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Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the $\gamma 2$ subunit of the γ-aminobutyrate type A receptor

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Abstract

The γ subunit of the γ-aminobutyric acid type A receptor ($GABA_A$-R) is essential for bestowing both normal single channel conductance and sensitivity to benzodiazepines on native $GABA_A$-Rs. The long splice variant of the $\gamma 2$ subunit ($\gamma 2L$) has been postulated to be essential in mediating the modulatory actions of ethanol at the $GABA_A$-R. In order to evaluate this hypothesis, gene targeting was used to delete the 24bp exon which distinguishes $\gamma 2L$ from the short splice variant ($\gamma 2S$). Mice homozygous for this exon deletion ($\gamma 2L^{-/-}$) are viable and indistinguishable from wild-type ($\gamma 2L^{+/+}$) mice. No $\gamma 2L$ mRNA was detected in these mice, nor could $\gamma 2L$-containing $GABA_A$-R protein be detected by specific antibodies. Radioligand binding studies showed the total amount of $\gamma 2$ subunit protein to be not significantly changed, suggesting that $\gamma 2S$ replaces $\gamma 2L$ in the brains of the knockout animals. Electrophysiological recordings from dorsal root ganglion neurons revealed a normal complement of functional receptors. There was no difference in the potentiation of GABA currents by ethanol (20–200 mM) observed in neurons from $\gamma 2L^{+/+}$ or $\gamma 2L^{-/-}$ mice. Several behavioral effects of ethanol, such as sleep time, anxiolysis, acute functional tolerance, chronic withdrawal hyperexcitability and hyperlocomotor activity were also unaffected by genotype. It is concluded that $\gamma 2L$ is not required for ethanol's modulatory action at the $GABA_A$-R or whole animal behavioral effects.

Keywords: Ethanol; $GABA_A$ receptor; Gene knockout; GABA

1. Introduction

γ-Aminobutyric acid type A receptors ($GABA_A$-Rs) mediate the bulk of inhibitory synaptic signalling in the central nervous system (Sieghart, 1995). The ubiquity of GABAergic transmission entails a role for GABA in diverse behavioral activities and also as a likely target for modification by sedative/hypnotic and anesthetic drugs, including the benzodiazepines, barbiturates and ethanol (Tanelian et al., 1993; Sieghart, 1995). $GABA_A$-Rs are heteromeric membrane proteins which permit

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permeation by chloride ions when activated by GABA, resulting in neuronal hyperpolarization and consequent inhibition. Multiple $GABA_A$-R subunits (α, β, γ, δ, ε) have been cloned, and multiple isoforms of these exist (e.g. α1-6, β1-3, γ1-3). Native $GABA_A$-Rs are believed to be mainly pentamers of stoichiometry: $\alpha\alpha\beta\beta\gamma$ (Chang et al., 1996; McKernan and Whiting, 1996). The γ2 subunit is expressed at high levels throughout developing and adult brain and spinal cord and is the predominant γ subunit produced (Laurie et al., 1992; Ma et al., 1993). Widespread evidence indicates that the γ subunits are required for benzodiazepine modulation of the $GABA_A$-R. Gene knockout mice that completely lack all γ2 gene products have confirmed this (Gunther et al., 1995). These mice lack 94% of benzodiazepine binding sites and consequently benzodiazepine sensitivity at all levels of analysis is abolished.

The γ2 subunit exists in two variant forms (Whiting et al., 1990; Kofuji et al., 1991). The long variant of γ2 (γ2L) differs from the short variant (γ2S) as a result of alternate splicing of the γ2 mRNA such that γ2L contains an additional 24 nucleotides that encode the 8 amino acids LLRMFSFK corresponding to residues 338-345 (Whiting et al., 1990; Kofuji et al., 1991). These additional amino acids are thought to contribute to the intracellular loop between the third and fourth putative transmembrane domains. Included within these eight amino acids is a consensus site for protein kinase C phosphorylation which has been shown to influence $GABA_A$-R function (Krishek et al., 1994). Distribution of γ2L and γ2S is remarkably different in many brain regions (Gutierrez et al., 1994).

A requirement for γ2L in the potentiation of GABA by ethanol has been proposed (Wafford et al., 1991) and has been an active and controversial area. Early work suggested an absolute requirement of the γ2L-specific site for phosphorylation by protein kinase C in ethanol potentiation of GABA (Wafford and Whiting, 1992). However, other studies have shown ethanol potentiation of GABA in receptors containing either γ2L or γ2S (Sigel et al., 1993; Marszalec et al., 1994; Mihic et al., 1994). The subject has been addressed many times, with frequently conflicting results, even between different expression systems (Harris et al., 1997).

As mentioned, a global knockout of the γ2 gene has been achieved (Gunther et al., 1995), producing mice which are behaviorally insensitive to benzodiazepines. These animals have a very brief lifespan and, since they lack both γ2S and γ2L they offer little insight into specific roles of the two splice variants of γ2. Therefore, in order to illuminate many of the unresolved issues regarding the pharmacologic significance of γ2L and to uncover any specific physiologic functions associated with alternate splicing of this gene, we have created a line of mice which is genetically incapable of producing the γ2L subunit, but do produce γ2S. Portions of this work were previously presented in abstract form (Homanics et al., 1997a).

2. Methods

2.1. Production of γ2L knockout mice

A $\sim$9 kb BglII restriction fragment of Strain 129/SvJ mouse genomic DNA that encompassed exon 9 (Fig. 1A) was subcloned from a P1 phage clone (Genome Systems, St. Louis, MO). A replacement type DNA targeting vector was constructed in which the bicistronic selectable marker gene PGKneoNTRtkpA (Wu et al., 1994) was flanked on the 5′ side by a $\sim$1.8 kb SacI-XbaI fragment and on the 3′ side by a $\sim$4.7 kb XbaI-BglII fragment (Fig. 1B). The targeting vector was linearized with SalI in the vector backbone before being electroporated into R1 (Nagy et al., 1990) embryonic stem (ES) cells as described (Homanics et al., 1997c). Transformants were selected with G418 (220 μg/ml; Life Technologies) for $\sim$10–12 days. Genomic DNAs prepared with Instagene (Bio-Rad, Hercules,

