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Benzodiazepine-insensitive mice generated by targeted disruption of the γ_2 subunit gene of γ-aminobutyric acid type A receptors

(gene targeting/endogenous benzodiazepines/assembly of ligand-gated ion channels/single channel recording)

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ABSTRACT **Vigilance, anxiety, epileptic activity, and muscle tone can be modulated by drugs acting at the benzodiazepine (BZ) site of γ-aminobutyric acid type A ($GABA_A$) receptors. *In vivo*, BZ sites are potential targets for endogenous ligands regulating the corresponding central nervous system states. To assess the physiological relevance of BZ sites, mice were generated containing $GABA_A$ receptors devoid of BZ sites. Following targeted disruption of the γ_2 subunit gene, 94% of the BZ sites were absent in brain of neonatal mice, while the number of GABA sites was only slightly reduced. Except for the γ_2 subunit, the level of expression and the regional and cellular distribution of the major $GABA_A$ receptor subunits were unaltered. The single channel main conductance level and the Hill coefficient were reduced to values consistent with recombinant $GABA_A$ receptors composed of α and β subunits. The GABA response was potentiated by pentobarbital but not by flunitrazepam. Diazepam was inactive behaviorally. Thus, the γ_2 subunit is dispensable for the assembly of functional $GABA_A$ receptors but is required for normal channel conductance and the formation of BZ sites *in vivo*. BZ sites are not essential for embryonic development, as suggested by the normal body weight and histology of newborn mice. Postnatally, however, the reduced $GABA_A$ receptor function is associated with retarded growth, sensorimotor dysfunction, and drastically reduced life-span. The lack of postnatal $GABA_A$ receptor regulation by endogenous ligands of BZ sites might contribute to this phenotype.**

γ-Aminobutyric acid type A ($GABA_A$) receptors, encoded by at least 15 subunit genes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, and ρ_1 and ρ_2), are heteromeric GABA-gated ion channels mediating the major inhibitory neurotransmission in the mammalian central nervous system (CNS) (1–3). Most receptors are assembled from α and β subunit variants in combination with the γ_2 subunit. Coexpression of α and β subunits is sufficient to form GABA-gated ion channels, as shown in recombinant receptors, but the addition of the γ_2 subunit is required for expression of $GABA_A$ receptors containing benzodiazepine (BZ) sites (4, 5). By modulating $GABA_A$ receptor function, ligands of the BZ site can enhance or inhibit a wide variety of CNS states, including vigilance, anxiety, epileptic activity, and memory. Endogenous BZ-like ligands (6, 7) were found to be operative in pathological states such as hepatic encephalopathy and idiopathic stupor (8–10). However, the physiological relevance of the BZ site is unresolved.

To investigate the significance of the BZ site for brain development and function, an attempt was made to generate mice containing $GABA_A$ receptors devoid of BZ sites. Targeted disruption of the γ_2 subunit gene was anticipated to result in mice that largely lack BZ binding sites but retain the ability to form functional GABA-gated ion channels, providing insight into the physiology and pathophysiology of $GABA_A$ receptors and their BZ sites and into the process of subunit assembly.

