Hoffmann-La Roche), respectively. Labeling densities of several brain regions was quantitated from the films by using MCID M4 image analysis system (Imaging Research, St. Catharines, Ontario, Canada), with the reference to simultaneously exposed  $^3H$ -plastic standards (Amersham) (18).

**Electrophysiology.** Dorsal root ganglion neurons (DRGs) were dissociated from postnatal day 1 mouse pups as described by White (19), plated onto poly-D-lysine coated coverslips and used for recordings within 12 h. Coverslips containing neurons were transferred to a chamber which was continuously perfused with extracellular medium containing 145 mM NaCl, 3 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 6 mM D-glucose, and 10 mM Hepes/NaOH (pH 7.4). Recordings were made using the whole-cell patch-clamp technique. Patch pipettes contained 145 mM *N*-methyl D-glucamine hydrochloride, 5 mM K<sub>2</sub>ATP, 1.1 mM EGTA, 2 mM MgCl<sub>2</sub>, 5 mM Hepes/KOH (pH 7.2), and 0.1 mM CaCl<sub>2</sub>; pipette resistance was 4–5 M $\Omega$ . Neurons were voltage clamped at –60 mV. GABA was rapidly applied (>1 ml/min) to the cell by local perfusion (20) using a motor-driven solution exchange device (Rapid Solution Changer 100; Molecular Kinetics, Pullman, WA). Responses were digitized (TL-1–125 interface; Axon Instruments, Foster City, CA) using PCLAMP, version 5 (Axon Instruments). Numerical data are presented throughout as mean  $\pm$  SEM.

**Electroencephalogram (EEG) Analysis.** Seven mice of each genotype, 2–6 months of age, had EEG recordings performed via epidural screw electrodes implanted above right and left parietal cortex, and a reference electrode in the nasal bone.

## RESULTS AND DISCUSSION

**Production and Molecular Characterization of Mice.** We used homologous recombination in mouse ES cells to disrupt the  $\beta 3$  gene (see Fig. 1 *a* and *b*). Of 71 ES cell clones analyzed, 4 displayed predicted restriction fragment lengths indicative of correct gene targeting. The 3' probe hybridized only to a  $\approx 16.5$ -kb *EcoRV* fragment from the wild-type allele in the parental ES cell line (R1) and wild-type mice. This probe also hybridized to an  $\approx 14.5$ -kb fragment from a correctly targeted allele. Following transmission of the targeted  $\beta 3$  gene through the mouse germ line, heterozygous ( $\beta 3^{+/-}$ ) mice were intercrossed to produce wild-type ( $\beta 3^{+/+}$ ),  $\beta 3^{+/-}$ , and homozygous ( $\beta 3^{-/-}$ ) mice for analysis.

The modified  $\beta 3$  gene was verified to be nonfunctional (i.e., a null allele) by Northern blot analysis. Hybridization of neonatal whole brain poly(A)<sup>+</sup> RNA with probes specific for exons 1–2 and exon 9 revealed the complete absence of the  $\approx 6.0$ -kb  $\beta 3$  mRNA in  $\beta 3^{-/-}$  mice (Fig. 1*c*). Densitometric analysis of autoradiographs indicated an  $\approx 30\%$  reduction in  $\beta 3$  mRNA in  $\beta 3^{+/-}$  mice.

**Neonatal Mortality and Cleft Palate.** Approximately 90% of  $\beta 3^{-/-}$  mice die within 24 h of birth. This frequency is similar to that observed in two known radiation-induced pink-eyed dilution (*p*) mutants ( $p^{cp}$  and  $p^{4THO-II}$ ) that lack the  $\beta 3$ ,  $\alpha 5$ , and  $\gamma 3$  subunits of the GABA<sub>A</sub>-R (5, 21, 22). The cause of death in these *p* locus mutants has been attributed to feeding problems that result from cleft palate. This phenotype can be rescued by transgenic reintroduction of the  $\beta 3$  subunit cDNA in some homozygous  $p^{4THO-II}$  mice (6). Interestingly, in the  $\beta 3^{-/-}$  mice described here, penetrance of cleft palate was only  $\approx 57\%$  (40/70), markedly lower than the 95% observed in the radiation-induced mutants. This may reflect differences in genetic background or the contribution of other genes deleted in the radiation-induced mutants.