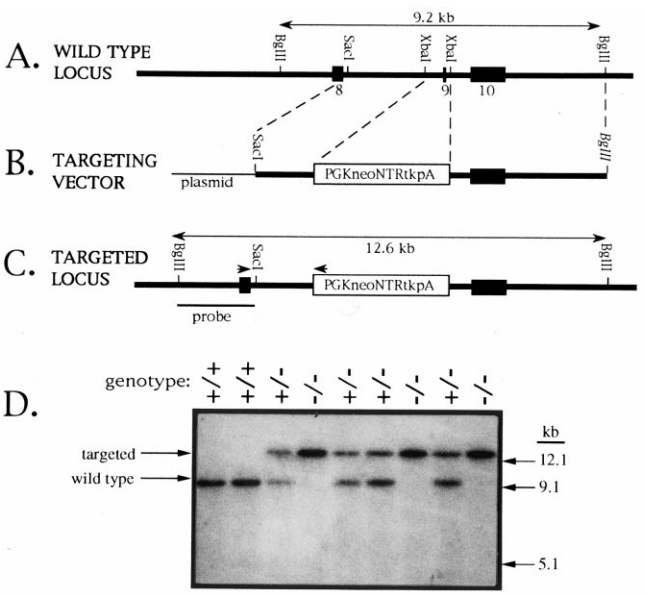

Fig. 1. Gene targeting strategy used to produce a γ2 allele that is incapable of producing the γ2L splice variant. (A) Genomic organization of the wild type γ2 locus around the 24 bp exon 9. (B) Replacement type DNA targeting vector that was used to target the γ2 locus and replace exon 9 with the selectable marker gene (PGKneoNTRtk). (C) Predicted genomic structure of the γ2 locus following homologous recombination with the targeting construct. (D) Southern blot analysis of BglII digested mouse genomic DNA demonstrating the wild type (+/+), heterozygous (+/−), and homozygous (−/−) genotypes. *Marker*, 1 kb DNA ladder (Life Technologies); *numbered black boxes*, exons; *thick lines*, intronic DNA; *thin lines*, plasmid backbone; *arrows*, BglII restriction fragments that hybridize with the probe; *arrowheads*, PCR primer binding sites used to screen for homologous recombinants. Note: restriction sites in italics were destroyed during vector construction.

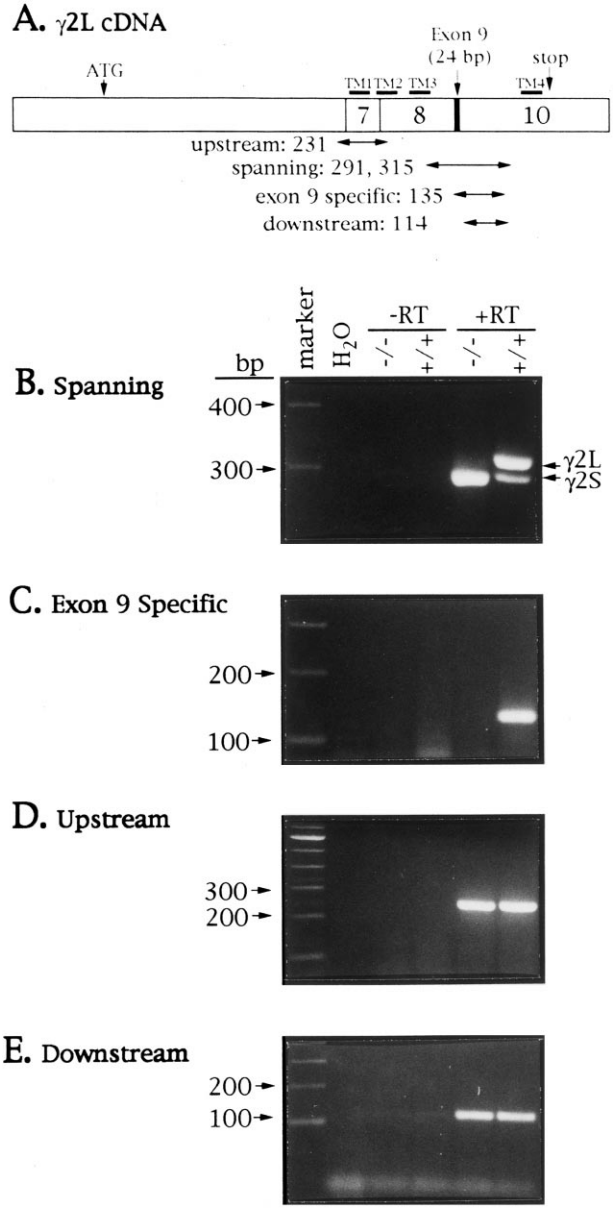

Fig. 2. RT-PCR analysis of cortical $\gamma 2$ mRNA. (A) Schematic representation of the $\gamma 2$L cDNA with exon-exon boundaries shown for exons 7-10. Shown below the cDNA are the names and locations of the amplicons that were analyzed along with their expected size (in bp). *ATG*, translational start site; *TM1-TM4*, putative transmembrane spanning domains; *stop*, translational termination site. (B) Ethidium bromide stained agarose gel of RT-PCR products derived from the 'spanning' amplicon. Reverse transcription reactions were conducted in the absence ($-$RT) or presence ($+$RT) of reverse transcriptase. Reverse transcription reactions conducted in the absence of RNA template (H_2O) served as negative controls. Note in wild type mice ($+/+$) both the 291 and 315 bp RT-PCR products that are derived from the $\gamma 2$S and $\gamma 2$L mRNAs, respectively, are readily detectable. In contrast, only the $\gamma 2$S derived RT-PCR product is detectable in homozygous mice ($-/-$). (C) Ethidium bromide stained agarose gel of RT-PCR products derived from the 'exon 9 specific' amplicon. Note presence of RT-PCR product in wild type mice and the absence of this product in homozygous mice. (D) Ethidium bromide stained agarose gel of RT-PCR products derived from the 'upstream' amplicon. Note the RT-PCR product is readily detectable in mice of both genotypes. (E) Ethidium bromided stained agarose gel of RT-PCR products derived from the 'downstream' amplicon. Note the RT-PCR product is readily detectable in mice of both genotypes.

CA) from pools of 4-6 G418 resistant clones were screened for targeting by PCR (Fig. 1C) using a $\gamma 2$ exon 8 specific primer (Genbank accession no.: M62374; nucleotides 1373-1397) and a primer (5′-TAAAGCGCATGCTCCAGACT-3′) specific to the promoter of the selectable marker gene. Reactions were performed with the Elongase Amplification System (Life Technologies). PCR products were subjected to agarose gel electrophoresis and Southern blot analysis. Individual clones from PCR positive pools were subjected to the same analysis. Individual PCR positive clones were analyzed for targeting by Southern blot analysis of genomic DNA following digestion with BglII and hybridization with an $\sim$2.3 kb BglII-SacI restriction fragment that is external to the targeting construct (Fig. 1C).