MATERIAL AND METHODS

Generation of Mutant Mice. A targeting vector (Fig. 1*a*) was constructed containing a 6.4-kb genomic region including exons 7, 9, and 10 of the γ_2 subunit gene isolated from a 129SV mouse genomic library. A 1.2-kb genomic *Pvu* II–*Nco* I fragment including exon 8 (coding for amino acids 306–375 of the γ_2 polypeptide) was replaced with the phosphoglycerate kinase (PGK)-neo cassette (11), and a tk expression cassette (12) was added at the 3′ end of the γ_2 sequence. Splicing from exon 7 to exon 9 would result in a stop of the translational reading frame and prohibit expression of sequences downstream of exon 7. Before electroporation into E14 ES cells (13), the plasmid was linearized at a polylinker site adjacent to the 5′ end of the γ_2 genomic sequence. E14 ES cells were cultured on irradiated G418-resistant feeder cells obtained from CD1-M-TKneo2 mouse embryos [BRL, Füllinsdorf (Basel)] in GMEM (Glasgow modification of Eagle's medium; Flow Laboratories) containing 10% total calf serum and leukemia inhibitory factor (10^3 units/ml, Life Technologies). The cells were transfected and screened for homologous recombinants (14) by using PCR and the primers $\gamma_{2.19}$ (5′-CATCT CCATC GCTAA GAATG TTCGG GAAGT-3′) derived from γ_2 sequences upstream of the targeting vector and $\gamma_{2.20}$ (5′-ATGCT CCAGA CTGCC TTGGG AAAAG C-3′) derived from PGK promoter sequences (11). Chimeric mice were generated (15) and mated to C57BL/6 females, and the offspring were genotyped by PCR amplification of tail DNAs. Reactions specific for the disrupted γ_2 allele [(0) in Fig. 1 *a* and *c*] were as described above; those specific for the wild-type (+) allele used exon 8-derived primers $\gamma_{2.21}$ (5′-TACTG TCCTG ACCAT GACAA CTTT-3′) and $\gamma_{2.22}$ (5′-GAGGG TTTTT CTTCT TTTTG TCTT-3′). Heterozygous mice ($\gamma_2^{0/+}$) were intercrossed to obtain homozygous mutants ($\gamma_2^{0/0}$).

Western Blot and Binding Assays. Of each genotype, 10–15 brains of newborn mice were pooled for membrane preparation. Aliquots were subjected to Western blotting (40 μg of protein per lane) using antisera specific for the different $GABA_A$ receptor subunits (16) or used for binding assays with [^{3}H]SR 95531 (0.5–20 nM) or [^{3}H]flumazenil (0.1–15 nM) (17) followed by Scatchard analyses using the LIGAND program (18).

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Abbreviations: BZ, benzodiazepine; GABA, γ-aminobutyric acid; DRG, dorsal root ganglion (ganglia); ES, embryonic stem; E, embryonic day; P, postnatal day.

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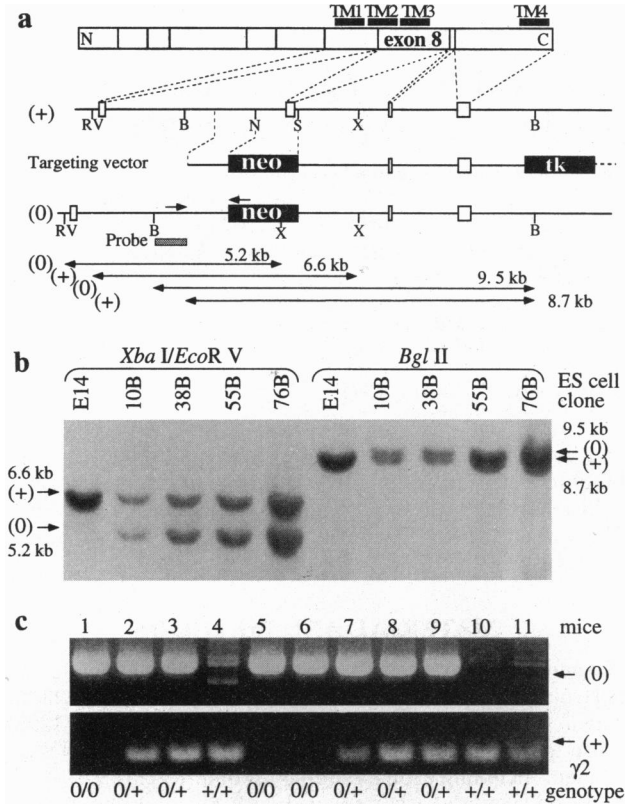