We also compared the size of the cleft between  $\beta 3^{-/-}$  and  $p^{cp}$  homozygotes and surprisingly found no significant differ-

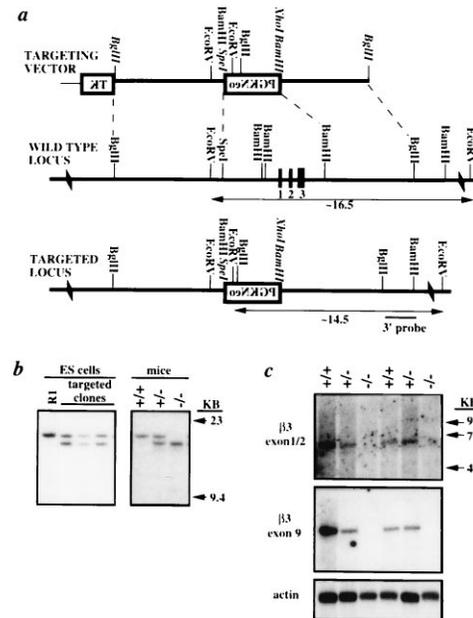


FIG. 1. Targeted disruption of the  $\beta 3$  locus. (*a*) A replacement vector was constructed to replace  $\approx 2.8$  kb of  $\beta 3$  genomic DNA including  $\approx 1.8$  kb of promoter and exons 1–3 with the selectable marker neomycin phosphotransferase (PGKneo). (Note that restriction sites set in italics were destroyed during vector construction.) (*b*) Southern blot analyses of the parental cell line (R1), three correctly targeted ES cell clones, and mice demonstrating all genotypes. (*c*) Northern blot analysis of neonatal mouse brain poly(A)<sup>+</sup> RNA that demonstrates the absence of  $\beta 3$  mRNA in brain of  $\beta 3^{-/-}$  mice.

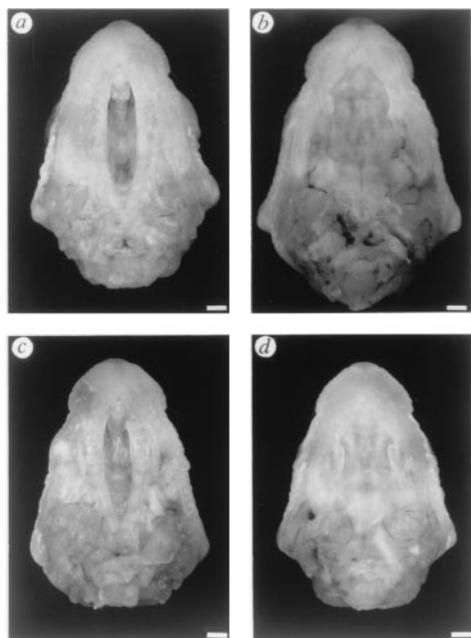


FIG. 2. Palate morphology of  $p^{cp}$  and targeted  $\beta 3$  neonates. (a)  $p^{cp}/p^{cp}$ . (b)  $p^{cp}/p^{6H}$ . (c)  $\beta 3^{-/-}$ . (d)  $\beta 3^{+/-}$ . The  $p^{cp}/p^{6H}$  and the  $\beta 3^{+/-}$  mice each have one wild-type  $\beta 3$  gene and do not exhibit cleft palate. (Bar = 1 mm.)