Correctly targeted ES cells were microinjected into C57BL/6J blastocysts to produce chimeric mice. Highly chimeric males were mated to C57BL/6J females. Agouti offspring that were heterozygous for the targeted allele ($\gamma 2L^{+/-}$) were interbred to produce mice that were wild type ($\gamma 2L^{+/+}$), $\gamma 2L^{+/-}$, or homozygous ($\gamma 2L^{-/-}$) at the $\gamma 2$ locus. The mice used for the studies reported here were derived from the $\gamma 2$-73b ES cell clone. The genetic background of all mice is C57BL/6J X Strain 129 F_2-F_4. The strain designation of these mice is *Gabrγ2L*^{tm1Geh} and they can be obtained from the Induced Mutant Resource at the Jackson Laboratory (Bar Harbor, ME).

2.2. RT-PCR

Production of $\gamma 2$ mRNA in cerebral cortex of $\gamma 2L^{-/-}$ mice was compared with $\gamma 2L^{+/+}$ mice using RT-PCR. Total RNA was isolated from cortex of individual mice using TRIzol (Life Technologies). Amplification grade Dnase I (Life Technologies, Inc.) was used to remove contaminating DNA from each RNA preparation. For each sample, reverse transcription was performed using the Superscript Preamplification System (Life Technologies, Inc.) and 5 μg of total RNA in a total reaction volume of 20 μl using random hexamers as primers. Parallel control reactions were performed, which lacked either reverse transcriptase or RNA. The cDNAs generated were analyzed by PCR using four different $\gamma 2$-specific primer sets (Fig. 2A). The corresponding nucleotides of the $\gamma 2$L mRNA (GENBANK accession no.: M62374) used for oligonucleotide primers were: upstream amplicon, 1027-1048 and 1257-1239; downstream amplicon, 1583-1605 and 1696-1677; spanning amplicon, 1395-1424 and 1709-1683; exon 9 specific amplicon, 1473-1496 and 1607-1588. PCR reactions (25 μl total volume) contained 1 μl of each RT reaction (or H_2O), 1.0 unit Taq DNA polymerase (Life Technologies), 1X reaction buffer, 2.0 mM $MgCl_2$, 0.39 μg of each primer, and 0.3 mM of each

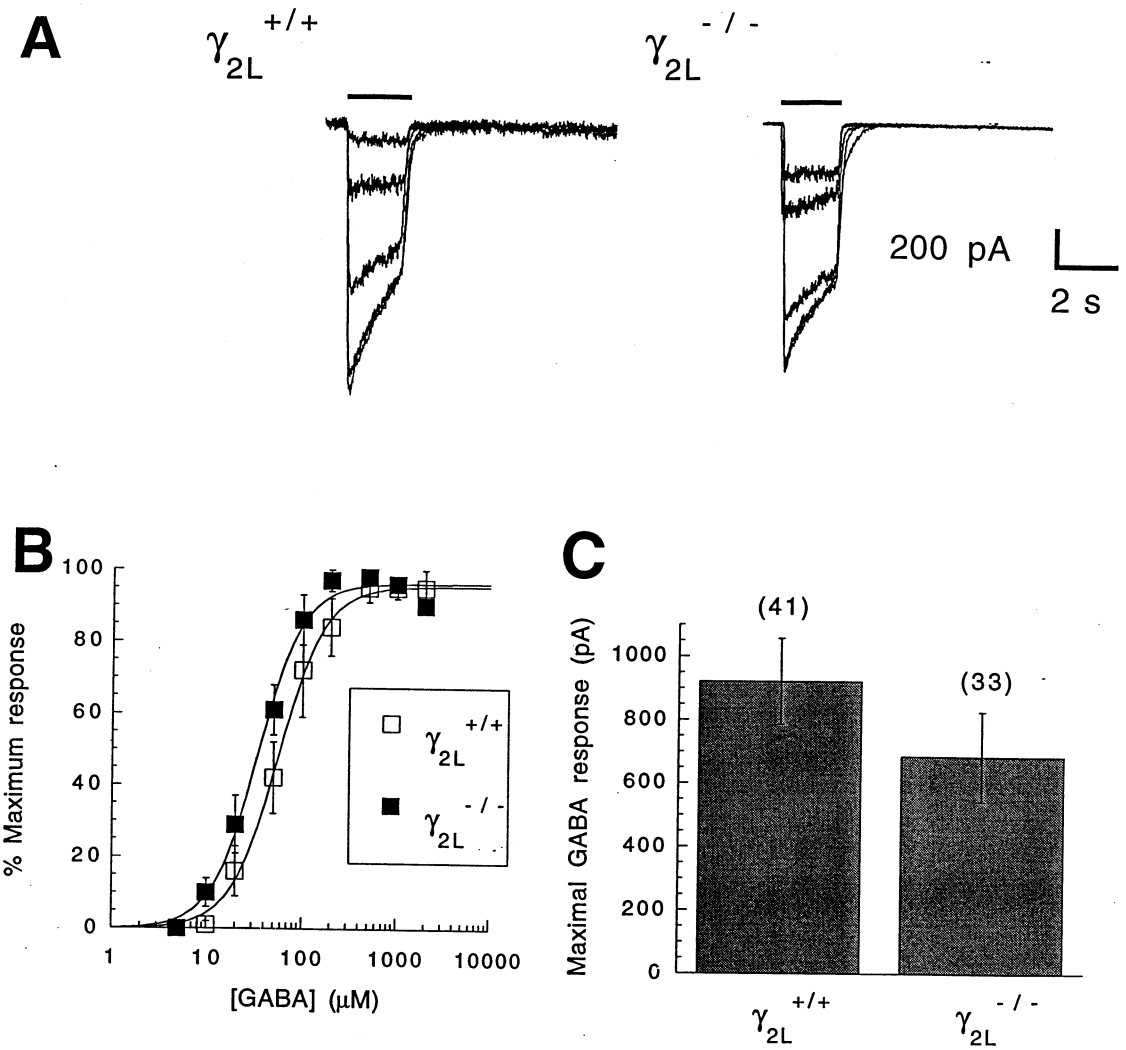

Fig. 3. Electrophysiological characterization of GABA responses in dorsal root ganglion (DRG) neurons from wildtype ($\gamma 2L^{+/+}$) and knockout ($\gamma 2L^{-/-}$) mice. (A) Whole-cell patch clamp recordings from acutely isolated individual DRG neurons: in each case, GABA was applied at 10, 20, 100, 1000 and 2000 μM. (B) Pooled GABA concentration-response curves for neurons from $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice. Data is pooled from eight neurons from three $\gamma 2L^{+/+}$ mice and ten neurons from three $\gamma 2L^{-/-}$ mice. (C) Pooled maximal amplitude data show no significant differences between the total current amplitudes in neurons from the $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ animals (number of neurons shown in parentheses). Maximal amplitude data is from nine $\gamma 2L^{+/+}$ mice and 7 $\gamma 2L^{-/-}$ mice.

deoxynucleotide triphosphate. Thermal cycling consisted of 94°C for 10 s, followed by 30 cycles of denaturation (92°C, 30 s), annealing (65°C, 40 s), and extension (72°C, 90 s), followed by a final extension at 72°C for 5 min. Following thermal cycling, aliquots of upstream, downstream, and exon 9 specific PCR reactions were analyzed on ethidium bromide stained agarose (2%) gels. PCR reactions with the spanning primer set were analyzed on Metaphore agarose (3%; FMC Bioproducts, Rockland, ME).