FIG. 1. Inactivation of the γ_2 subunit gene of $GABA_A$ receptors in embryonic stem (ES) cells and generation of mutant mice. (*a*) Targeting strategy. The γ_2 subunit polypeptide is indicated (top) with exon–exon boundaries and positions of putative transmembrane domains (TM1–4). Below, the relevant region of the wild-type (+) γ_2 subunit gene, of the targeting vector, and of the mutant (0) γ_2 locus is shown with the location of exons 7–10 and the positions of the neomycin (neo) (11) and thymidine kinase (tk) (12) cassettes for positive and negative selection. The position of mini-exon 9 is arbitrary. PCR primers (short arrows) used for screening of mutant ES cells and restriction fragments (long arrows) and restriction sites used for genomic mapping are indicated: B, *Bgl* II; RV, *Eco*RV; N, *Nco* I; P, *Pvu* II; S, *Sac* I; X, *Xho* I. (*b*) Southern blot analysis of ES cell isolates. Genomic DNA from four ES cell clones and from parental embryonic day 14 (E14) cells was digested with *Xba* I/*Eco*RV or *Bgl* II and analyzed by Southern blot using a genomic probe (*Bgl* II–*Pvu* II) derived from sequences located 5′ of the targeting vector as indicated in 4*a*. (*c*) Genotyping of tail DNAs of newborn offspring from a $\gamma_2^{0/+}$ intercross. Sequential PCRs specific for mutant (0) and wild-type (+) alleles were performed and analyzed on an agarose gel stained with ethidium bromide.

Autoradiography and Immunohistochemistry. Cryostat brain sections (15 μm) were incubated with 20 nM [^{3}H]SR 95531 or 5 nM [^{3}H]flumazenil in the absence or presence of 0.1 mM GABA or 1 μM clonazepam, respectively, to determine nonspecific binding (19). For immunohistochemistry, free-floating sections were processed for immunoperoxidase staining with antisera specific for the $GABA_A$ receptor subunits α_1, α_2, α_5, and $\beta_{2/3}$ as described (16, 20). Sections from dorsal root ganglia (DRG) were processed for immunofluorescence staining with the same antibodies and visualized by confocal laser microscopy (Leica TCS 4D).

Electrophysiological Analyses. Individual somata of acutely dissociated DRG neurons (21) from newborn mice were held at −60 mV under conventional whole cell patch clamp conditions, with $[Cl^-]_i = [Cl^-]_o$. GABA was applied by a multibarrelled microapplicator (22) and dose–response curves for individual neurons were fitted separately using the logistic equation, normalized to the calculated maximum, averaged and refitted. Single channel currents were low-pass filtered (1 kHz) and digitized (5-kHz sampling rate). Amplitude histograms were constructed by binning the channel openings (0.025 and 0.05 pA per bin width). To determine the effects of sodium pentobarbital, flunitrazepam, and Zn^{2+} ions, the test substances were preapplied for 2 min and the response to a 2-s pulse of GABA (5 μM) mixed with the test substance was compared with the response evoked by GABA alone.

RESULTS

Generation of Mutant Mice. To disrupt the γ_2 subunit gene, a targeting vector encoding exons 7, 9, and 10 but lacking exon 8 of the γ_2 subunit gene was used to transfect ES cells. Exon 8 codes for most of the putative second and third transmembrane region (TM2 and TM3) of the γ_2 polypeptide (Fig. 1*a*). Electroporation into ES cells and screening for targeted cells resulted in 10 positive clones from 192 analyzed. Southern blot analysis of *Xba* I/*Eco*RV-cut genomic DNA of four of these targeted clones with a 5′ flanking genomic probe resulted in two bands of 6.6 and 5.2 kb, indicating the wild-type (+) and mutant (0) alleles of the γ_2 genomic locus (Fig. 1 *a* and *b*). Similarly, *Bgl* II-cut genomic DNA, hybridized with the same probe, resulted in bands of 8.7 and 9.5 kb, indicating that no alteration of 3′ flanking sequences had occurred. Two clones (55B and 76B) were injected into blastocysts and chimeric founders derived from both clones were crossed with C57BL/6 mice. Heterozygous ($\gamma_2^{0/+}$) offspring were intercrossed and homozygous mutants ($\gamma_2^{0/0}$, Fig. 1*c*) were detected at the expected Mendelian ratio (24.2%, n = 178).

Expression of $GABA_A$ Receptor Subunits and Binding Sites. In newborn $\gamma_2^{0/0}$ mice, $GABA_A$ receptor subunit expression was unaltered except for the lack of the γ_2 subunit and a slight but inconsistent reduction in $\beta_{2/3}$ subunit immunoreactivity as shown by Western blotting of brain membranes using antisera directed against subunits α_2, α_3, β_1, $\beta_{2/3}$, γ_1, γ_2, and γ_3 (Fig. 2*a*). Thus, disruption of the γ_2 subunit gene did not result in compensatory changes in the expression of the other subunits tested. A truncated γ_2 polypeptide was not detected.