ence between these two mutants (Fig. 2). Mean and SEM of ratios obtained for  $\beta 3^{-/-}$  and  $p^{cp}/p^{cp}$  were  $0.23 \pm 0.01$  ( $n = 10$ ) and  $0.24 \pm 0.01$  ( $n = 10$ ), respectively. The ranges of direct measurements of each group are  $L = 4.5$ – $5.7$  mm,  $W_{1/2} = 0.89$ – $1.6$  mm ( $\beta 3^{-/-}$ ) and  $L = 4.4$ – $6.0$  mm,  $W_{1/2} = 0.86$ – $1.6$  mm ( $p^{cp}/p^{cp}$ ). The observation that the lower penetrance of cleft palate has little effect on severity of the palate lesion may suggest that induction of cleft palate is regulated by threshold mechanisms. Reaching the threshold to induce cleft palate may be fulfilled by multiple factors, whose effects are augmented by the absence of  $\beta 3$  gene product, somehow more strongly in  $p^{cp}$  and  $p^{4THO-II}$ , possibly due to the additional loss of the  $\alpha 5$  and  $\gamma 3$  subunits. This multiple factor threshold hypothesis is appealing, since toxicological studies of cleft palate induction suggest that there are multiple pathways involved in the formation of cleft palate (see ref. 23–26). As noted by Culiati *et al.* (6, 22), paradoxically both the loss of a GABA<sub>A</sub>-R as well as the addition of GABA (27) or the GABA agonist, diazepam (28), all promote cleft palate. Taken together these studies indicate the importance of the GABAergic system in normal palate development.

We do not know at this time why  $\approx 30\%$  of  $\beta 3^{-/-}$  mice with apparently normal palates die neonatally. The fact that these mice do die in the same time frame as those with palate defects calls into question the assumption that it is the clefting defect alone that results in the lethal phenotype. The cause of death cannot be attributed to defects in gross morphology, as the brains of these mice are morphologically normal (data not shown). This confirms the observation reported for  $p^{cp}$  homozygous brain (5).

**GABA<sub>A</sub>-R Pharmacology.** The contribution that  $\beta 3$  subunits make to the population of heteropentameric GABA<sub>A</sub>-Rs was studied by comparing binding of [ $^3$ H]muscimol and [ $^3$ H]Ro15-4513 as ligands to label GABA and benzodiazepine sites, respectively. GABA<sub>A</sub>-R density is remarkably reduced in  $\beta 3^{-/-}$  mice. Binding of [ $^3$ H]muscimol and [ $^3$ H]Ro15-4513 was approximately halved in brain homogenates of  $\beta 3^{-/-}$  mice compared with  $\beta 3^{+/+}$ , without any change in affinity (Table 1). Autoradiographic analysis of newborn mouse brains also indicated that  $\beta 3^{-/-}$  mice have lower binding for both ligands

Table 1. Binding of [ $^3$ H]muscimol and [ $^3$ H]Ro15-4513 to whole brain homogenates prepared from postnatal day 1 (pups) or 8- to 12-week-old (adult) mice

Binding parameter	Wild type ( $\beta 3^{+/+}$ )	Homozygous ( $\beta 3^{-/-}$ )	<i>P</i>
<b>[<math>^3</math>H]Muscimol (pups)</b>			
$B_{max}$ , pmol/mg	$3.0 \pm 0.2$	$1.5 \pm 0.2$	$<0.001$
$K_d$ , nM	$10.8 \pm 1.1$	$9.1 \pm 3.3$	0.62
<b>[<math>^3</math>H]Ro15-4513 (pups)</b>			
$B_{max}$ , pmol/mg	$1.5 \pm 0.1$	$0.7 \pm 0.1$	$<0.001$
$K_d$ , nM	$8.0 \pm 2.9$	$8.8 \pm 3.4$	0.86
<b>[<math>^3</math>H]Ro15-4513, adult</b>			
$B_{max}$ , pmol/mg	$3.1 \pm 0.3$	$1.7 \pm 0.1$	0.01
Zolpidem + [ $^3$ H]Ro15-4513 (pups) IC <sub>50</sub> , $\mu$ M	$0.54 \pm 0.09$	$0.15 \pm 0.04$	$<0.001$