2.3. Antibodies to the rat γ2S, γ2L, and γ2 subunits of $GABA_A$-Rs

The γ2S, γ2L, and γ2 antibodies were raised in rabbits using γ2S peptide KKKKNPAPTIDI corresponding to residues 332-343 (Shivers et al., 1989), γ2L peptide (NPLLRMFSFKAP) corresponding to residues 336-347 (Whiting et al., 1990; Kofuji et al., 1991), and γ2 peptide (Pyroglutamate-KSDDDYEDYASNKT) corresponding to residues 1–15. The γ2S and γ2L antibodies were characterized as described (Gutierrez et al., 1994; Khan et al., 1994). The γ2 antibody was similarly characterized (unpublished results).

2.4. Immunoprecipitation of [^{3}H]flunitrazepam binding sites

Membranes were prepared from adult $\gamma 2L^{+/+}$ ($n = 8$) and $\gamma 2L^{-/-}$ ($n = 8$) mouse whole brain. $GABA_A$-R solubilization, immunoprecipitation and radioligand binding procedures have been described elsewhere

Table 1
Immunoprecipitation of [^{3}H]flunitrazepam (10 nM) binding (% cpm) from mouse whole brain by anti-2γL, anti-γ2S and anti-γ2 antibodies[a]

	Anti-γ2L	Anti-γ2S	Anti-γ2
$\gamma2L^{+/+}$	32 ± 6 (5)	33 ± 6 (6)	63 ± 4 (3)
$\gamma2L^{-/-}$	0 ± 1** (3)	79 ± 6** (6)	60 ± 3 (3)

[a] Immunoprecipitations were done with 60 µl of anti-γ2L, 25 µl of anti-γ2S and 50 µl of anti-γ2 antibodies. Values are percentage of cpm in the pellet. The 100% values of the binding of [^{3}H]flunitrazepam (10 nM) to the solubilized receptors were 7544 ± 1318 and 8260 ± 1487 cpm for $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ mouse brain, respectively. No immunoprecipitation was detected with preimmune sera. Clonazepam (10 µM) was used for non-specific binding. Values are mean ± SD. The number of experiments are indicated in parenthesis. Each experiment was done in triplicate.
** $P<0.01$

(Khan et al., 1996). Briefly, 400 µl of solubilized $GABA_A$-Rs (0.3–0.4 pmol of [^{3}H]flunitrazepam binding) in buffer containing protease inhibitors, were incubated with antiserum overnight at 4°C. The receptor-antibody complexes were recovered by incubation with protein A-agarose followed by centrifugation. Immunoprecipitation was quantified by determining the binding of [^{3}H]flunitrazepam (10 nM) to the pellet and supernatant. Clonazepam (10 µM) was used to define non-specific binding. Each assay was carried out in triplicate. Results are expressed as mean ± S.D. Student's *t*-test was used for statistical analysis. For sequential immunoprecipitation, the supernatant of the first immunoprecipitation was incubated with the same volume of antibody, and the immunoprecipitation procedure was repeated.

2.5. Electrophysiology

Dorsal root ganglion neurons (DRGs) were dissociated from 4–6-week-old mice, as described by White (1992), plated onto poly-D-lysine coated coverslips and used for recordings within 12 h. On any given day, recordings were made from similar numbers of cells from $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ littermates to minimize possible day to day variations in dissection and culturing conditions. Genotyping was performed prior to the experiments and tails were clipped post mortem as a source of DNA for genotype confirmation by Southern analysis as described above. Cover slips containing neurons were transferred to a chamber which was continuously perfused with extracellular medium containing (in mM): 145 NaCl, 3 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 6 D-glucose, 10 HEPES/NaOH (pH 7.4). Recordings were made using the whole-cell patch-clamp technique, as described in previous publications (Krasowski et al., 1997). Patch pipettes contained (in mM): 145 *N*-methyl D-glucamine hydrochloride, 5 K_2ATP, 1.1 EGTA, 2 $MgCl_2$, 5 HEPES/KOH (pH 7.2), 0.1 $CaCl_2$; pipette resistance was 4–5 MΩ. Neurons were voltage clamped at −60 mV. GABA and ethanol were rapidly applied (> 1 ml/min) to the cell by local perfusion (Krasowski et al., 1997) using a motor-driven solution exchange device. Responses were digitized (TL-1-125 interface; Axon Instruments, Foster City, CA) using pCLAMP 5 (Axon Instruments). In each population, GABA (Sigma) concentration-response curves were first constructed, then an EC_{10} concentration of GABA appropriate for that population of $GABA_A$-Rs was selected for studies of GABA potentiation by ethanol. Ethanol (Aaper Alcohol and Chemical, Shelbyville, KY) was applied at concentrations between 10 to 200 mM and GABA applications retested in the presence of ethanol before returning to the control bathing solution and re-establishing the control GABA responses. Receptor modulation by ethanol was expressed as percentage change of the response to the EC_{20} concentration of GABA relative to the control, as described elsewhere (Krasowski et al., 1997). Receptor modulation by zinc (Sigma) was expressed as percentage change of the response to the EC_{50} concentration of GABA. Numerical data are presented throughout as mean ± SEM. Midazolam was obtained as Versed injection (Roche Pharmaceuticals, Manati, P.R.).

2.6. Behavioral characterization of $\gamma2L^{-/-}$ animals

All animal experiments were approved by the Institutional Animal Care and Use Committee. Both male and female mice that were at least 7 weeks of age were used for all assays. Neither age or body weight differed with respect to genotype within any experiment. All experiments were performed by an investigator who was unaware of the genotype being assayed.