[^{3}H]SR 95531 and [^{3}H]flumazenil were used as radioligands to monitor the presence of GABA and BZ sites in newborn brain. In membranes from $\gamma_2^{0/0}$ brains, the number of GABA sites (B_{max}) was found to be reduced by 22% ± 8%, while the number of [^{3}H]flumazenil sites was reduced by 94% ± 2% (Fig. 2*b*) compared to wild-type controls. The affinities of the

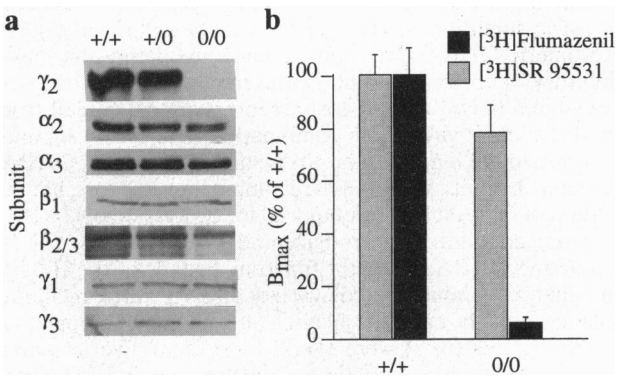

FIG. 2. $GABA_A$ receptor subunits and binding sites expressed in brains of newborn γ_2 subunit mutant mice. (*a*) Western blot analyses of the $GABA_A$ receptor subunits in $\gamma_2^{+/+}$, $\gamma_2^{0/+}$, and $\gamma_2^{0/0}$ mice. (*b*) Scatchard analyses of binding data using [^{3}H]SR 95531 (GABA site) and [^{3}H]flumazenil (BZ site) as ligands. The K_d values for [^{3}H]SR 95531 in $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ membranes were 10 ± 2.6 and 11 ± 1.4 nM and the B_{max} values were 0.67 ± 0.05 and 0.53 ± 0.07 pmol/mg of protein, respectively. K_d values for [^{3}H]flumazenil in $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ membranes were 0.9 ± 0.1 and 1.2 ± 0.3 nM and the B_{max} values were 0.87 ± 0.09 and 0.052 ± 0.015 pmol/mg, respectively. Data represent means ± SD from three experiments.

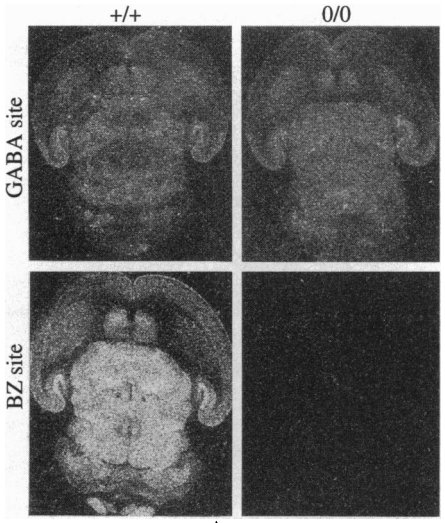

FIG. 3. Distribution of GABA and BZ binding sites in $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ brains: Autoradiographs of brain sections from newborn mice incubated with [^{3}H]SR 95531 (GABA site) and [^{3}H]flumazenil (BZ site). (×4.5.)

radioligands remained unchanged. When monitored autoradiographically in brain sections, the signal intensity and distribution of [^{3}H]SR 95531 binding were nearly unchanged, while [^{3}H]flumazenil binding was not detectable in $\gamma_2^{0/0}$ brains (Fig. 3). Thus, in the absence of the γ_2 subunit, $GABA_A$ receptors were formed in almost unchanged numbers with normal distribution, but they largely lacked BZ sites.