(Fig. 3 A and C). [ $^3$ H]Ro15-4513 labeled less sites in  $\beta 3^{-/-}$  mice than in  $\beta 3^{+/+}$  or  $\beta 3^{+/-}$  mice, the labeling being significantly lower in all regions quantitated (Table 2). A saturating concentration (30  $\mu$ M) of zolpidem, a GABA<sub>A</sub>-R isoform selective benzodiazepine site agonist that is unable to bind to  $\alpha 5$  and/or  $\gamma 3$  subunit-containing receptors (29, 30), displaced similar proportions of benzodiazepine binding in all three genotypes (Fig. 3B). This indicates that some zolpidem-insensitive receptors, presumably containing  $\alpha 5$  and  $\gamma 3$ , persist

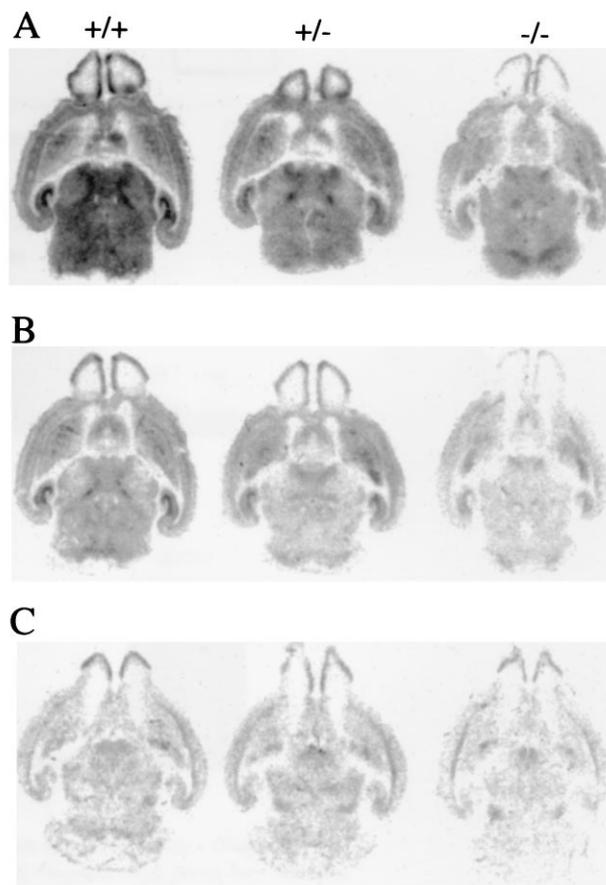


FIG. 3. Autoradiographic binding of a GABA site ligand ([ $^3$ H]muscimol) and a benzodiazepine site ligand ([ $^3$ H]Ro15-4513) to representative horizontal brain sections from newborn wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice. (A) Total binding of 5 nM [ $^3$ H]Ro15-4513. (B) Binding of [ $^3$ H]Ro15-4513 in the presence of 30  $\mu$ M zolpidem. (C) Total binding of 20 nM [ $^3$ H]muscimol. All images represent specific binding since [ $^3$ H]Ro15-4513 and [ $^3$ H]muscimol failed to label any sites in the presence of 10  $\mu$ M flumazenil and 100  $\mu$ M GABA, respectively.

Table 2. Binding of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]Ro15-4513 to postnatal day 1 mouse brain sections as revealed by autoradiography

Brain region	[ <sup>3</sup> H]Muscimol, nCi/mg	[ <sup>3</sup> H]Ro15-4513, nCi/mg
Frontoparietal cortex		
$\beta_3^{+/+}$	0.80 ± 0.14	3.70 ± 0.84
$\beta_3^{+/-}$	0.72 ± 0.10	2.58 ± 0.33
$\beta_3^{-/-}$	0.58 ± 0.09*	2.59 ± 0.52*
Olfactory bulbs		
$\beta_3^{+/+}$	0.65 ± 0.08	2.43 ± 0.40
$\beta_3^{+/-}$	0.55 ± 0.07	2.16 ± 0.46
$\beta_3^{-/-}$	0.59 ± 0.05	1.00 ± 0.08†
Caudate/putamen		
$\beta_3^{+/+}$	0.66 ± 0.07	3.42 ± 0.63
$\beta_3^{+/-}$	0.64 ± 0.14	3.15 ± 0.34
$\beta_3^{-/-}$	0.53 ± 0.10	2.57 ± 0.37*
Thalamus		
$\beta_3^{+/+}$	0.83 ± 0.16	4.92 ± 0.62
$\beta_3^{+/-}$	0.81 ± 0.22	3.79 ± 0.40
$\beta_3^{-/-}$	0.52 ± 0.06*	2.51 ± 0.85†
Hippocampus		
$\beta_3^{+/+}$	0.70 ± 0.11	3.68 ± 0.90
$\beta_3^{+/-}$	0.64 ± 0.14	2.96 ± 0.80
$\beta_3^{-/-}$	0.57 ± 0.16	2.22 ± 0.48*