Table 2
Accelerating rotarod performance[a]

Genotype	*n*	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8
$\gamma2^{+/+}$	19	79.9 ± 6.9 (0)	125.9 ± 11.4 (26)	152.2 ± 11.8 (59)	157.4 ± 8.2 (65)	163.4 ± 7.8 (71)	162.9 ± 9.4 (82)	171.8 ± 5.6 (82)	174.5 ± 3.3 (82)
$\gamma2^{-/-}$	20	70.7 ± 8.3 (0)	113.6 ± 9.5 (15)	143.1 ± 10.7 (44)	150.8 ± 11.8 (61)	160.7 ± 8.7 (61)	166.9 ± 6.3 (67)	166.6 ± 7.5 (78)	168.7 ± 6.5 (78)

[a] Results in (s) are presented as mean ± SEM. Numbers in parentheses indicate the precentage of mice remaining on rotarod for the entire 180 s of each trial.

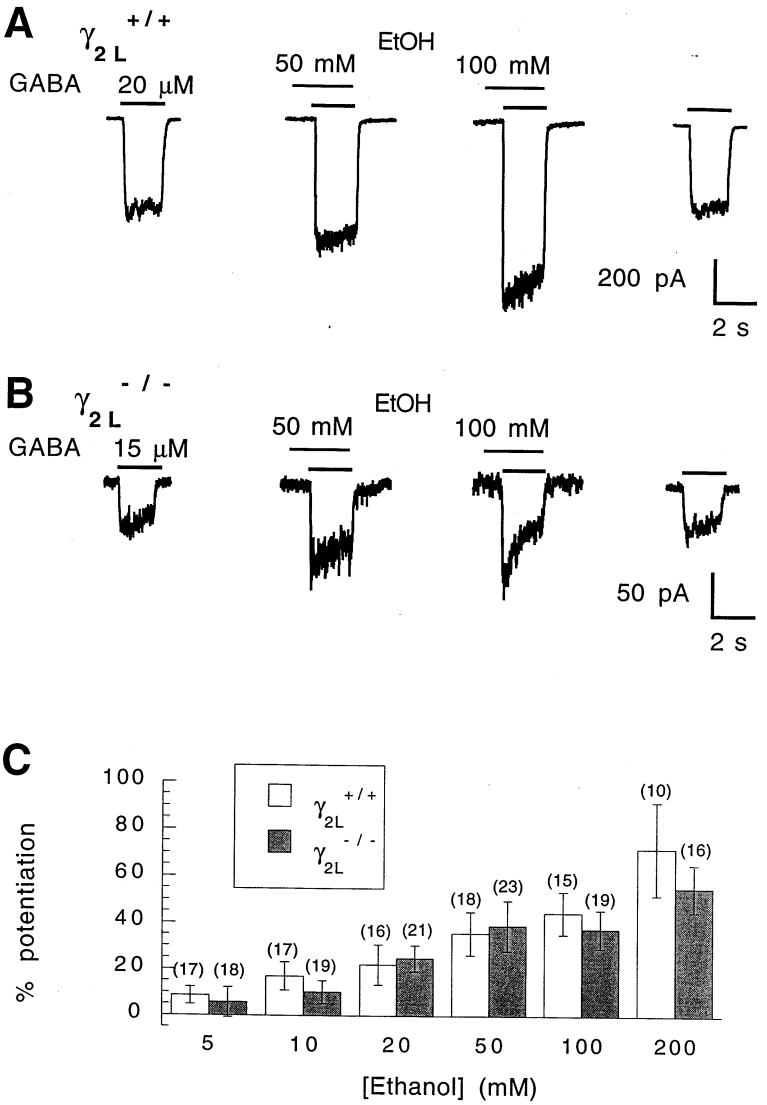

Fig. 4. Submaximal GABA currents in DRG neurons from both wild type ($\gamma 2L^{+/+}$) and knockout ($\gamma 2L^{-/-}$) mice were potentiated by ethanol. (A) Representative trace from a DRG neuron from a $\gamma 2L^{+/+}$ mouse. Submaximal responses to GABA were potentiated by 50 and 100 mM ethanol in a concentration-dependent manner. Bars above the traces indicate when drugs were applied. (B) Representative trace from a DRG neuron from a $\gamma 2L^{-/-}$ mouse. Submaximal responses to GABA were potentiated by 50 and 100 mM ethanol in a concentration-dependent manner. (C) Pooled ethanol potentiation data showing that there is no significant difference between $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice in the magnitude of ethanol-induced potentiation in DRG neurons, over a range of 5–200 mM ethanol. A significant effect of ethanol was observed at concentrations $\geq$ 20mM in both cases. Numbers in parentheses state number of neurons studied. Neurons were from four to six $\gamma 2L^{+/+}$ mice and 5-6 $\gamma 2L^{-/-}$ mice for all ethanol concentrations examined.

Effects of the gene knockout on motor coordination were tested using an accelerating rotarod assay. Briefly, mice were acclimated to the nonrotating rotarod (Basile Rota-Rod Treadmill, Model 7650; Stoelting, Wood Dale, IL) for 3 min on the day prior to the start of daily testing sessions. For eight consecutive days, each mouse was placed on the stationary rod and approximately 30 s later, the rod accelerated from 3 to 19 rpm over 180 s. The duration that each mouse remained on the rod was recorded. Effect of genotype on time on rod per trial was compared by Student's *t*-test.

Responses to ethanol were investigated with a battery of assays that assess several different behavioral endpoints. To ensure that clearance and metabolism of ethanol was not altered in these mice, both $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice were injected with ethanol (3.5 g/kg; ip). Ethanol concentrations in serum collected from the retroorbital sinus at 10, 30, 60, 90, 150, 180, and 225 min postinjection were determined using an enzymatic assay (Sigma: Procedure No. 333-UV) to determine peak blood ethanol concentrations (BEC). In addition, linear regression analysis was used to determine the rate of ethanol clearance between 30 and 225 min.

Table 3
Elevated plus-maze assay[e]

Genotype (n)	Treatment	Total arm entries[a]	% Entries open arms[b,c]	% Time on open arms[c,d]
$\gamma 2L^{+/+}$(19)	Vehicle	12.0 ± 1.1	25.6 ± 3.7	21.7 ± 4.4
$\gamma 2L^{-/-}$(19)	Vehicle	12.0 ± 0.9	17.4 ± 3.1	13.8 ± 3.2
$\gamma 2L^{+/+}$(20)	Ethanol	17.4 ± 1.9	37.8 ± 3.7	41.0 ± 5.4
$\gamma 2L^{-/-}$(20)	Ethanol	18.9 ± 1.7	31.8 ± 3.6	29.7 ± 4.8

[a] Significant effect of treatment ($P < 0.001$).
[b] Significant effect of genotype ($P < 0.05$).
[c] Significant effect of treatment ($P < 0.001$).
[d] Significant effect of genotype ($P < 0.04$).
[e] Results are mean $\pm$ SEM.