Distribution of γ_2 Subunit-Deficient $GABA_A$ Receptors. Potential alterations in the regional or cellular distribution of $GABA_A$ receptor subunits in $\gamma_2^{0/0}$ mice were analyzed immunohistochemically in sections of neonatal brain and DRG using antibodies specific for the α_1, α_2, α_5, and $\beta_{2/3}$ subunits. No changes in distribution or staining intensity of these subunits were detected in mutants compared to wild-type. In particular, in the neocortex, the α_1 subunit delineated the boundaries of the primary somatosensory area and of the entorhinal cortex in mutant and wild-type mice. In the striatum, the α_2 and α_5 subunit immunoreactivity was particularly intense in striosomes, whereas the matrix was only lightly stained (Fig. 4*a*). Most strikingly, in the spinal trigeminal sensory nucleus, all three α subunits stained the barrelettes (Fig. 4*b*), indicating that the somatotopic organization of the whisker-to-barrel pathway was conserved. The $\beta_{2/3}$ subunit immunoreactivity was prominent throughout the brain, suggesting the presence of high densities of $GABA_A$ receptors in both types of mice. Thus, the regional distribution of $GABA_A$ receptors in γ_2 subunit-deficient mice is properly established at postnatal day 0 (P0). On the cellular level, a distinct staining including hot spots on the cell membrane was observed by high-resolution confocal laser microscopy in both mutant and control mice, as illustrated for DRG cells (Fig. 4*c*). Thus, the mechanisms governing the expression, transport, and integration of the receptors into the cell membrane were not dependent on the γ_2 subunit.

Electrophysiology of γ_2 Subunit-Deficient $GABA_A$ Receptors. While $GABA_A$ receptors in $\gamma_2^{0/0}$ mice were present in nearly unchanged numbers and with normal distribution, it was essential to test whether they had retained the ability to be gated by GABA and modulated by drugs other than ligands of the BZ site. Whole cell patch clamp analysis and single channel recordings were thus performed on DRG neurons of newborn mice. These cells display a homogeneous subunit repertoire (α_2, α_3, β_3, γ_2) and are therefore suitable for electrophysiological receptor assays. Compared to wild-type neurons, the GABA dose–response curve in $\gamma_2^{0/0}$ cells showed a slightly lower EC_{50} value for GABA (Fig. 5*a*). Single channel analysis revealed a main conductance state of 28.3 ± 0.9 pS (Fig. 5*b*) corresponding to that of recombinant receptors assembled from $\alpha_x\beta_y\gamma_2$ subunits ($\alpha_1\beta_2\gamma_2$, 32 pS; $\alpha_1\beta_1\gamma_2$, 29 pS) (23, 24). In $\gamma_2^{0/0}$ mice, a main conductance of 11.3 ± 1.0 pS was found, corresponding to that of recombinant $\alpha\beta$ receptors ($\alpha_1\beta_2$, 11 pS; $\alpha_1\beta_1$, 15 pS) (23, 24). A Hill coefficient of 1.0 (Fig. 5*a*) and an increased sensitivity to Zn^{2+} (Fig. 5*c*) were also consistent with $\alpha\beta$ receptors (23, 25). In $\gamma_2^{0/0}$ DRG neurons, all receptors appeared to be operative since, in the absence of overt changes in channel kinetics, the 2.5-fold reduction in the channel main conductance state (Fig. 5*b*) corresponded to the 2.7-fold reduction in the whole cell GABA current ($\gamma_2^{+/+}$, 414 ± 350 pA, n = 15; $\gamma_2^{0/0}$, 155 ± 89 pA, n = 13; 10 μM GABA). Pharmacologically, the GABA response in $\gamma_2^{0/0}$ neurons was inhibited by bicuculline and picrotoxin and potentiated by pentobarbital as in wild-type neurons (Fig. 5*c*). However,