Optical density values in relation to radioactivity standards are means ± SEM for three to six brains. THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) (10 μM) was able to reduce [<sup>3</sup>H]muscimol binding to the background level (data not shown). The statistical significance of differences between  $\beta_3^{+/+}$ ,  $\beta_3^{+/-}$ , and  $\beta_3^{-/-}$  mice was determined using one-way ANOVA followed by Bonferroni post test (\*,  $P < 0.05$ ; †,  $P < 0.001$ ).

in the  $\beta_3^{-/-}$  mice. The more quantitative measurements on total brain homogenates showed a significant reduction in the IC<sub>50</sub> (increased potency) for zolpidem displacement of benzodiazepine in  $\beta_3^{-/-}$  mice (Table 1), indicating that some  $\alpha_5$  and  $\gamma_3$  subunits depend on the presence of  $\beta_3$  for incorporation into GABA<sub>A</sub>-R heteropentameric isoforms.

The binding of [<sup>3</sup>H]muscimol assayed by autoradiography also was decreased in  $\beta_3^{-/-}$  mice, but significant decreases were observed only in the cortex and thalamus. [<sup>3</sup>H]Muscimol binding may, however, detect only high affinity subpopulations of GABA<sub>A</sub>-Rs (16), with presently unknown subunit composition. Because benzodiazepine binding is decreased more widely than [<sup>3</sup>H]muscimol binding, the latter may emphasize GABA<sub>A</sub>-R isoforms that do not contain  $\beta_3$ , as suggested by studies on  $p^{cp}$  mice (8).

The autoradiographic data indicate that the  $\beta_3$  subunit is widely expressed in the mouse brain, possibly assembling with several  $\alpha$  and  $\gamma$  subunit variants. The reduced binding observed for the GABA and benzodiazepine ligands demonstrates that another subunit such as  $\beta_2$  is unable to substitute for the  $\beta_3$  subunit. This results in a marked deficit in GABA<sub>A</sub>-R level in the  $\beta_3^{-/-}$  animals. These observations are consistent with largely separate receptor populations containing  $\beta_2$  and  $\beta_3$  subunits in the rat brain (31).

**Electrophysiology.** *In situ* hybridization studies of neonatal rat have demonstrated that sensory neurons of the DRG express high levels of  $\beta_3$  mRNA (32). Electrophysiologic recordings from sensory neurons isolated from the DRG of neonatal mice showed a dramatic (~80%) decrease in the maximal amplitude of GABA-activated chloride currents in a population of neurons from  $\beta_3^{-/-}$  mice relative to cells taken from  $\beta_3^{+/+}$  siblings ( $P < 0.001$ , Student's *t* test; Fig. 4A and C). In addition, there was a significant decrease (25%) in the GABA current amplitude in neurons from  $\beta_3^{+/-}$  mice relative to  $\beta_3^{+/+}$  mice ( $P < 0.05$ , Student's *t* test; Fig. 4C). A large fraction (27/36) of the neurons tested from  $\beta_3^{-/-}$  animals responded to GABA with <100 pA of current, corresponding