The hypnotic effects of ethanol were compared between genotypes using the standard sleep time assay. Briefly, mice were injected with ethanol (3.5 g/kg; ip) and the duration of the loss of the righting reflex was measured (Homanics et al., 1997c). Normothermia was maintained with the aid of a heat lamp. Effect of genotype was compared by Student's t-test. In all sleep time assays, mice that failed to lose the righting reflex (i.e., sleep time = 0 min) and mice that failed to regain the righting reflex within 2 SD's of the mean sleep time for that genotype were excluded from the analysis.

Tolerance to the acute ataxic effects of ethanol on motor coordination was assessed using the acute functional tolerance (AFT) assay (Gallaher et al., 1982). Briefly, mice were acclimated to a stationary rod. Mice were then injected with ethanol (1.75 g/kg; ip) and placed on the rod until loss of balance. When the mice regained the ability to remain on the stationary rod for 1 min (referred to as t_1), a blood sample was collected for determination of BEC. Subsequently, mice were immediately injected with a second dose of ethanol (2.0 g/kg; ip) that induced ataxia. Mice were periodically tested until they could remain on the rod for 1 min (referred to as t_2) and a second blood sample was collected for BEC determination. AFT is defined in terms of the difference in BEC at t_2 and t_1. AFT, t_1, t_2, and BEC at t_1 were compared between genotypes by Student's t-test.

Alcohol dependence following termination of chronic ethanol exposure was examined by comparing withdrawal hyperexcitability in $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice. Mice were exposed to ethanol vapors in inhalation chambers as described (Homanics et al., 1998). Control (CONT) treated mice of both genotypes were tested in parallel to ethanol (EtOH) treated mice with the exception that CONT mice were exposed to air instead of ethanol. Ethanol exposure followed the multiple withdrawal paradigm (Becker and Hale, 1993) and consisted of three 16 h periods of exposure with each exposure separated by 8 h of abstinence. Immediately prior to each exposure cycle, all EtOH treated mice were pretreated with pyrazole (1mmol/kg; ip), an inhibitor of alcohol dehydrogenase, and a priming dose of ethanol (1.6 g/kg; ip). Control mice were also pretreated with pyrazole but were injected with saline instead of ethanol. BEC was determined on serum samples collected from all mice at the conclusion of the third exposure cycle. EtOH treated mice were excluded from the analysis if BEC was < 50 or > 300 mg/dl. Body weights were recorded immediately prior to the first treatment cycle and at the conclusion of the final treatment cycle.

Dependence on ethanol was assessed by scoring handling induced convulsions (HIC) to provide an index of withdrawal hyperexcitability. Mice were scored hourly for the first 10 h following termination of the final exposure cycle and again at 23 and 24 h into the withdrawal period for HIC using the scale of Becker and Hale (1993). Hourly HIC scores for each genotype-treatment combination were used to generate 24 h HIC curves, which were numerically integrated. Body weight,% body weight change, age, BEC, and area under HIC curves, were compared between genotypes by analysis of variance with Fisher's Protected Least Significant Difference post-hoc test when appropriate (StatView, Abacus Concepts).

The elevated plus-maze was used to assess basal levels of locomotor activity and anxiety, as well as the anxiolytic and locomotor stimulatory effects of a low dose of ethanol. The design of the apparatus and the experimental protocol were essentially as originally described (Lister, 1987). The apparatus consisted of two enclosed arms and two open arms that extended from a central platform. Mice were injected with either saline or ethanol (1.5 g/kg; ip) 30 min prior to being placed on the central platform facing an open arm. Each mouse was observed for 5 min and time spent on each arm, as well as the number of entries onto each arm, were recorded. A mouse was considered to have entered an arm when all four paws were on the arm. Effect of genotype and treatment on the percent of entries onto open arms, percent of time on open arms, and total number of arm entries were compared by analysis of variance.

Table 4
Ethanol response data[a]

	Peak BEC (mg/dl)	Clearance (mg/dl min^{-1})	t_1 (min)	BEC @ t_1 (mg/dl)	t_2 (min)	Acute functional tolerance (mg/dl)
$\gamma2L^{+/+}$	452.8 ± 7.5	0.79 ± 0.08	16.9 ± 1.5	217.6 ± 6.3	143.7 ± 6.8	101 ± 9.8
n	5	5	17	17	17	17
$\gamma2L^{-/-}$	434.0 ± 18.7	0.84 ± 0.10	14.9 ± 1.9	214.0 ± 4.7	154.8 ± 5.9	107.4 ± 6.2
n	5	5	19	19	19	19

[a] Results are presented as mean ± SEM.

Table 5
Withdrawal hyperexcitability testing[a]

Genotype/treatment	*n*	BEC (mg/dl)	Initial body weight (g)	% Body weight change
$\gamma2L^{+/+}$/CONT	21	Non detectable	23.5 ± 0.7	0.4 ± 1.0
$\gamma2L^{-/-}$/CONT	20	Non detectable	25.6 ± 0.9	1.1 ± 1.1
$\gamma2L^{+/+}$/EtOH	18	201.5 ± 14.8	24.5 ± 1.1	−0.6 ± 1.0
$\gamma2L^{-/-}$/EtOH	20	178.3 ± 13.2	24.4 ± 0.7	0.7 ± 0.9

[a] Results are presented as mean ± SEM.

3. Results

3.1. Generation and molecular characterization of knockout mice

Our approach to creating gene knockout mice that specifically lack only the long splice variant of the $\gamma2$ subunit of the $GABA_A$-R is illustrated in Fig. 1. The net result of replacing the endogenous $\gamma2$ locus (Fig. 1A) with the replacement type targeting vector (Fig. 1B) is the replacement of a 0.6 kb XbaI fragment which includes the 24 bp exon 9 and associated splice signals with the bicistronic selectable marker gene (PGKneoN-TRtkpA). G418-resistant ES cell clones derived from electroporation of ES cells with the targeting construct were screened for homologous recombination at the $\gamma2$ locus by PCR (data not shown). PCR positive ES cell clones were analyzed in greater detail by Southern blot analysis to determine the fidelity of the targeting event. Of 1584 ES cell clones screened, seven had restriction fragment length polymorphisms indicative of gene targeting at the $\gamma2$ locus. Southern blot analysis of BglII digested genomic DNA from ES cell clones that harbor wild type $\gamma2$ alleles display a hybridizing band of ~9.2 kb (Fig. 1A, D). In contrast, in ES cell clones that have undergone replacement of the endogenous $\gamma2$ gene with the targeting vector, the probe also hybridizes to a ~12.6 kb BglII restriction fragment (Fig. 1C,D). Analysis with additional restriction enzymes as well as hybridization with different probes confirmed the structure of the targeted allele (data not shown).