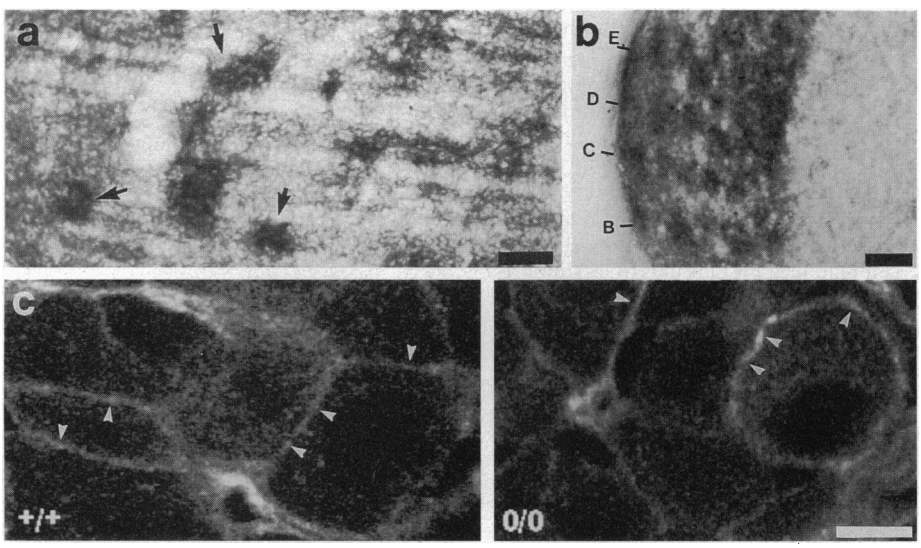

FIG. 4. Immunohistochemical distribution of $GABA_A$ receptor subunits in newborn $\gamma_2^{0/0}$ mice. (*a*) Differential α_2 subunit staining of patch and matrix compartments of the striatum (arrows point to intensely stained neurons in striosomes). (*b*) Barrelettes in the spinal trigeminal nucleus, pars interpolaris, stained with the α_5 subunit antiserum. The somatotopic organization of four rows of whiskers is evident (rows B–E). Similar features were also detected in $\gamma_2^{+/+}$ and $\gamma_2^{+/0}$ mice (not shown). (*c*) Cellular distribution of the $\beta_{2/3}$ subunit immunoreactivity (monoclonal antibody bd17) in DRG neurons of $\gamma_2^{+/+}$ (*Left*) and $\gamma_2^{0/0}$ (*Right*) mice. Arrowheads point to cell membrane staining. (*a* and *b*, bars = 100 μm; *c*, 10 μm.)

GABA-gated currents of $\gamma_2^{0/0}$ neurons showed no response to flunitrazepam (Fig. 5*c*). Likewise, diazepam (10 mg/kg p.o.) failed to induce sedation and loss of righting reflex in $\gamma_2^{0/0}$ animals tested at P14, in contrast to wild-type mice. These results are consistent with the view that in γ_2 subunit-deficient mice functional $GABA_A$ receptors are formed from α and β subunit variants. In addition, the findings suggest that in wild-type receptors the γ_2 subunit contributes to (*i*) the sensitivity of the receptor to GABA, (*ii*) the cooperativity of GABA in gating the channel, (*iii*) the formation of the main conductance state of 28.3 pS, (*iv*) low sensitivity to zinc ions, and (*v*) the presence of BZ sites on the receptor.

Histology and Life Expectancy of γ_2 Subunit-Deficient Mice. Examination of Nissl-stained sections failed to reveal any obvious alterations in brain cytoarchitecture of newborn $\gamma_2^{0/0}$ mice as compared to wild-type littermates. In particular, the lamination of cortical structures was normal, the developing granule cell layers were present in the cerebellum, and the foliation of this structure was unaltered. These observations, together with the lack of alteration in $GABA_A$ receptor distribution (see above), suggest that there was no retardation in brain maturation in mutant mice.

The majority of $\gamma_2^{0/0}$ mice died within a few days after birth, although their birth weight was normal and the major peripheral organs, including those known to express $GABA_A$ receptors (pituitary, pancreas, adrenal gland), did not reveal any pathological changes. The surviving mutants exhibited sensorimotor deficits manifested first by an excessive hyperactivity in body and limb movement following birth and later by impaired grasping and righting reflexes and abnormal gait (duck walk). They failed to thrive and none survived beyond P18, although feeding was apparently not impaired, as evidenced by the presence of milk in the stomach and the lack of overt atrophies of the intestinal tract. Likewise, the immunostaining of anterior pituitary hormones (prolactine, corticotropin, and growth hormone) was unaltered, pointing to a normal endocrine control. In contrast to homozygous mutants, heterozygous mice, in which the number of GABA sites was normal and BZ sites were reduced by only 20% ± 4%, developed normally to adulthood.