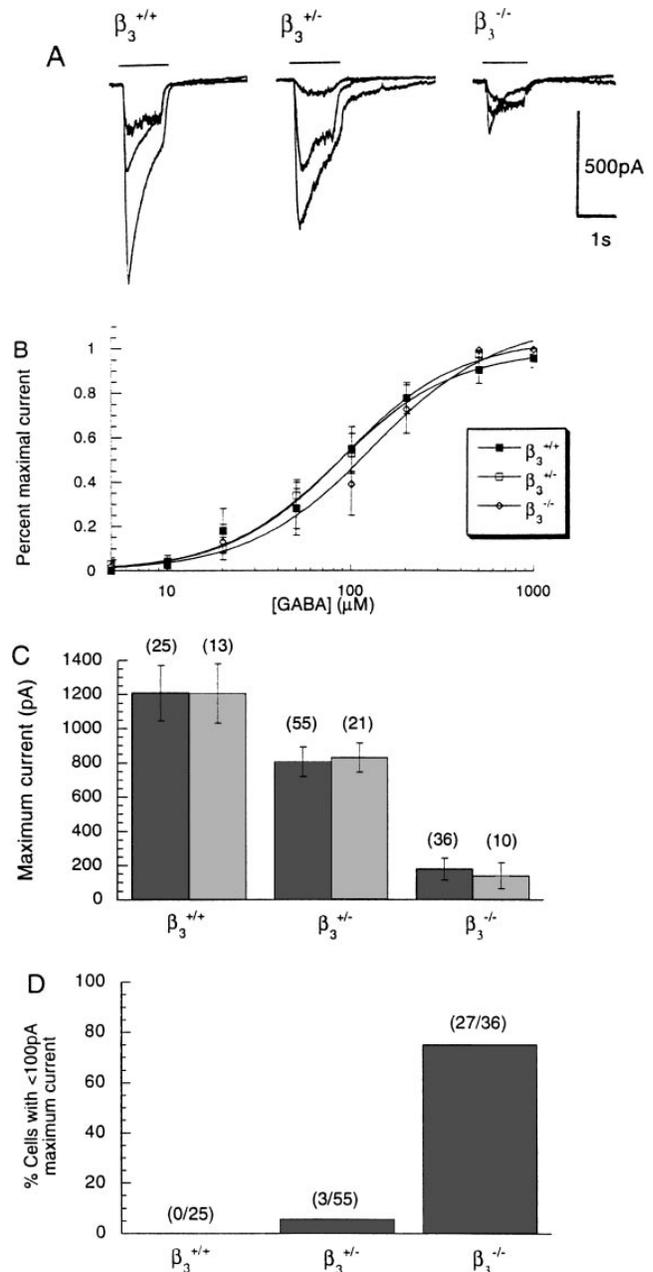


FIG. 4. GABA-evoked whole cell currents from acutely dissociated DRG neurons of newborn  $\beta_3^{+/+}$ ,  $\beta_3^{+/-}$ , and  $\beta_3^{-/-}$  mice. (A) Representative currents from individual neurons from each group in response to GABA (10 μM, 100 μM, and 1 mM). The bar above the trace represents duration of GABA application. (B) Pooled GABA concentration-response curves ( $4 < n < 7$ ), yielding EC<sub>50</sub> values of 86.1 ± 9.3 μM (+/+), 92.1 ± 6.0 μM (+/-), and 131.0 ± 23.0 μM (-/-). Hill coefficients were 1.4, 1.4, and 1.3. (C) Pooled data for maximal GABA current amplitudes (mean ± SEM) expressed for all neurons tested (dark shading) and for the average current from neurons in each mouse (light shading). (D) The majority of neurons from  $\beta_3^{-/-}$  mice produced <100 pA maximal current. In contrast, only a small minority of neurons from  $\beta_3^{+/-}$  mice and none from  $\beta_3^{+/+}$  animals produced such small currents in response to GABA.

to <10% of the mean from control neurons (Fig. 4D). No neurons from  $\beta_3^{+/+}$  animals fell into this range. Interestingly, however, a few neurons from  $\beta_3^{-/-}$  animals gave large currents. The concentration-response curve for GABA was not significantly different in neurons from the  $\beta_3^{+/-}$  or  $\beta_3^{-/-}$  mice (Fig. 4B).