The targeting strategy outlined in Fig. 1A–C was designed to be the first step of a two step double replacement gene targeting strategy (Wu et al., 1994). Our attempts to delete the selectable marker gene from the target locus with a second step targeting event were uniformly unsuccessful despite an extensive attempt with several different ES cell clones that each harbored the locus shown in Fig. 1C.

Correctly targeted ES cells were injected into recipient blastocysts and numerous highly chimeric mice were produced that transmitted the targeted $\gamma2$ allele to the ES cell-derived agouti offspring of the next generation. These $\gamma2L^{+/-}$ F_1 offspring appeared healthy and were indistinguishable from $\gamma2L^{+/+}$ littermates. However, breeding of $\gamma2L^{+/-}$ F_1 male mice revealed that many $\gamma2L^{+/-}$ males were sterile or had reduced fertility. This was not entirely unexpected since the selectable marker gene, which expresses neomycin phosphotransferase and herpes simplex virus thymidine kinase, is present in the genome of the mutant mice. Although expression of these marker genes is innocuous to most cell types, expression of thymidine kinase in spermatids can be lethal to those cells (Braun et al., 1990). A less plausible alternative explanation, which cannot be ruled out at this time, is that the $\gamma2L$ deletion itself is responsible for the reduced fertility of the male mice. Female $\gamma2L^{+/-}$ F_1 mice were of normal fertility when tested with $\gamma2L^{+/+}$ males.

To verify that the gene targeting event eliminated production of the $\gamma2L$ splice variant, RT-PCR analysis of cortical $\gamma2$ RNA was conducted. Using a 'spanning'

primer set that binds to mRNA sequences derived from exons 8 and 10 of the $\gamma 2$ gene (Fig. 2A), both the 291 bp $\gamma 2S$ and the 315 bp $\gamma 2L$ amplicons were readily detectable in RNA derived from $\gamma 2L^{+/+}$ mice (Fig. 2B). In contrast, only the $\gamma 2S$ mRNA was detectable in $\gamma 2L^{-/-}$ derived mRNA; no $\gamma 2L$ mRNA was detectable. Although this analysis was not designed to be quantitative, an apparent increase in $\gamma 2S$ message was noted in the $\gamma 2L^{-/-}$ sample compared to the $\gamma 2L^{+/+}$ sample (Fig. 2B). Using an 'exon 9-specific' primer set in which the 5′ primer binding site is located within exon 9, the readily detectable 135 bp amplicon that could be synthesized from $\gamma 2L^{+/+}$ cortical RNA was completely absent in PCR reactions from $\gamma 2L^{-/-}$ RNA (Fig. 2C). These results with the spanning and exon 9-specific primer sets establish that $\gamma 2$ exon 9-derived sequences are completely absent in homozygous $\gamma 2L$ knockout mice. To confirm that the gene targeting event did not prevent transcription of $\gamma 2$ sequences located 5′ or 3′ of exon 9, further RT-PCR analyses were conducted using 'upstream' and 'downstream' primer sets. In both $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ derived RNA, both the 231 bp upstream and the 114 bp downstream RT-PCR amplicons were readily detectable (Fig. 2D, 2E). All of these RT-PCR experiments were repeated with RNA isolated from cerebella with similar results (data not shown).

Radioligand binding studies with immunoprecipitated $GABA_A$-Rs confirm the absence of the $\gamma 2L$ subunit containing $GABA_A$-Rs in the $\gamma 2L^{-/-}$ mouse brain; as antibody specific to $\gamma 2L$ failed to immunoprecipitate [^{3}H]flunitrazepam binding sites from membrane prepared from mutant mice (Table 1). There were also significant differences between the immunoprecipitation values obtained with anti-$\gamma 2S$ antibodies in the $\gamma 2L^{-/-}$ mouse brain vs $\gamma 2L^{+/+}$ (Table 1); these studies suggest that there is ~ 2.4 fold upregulation of $\gamma 2S$ subunit containing $GABA_A$-Rs in the $\gamma 2L^{-/-}$ mouse brain compared to $\gamma 2L^{+/+}$ brain. The values obtained with sequential immunoprecipitations were approximately 15% higher than those obtained with a single immunoprecipitation (data not shown) as reported previously (Khan et al., 1994) but, the relative values obtained with cumulative immunoprecipitation were similar to the ones shown in Table 1.

In the mutant mouse brain, it seems that the $\gamma 2S$ subunit replaces $\gamma 2L$ because; (1) The immunoprecipitation values obtained with the anti-$\gamma 2$ antibody (which recognizes both $\gamma 2L$ and $\gamma 2S$) did not differ between $\gamma 2L^{+/+}$ mouse and $\gamma 2L^{-/-}$ mouse brain (Table 1) in spite of the absence of the $\gamma 2L$ subunit in the latter mice, and (II) the immunoprecipitation values obtained with anti-$\gamma 2S$ and anti-$\gamma 2$ antibodies in the $\gamma 2L^{-/-}$ mouse are similar to the summation values obtained with the $\gamma 2L$ and $\gamma 2S$ antibodies in $\gamma 2L^{+/+}$ mouse (79 and 60% vs. $32 + 33\%$, i.e., 65%).

Mice that completely lacked $\gamma 2L$ were viable, healthy, and indistinguishable from $\gamma 2L^{+/+}$ and $\gamma 2L^{+/-}$ littermates. Effect of the $\gamma 2L$ splice variant deletion on motor coordination was assessed by comparing the ability of $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice to maintain balance on an accelerating rotarod during consecutive single daily trials. As shown in Table 2, $\gamma 2L^{-/-}$ and $\gamma 2L^{+/+}$ mice did not differ in the ability to maintain balance on the rotarod during any of the daily trails.