DISCUSSION

The ubiquitous γ_2 subunit can be physically omitted from $GABA_A$ receptors *in vivo* without interfering with assembly of GABA-gated ion channels from the remaining subunits. The γ_2-deficient $GABA_A$ receptors are assembled from α and β subunit variants, a conclusion supported by the comparison of the receptors in γ_2 subunit-deficient mice with recombinant receptors assembled from α and β subunits *in vitro* (23, 24). The two types of receptors corresponded closely in single channel conductance, Hill coefficient, and the ability to be inhibited by bicuculline and pictrotoxin and potentiated by pentobarbital but not by BZ site ligands. In addition, α and β subunits were coexpressed in neural membranes of γ_2 subunit-deficient mice with a regional and cellular distribution corresponding to that in wild-type brain. Thus, the γ_2 subunit is not required for subunit assembly, transport, insertion into membranes, subcellular targeting, and clustering of $GABA_A$ receptors. These processes can be governed by α and β subunits alone. In contrast to the present findings on $GABA_A$ receptors, gene targeting of a particular subunit of other ligand-gated ion channels was associated with loss of the entire receptor complex, as shown for the NR1 subunit of the *N*-methyl-D-aspartate receptor (26, 27) or the β_2 subunit of the neuronal nicotinic acetylcholine receptor (28).

The lack of the γ_2 subunit abolishes the sensitivity of nearly all $GABA_A$ receptors to BZ site ligands *in vivo*, as demonstrated by the autoradiographical, electrophysiological, and behavioral evidence. Thus, a mammal has been generated that