The results from isolated DRG neurons suggest that the majority of the GABA<sub>A</sub>-Rs on the cell bodies of these sensory

neurons in the neonatal mouse are  $\beta 3$ -containing, consistent with *in situ* hybridization studies in the neonatal rat (32). The small GABA-evoked currents in neurons from  $\beta 3^{-/-}$  animals were sensitive to potentiation by the anticonvulsant loreclezole (5  $\mu\text{M}$ ;  $n = 4$ ; data not shown), consistent with the remaining functional GABA<sub>A</sub>-Rs being  $\beta 2$ -containing rather than  $\beta 1$ -containing (33). Since GABA<sub>A</sub>-Rs are found on the terminals of sensory afferents in the dorsal and ventral horn of the spinal cord, where they are responsible for presynaptic inhibition (34), we suggest that some of the motor manifestations of hyperexcitability in the  $\beta 3$ -deficient animals result from a decrease in the effectiveness of spinal presynaptic inhibition. This in turn is due to the decline in numbers of GABA<sub>A</sub>-Rs on the terminals of primary afferents, consistent with the loss of  $\beta 3$ -containing receptors observed at the cell body level in our experiments in the  $\beta 3^{-/-}$  animals. The smaller but statistically highly significant receptor deficit seen in the heterozygotes suggests the possibility of reduced spinal presynaptic inhibition in these animals also.

**Additional Abnormalities.** The  $\approx 5$ –10% of  $\beta 3^{-/-}$  mice that survive to adulthood display several behavioral and physiological abnormalities. The extent of these abnormalities varies greatly from mouse to mouse. Most  $\beta 3^{-/-}$  mice are runted until weaning but they achieve normal body weight by adulthood. The mice are very hyperactive and hyperresponsive to human contact and other sensory stimuli. Many, but not all,  $\beta 3^{-/-}$  mice have been observed to run continuously in tight circles for extended periods of time. When held by the tail, they hold all paws in like a ball, which is frequently a sign of neurological impairment. They have difficulty swimming, walking on grids, and repeatedly fall off platforms and rotarods. In marked contrast to the exceptional surviving *p* locus mutant mice mentioned above, which are also runted and nervous (5, 21, 22),  $\beta 3^{-/-}$  mice do not display jerky gait. Fertility is normal but  $\beta 3^{-/-}$  mothers fail to display appropriate nurturing behavior irrespective of the genotype of the offspring. Longevity of  $\beta 3^{-/-}$  mice that survive the neonatal period is reduced to an average age of 18 weeks (range, 9–41 weeks,  $n = 20$ ) in contrast to an expected 127 weeks; the cause of premature death is unknown.

**Seizures and EEG Analysis.** Male and female  $\beta 3^{-/-}$  mice were observed up to  $\approx 10$  months of age. They displayed recurring hyperactive behavior that was interrupted by periods of arrest lasting seconds to minutes. Myoclonic body jerks and epileptic seizures were observed in mice over 10 weeks of age, especially in older mice. Seizures varied in severity; the mildest seizures consisted of twitching of muscles of the mouth, face, whiskers, and ears. More severe seizures included head and bilateral forelimb jerks, with or without opisthotonos, arching of the tail, and falls. In the most severe seizures, convulsions were succeeded by a wild running/bouncing phase. Seizures were noted clinically in 10 of 24 mice by chance observation. Closer observation during EEG analysis revealed seizures in 4 of 4  $\beta 3^{-/-}$  mice. Thus, seizures are likely present in all  $\beta 3^{-/-}$  mice.

EEGs of  $\beta 3^{-/-}$  mice had relatively normal background, especially during maximal alertness, but were frequently interrupted by higher amplitude polymorphic slow and sharp waves, corresponding to behavioral quiescence (Fig. 5A). This abnormal EEG pattern stopped on arousal. High-amplitude generalized interictal spikes were frequently observed. These abnormal epileptiform patterns were seen in 7 of 7  $\beta 3^{-/-}$  animals examined. Seizures were associated with generalized spike/waves. A large spike frequently appeared immediately prior to or at the start of ictus and synchronized buildup, followed by postictal depression (Fig. 5B).  $\beta 3^{+/+}$  mice did not show these abnormal EEG patterns, whereas  $\beta 3^{+/-}$  mice exhibited patterns intermediate between  $\beta 3^{-/-}$  and  $\beta 3^{+/+}$  mice (Fig. 5A). Lack of seizures in wild-type mice is consistent with the well-known seizure resistance of C57BL/6J mice (35).

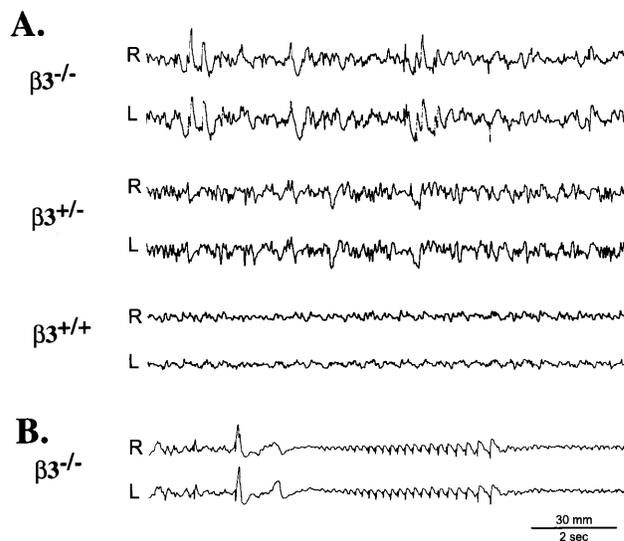


FIG. 5. Electroencephalographic recordings. (A) Three animals ( $\beta 3^{-/-}$ ,  $\beta 3^{+/-}$ ,  $\beta 3^{+/+}$ ) were simultaneously recorded at 2 months of age. The  $\beta 3^{-/-}$  record (Top) is abnormal, displaying abundant paroxysmal slow and sharp wave activity, in marked contrast to the normal pattern of  $\beta 3^{+/+}$  littermate controls (Bottom).  $\beta 3^{+/-}$  mice display a pattern intermediate between that of  $\beta 3^{-/-}$  and  $\beta 3^{+/+}$  records. (B) Seizure in  $\beta 3^{-/-}$  mouse at 5 months of age, followed by suppression of electrocortical activity.

As an additional control experiment, two Strain 129/SvJ mice (age 15 weeks) were recorded for 3 h on two different days; no EEG abnormalities occurred.

## CONCLUSION

To our knowledge, no previous study has established that alterations in GABA<sub>A</sub>-Rs underlie any type of animal or human epilepsy. The results presented here provide evidence that a reduction in certain GABA<sub>A</sub>-R subunit proteins, in this case the  $\beta 3$  subunit, can cause epilepsy. The human genetic disorder Angelman syndrome is characterized by severe mental retardation and epilepsy (36–38). This syndrome is produced by deletion or mutation in maternal chromosome 15q11–13 (39), an area to which the genes for the  $\alpha 5$ ,  $\gamma 3$ , and  $\beta 3$  subunits of the GABA<sub>A</sub>-R map (9). Studies in progress are comparing the features of the seizures in  $\beta 3^{-/-}$  knockout mice and other abnormal behaviors with those seen in human diseases, including Angelman syndrome.

Selective knockout of the  $\beta 3$  subunit of the GABA<sub>A</sub>-R also produces the cleft palate and neurological abnormalities seen in large genetic deletions that encompass numerous genes including a cluster of three GABA<sub>A</sub>-R subunits. The phenotypic characteristics of  $\beta 3^{-/-}$  mice are consistent with the expected results of a deficiency of GABA-mediated inhibition in adult brain and spinal cord and possibly a deficiency in developmental functions subserved by GABA. The suspected links between GABA and epilepsy and GABA and brain development are now more firmly established.

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