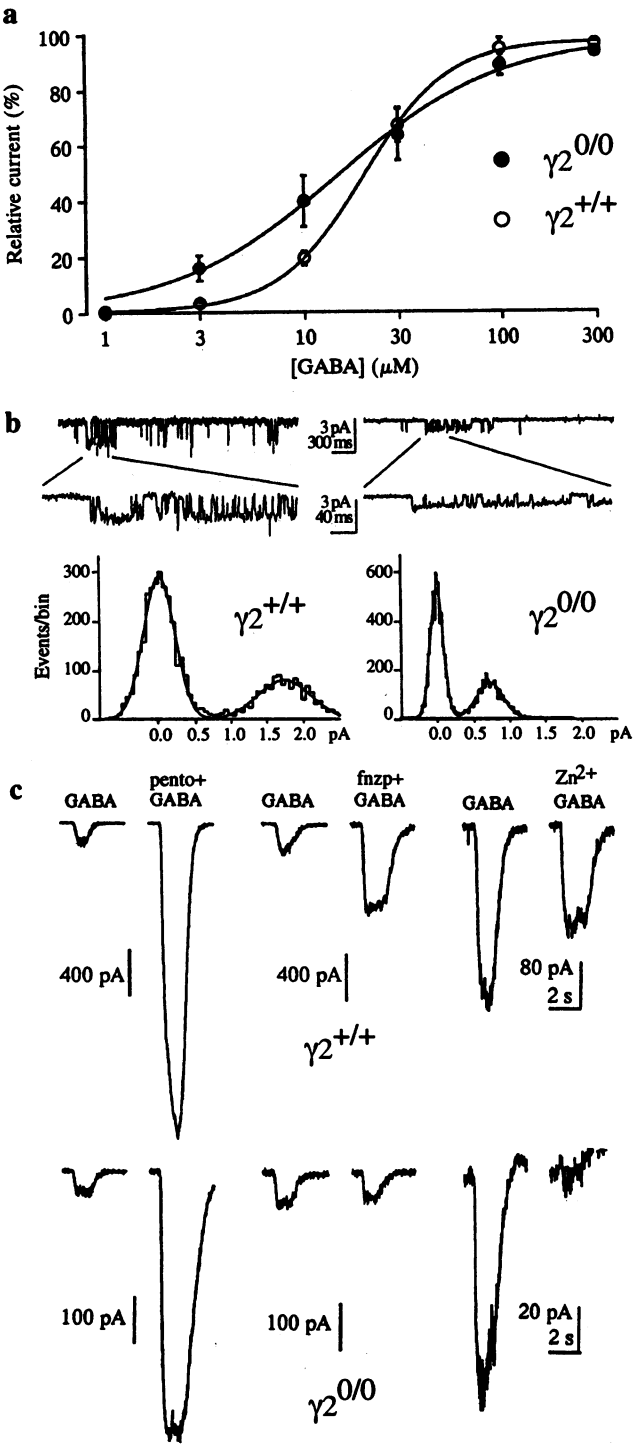

FIG. 5. GABA-evoked whole cell and single channel currents from DRG neurons of newborn $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ mice. (*a*) GABA dose–response curves, yielding EC_{50} values of 20.0 ± 0.5 μM (n = 7, $\gamma_2^{+/+}$) and 15.0 ± 3.5 μM (n = 5, $\gamma_2^{0/0}$). The Hill coefficients were 1.98 ± 0.07 ($\gamma_2^{+/+}$) and 1.05 ± 0.30 ($\gamma_2^{0/0}$). (*b*) Elementary currents evoked by GABA at low and high time resolution, illustrating the main conductance states. Single channel current amplitude histograms were fitted to the sum of two Gaussians representing the baseline current and the amplitude of the openings. From patches clamped at −60 mV, current amplitudes were derived (5 μM GABA) corresponding to single channel main conductance states of 28.3 ± 0.9 pS (n = 5, $\gamma_2^{+/+}$) and 11.3 ± 1.0 pS (n = 4, $\gamma_2^{0/0}$). The traces given correspond to 28.6 and 12.3 pS, respectively. (*c*) Current traces showing the effects on the currents evoked by GABA pulses (2 s, 5 μM) in $\gamma_2^{+/+}$ and $\gamma_2^{0/0}$ DRG neurons of the test substances indicated (pento, pentobarbital, 100 μM; fnzp, flunitrazepam, 1 μM; Zn^{2+}, $ZnSO_4$, 10 μM).

largely lacks the evolutionarily conserved BZ site and regulation by putative endogenous ligands (6, 7) of this site. The few BZ sites remaining in $\gamma_2^{0/0}$ brains are most likely due to a minor receptor population containing the γ_3 subunit (29, 30). The lack of the γ_2 subunit also alters the single channel characteristics and the GABA sensitivity of the receptor, whereby the reduction in the main conductance state might be offset, at least in part, by the decrease in the EC_{50} value for GABA. Thus, apart from the lack of BZ sites, a limited reduction of $GABA_A$ receptor function would be expected to arise. In keeping with this notion, prenatal brain development appears to be unaffected in γ_2-deficient mice, as evidenced by the normal brain cytoarchitecture at birth, by the normal emergence of modular brain structures such as the barrelettes in the brainstem, and by the unaltered spatio-temporal expression patterns of $GABA_A$ receptor subunits, including the developmental switch from α_2 to α_1 subunit expression around birth (31). In view of the postulated neurotrophic action of GABA during embryogenesis (32), γ_2-deficient receptors appear to be sufficient to mediate the nonsynaptic actions of GABA in prenatal brain. Thus, the BZ sites, expressed abundantly in prenatal rodent brain [starting at E14 in the rat (33)], do not appear to play an essential role for brain development.

However, postnatally, the alterations in $GABA_A$ receptor function result in severe growth retardation, sensorimotor and behavioral dysfunctions, and a drastic reduction in life-span, although no impairment in feeding or lack of anterior pituitary hormones for endocrine control is apparent. Rather, the striking postnatal phenotype may be linked to the onset of synaptogenesis and the formation of inhibitory circuits in the brain (34, 35). The γ_2-deficient $GABA_A$ receptors may not provide the precise control of GABAergic tone required in inhibitory synaptic transmission controlling neuronal excitability. Thus, the time at which most GABAergic synapses are expected to be operative coincides with the maximal life-span (P18) of $\gamma_2^{0/0}$ mice. It appears that the impairment of the receptor response to GABA and, possibly, the lack of receptor regulation by putative endogenous ligands of the BZ site contribute to this phenotype.

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