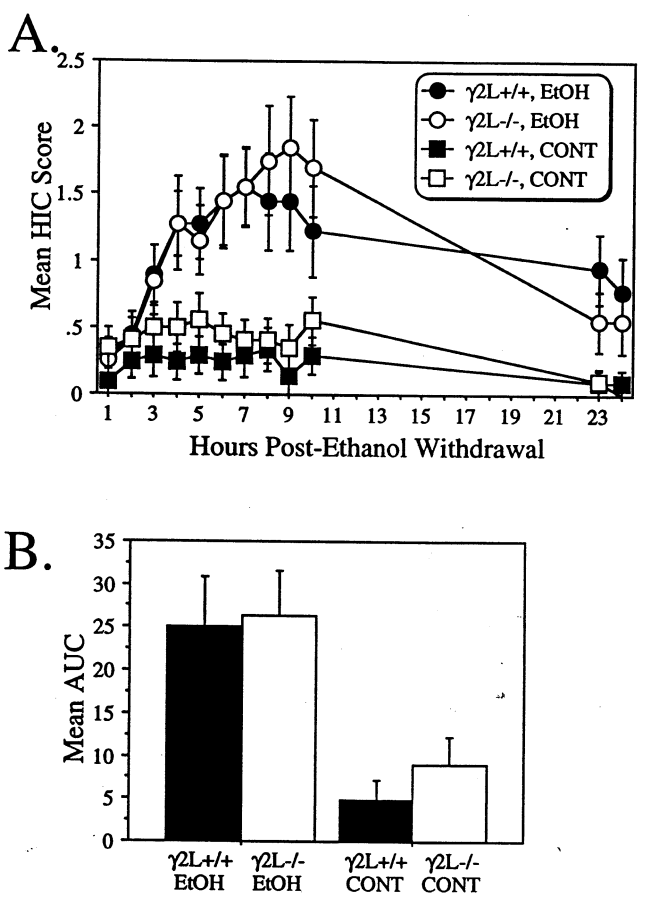

Fig. 5. Comparison of withdrawal hyperexcitability between wild type ($\gamma 2L^{+/+}$) and knockout ($\gamma 2L^{-/-}$) mice following chronic exposure to air (CONT) or ethanol vapors (EtOH). (A) Twenty four hour HIC scores. (B) Area under the 24 h HIC curves (mean units $\pm$ SEM). Effect of treatment was significant ($P < 0.0001$).

3.2. Electrophysiology

DRG neurons were chosen for study as postnatally they predominantly express $\gamma 2L$ containing $GABA_A$-Rs (Ma et al., 1993) and extensive work has been done on their GABA/ethanol pharmacology (Nakahiro et al., 1991). In experiments on $\gamma 2L^{-/-}$ mice, recordings from sensory neurons isolated from the DRG of 4–6-week-old mice showed no significant change in maximal GABA-activated chloride currents in $\gamma 2L^{-/-}$ mice relative to their $\gamma 2L^{+/+}$ littermates (Fig. 3A, C). Full concentration-response curves for GABA were con-

structed in neurons from both sets of animals (Fig. 3B). In cells from the $\gamma 2L^{+/+}$ animals, the EC_{50} for GABA was 55.8 ± 2.6 μM, with a Hill coefficient of 1.8 ± 0.1. In cells from the $\gamma 2L^{-/-}$ animals, the EC_{50} for GABA was 33.8 ± 2.6 μM, with a Hill coefficient of 1.8 ± 0.2. The EC_{50} values for GABA did not differ significantly between cells from $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ animals (unpaired t-test, $P = 0.18$). The effects of the benzodiazepine, midazolam, were studied on both sets of neurons to determine whether a γ subunit was expressed in the majority of neurons. Five micromolar midazolam enhanced the response to a EC_{20} concentration of GABA (10–30μM) by $147 \pm 24\%$ (n = seven neurons from three mice) in cells from the $\gamma 2L^{+/+}$ mice, and by $169 \pm 39\%$ (n = eight neurons from three mice) in cells from $\gamma 2L^{-/-}$ animals. This difference was not statistically significant.

We did however notice a significant increase in the degree of inhibition of GABA currents produced by 5μM zinc in neurons from $\gamma 2L^{-/-}$ animals. In cells from $\gamma 2L^{+/+}$ mice, 5μM zinc decreased GABA responses by $1.8 \pm 6.3\%$ (n = eight neurons from three mice), i.e., zinc had no effect. In constrast, in cells from $\gamma 2L^{-/-}$ mice a significant $27 \pm 9\%$ (n = seven neurons from three mice) inhibitory effect of zinc was observed. A high concentration of zinc chloride (500 μM), which would be expected to strongly inhibit both $\alpha\beta$ and $\alpha\beta\gamma$ $GABA_A$-Rs, had identical effects on neurons from both $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice: $89.5 \pm 8.6\%$ inhibition in $\gamma 2L^{+/+}$ mice (n = four neurons from two mice) compared to $79.0 \pm 7.0\%$ inhibition in $\gamma 2L^{-/-}$ mice (n = four neurons from two mice). The cells from $\gamma 2L^{-/-}$ mice were significantly more sensitive to inhibition by 5μM zinc than those from the $\gamma 2L^{+/+}$ animals ($P < 0.05$; Student's t-test). Inhibitory effects of such a low concentration of zinc are seen in experiments on recombinant $GABA_A$-Rs lacking the γ subunit (Draguhn et al., 1990). Although we did not obtain full concentration-response curves for zinc inhibition, this result might be taken to indicate the presence within DRG neurons from the $\gamma 2L^{-/-}$ animals of a population of zinc-sensitive receptors consisting only of $\alpha\beta$ subunits, co-existing with a large population of zinc-insensitive and midazolam-sensitive $\alpha\beta\gamma$ receptors. $\alpha\beta$ receptors have been described in DRG neurons from animals which are devoid of all $\gamma 2$ proteins (Gunther et al., 1995).

$GABA_A$-Rs are sensitive to modulation by alcohols, including ethanol (Nakahiro et al., 1991; Aguayo and Pancetti, 1994). In experiments with ethanol, we observed in DRG cells from control mice a modest but significant potentiation of the responses to submaximal GABA by concentrations of ethanol of 20 mM and above (Fig. 4A, C). However, as reported by others studying mouse sensory neurons (Aguayo and Pancetti, 1994), the degree and occurrence of potentiation was highly variable. An equal and variable degree of potentiation of GABA currents by ethanol was also observed in cells taken from $\gamma 2L^{-/-}$ animals (Fig. 4B, C). No significant differences were seen between cells from the two sets of animals at any concentration of ethanol. The results obtained in the knockout animals in this study are not consistent with an obligate role for the $\gamma 2L$ splice variant in the potentiation of receptor function by ethanol (Wafford et al., 1991). In fact, these results confirm that ethanol can enhance the action of submaximal GABA, independently of phosphorylation within the eight amino acid residue segment encoded by exon 9 of the $\gamma 2$ subunit gene. The present results clearly demonstrate in a neuronal context that native $GABA_A$-Rs which contain the $\gamma 2S$ subunit can be positively modulated by ethanol, as demonstrated in recombinant systems by others (Sigel et al., 1993; Marszalec et al., 1994; Mihic et al., 1994); indeed GABA responses in $GABA_A$-Rs consisting of only the α and β subunits have been demonstrated to be potentiated by ethanol (Mihic et al., 1994).

3.3. Ethanol induced ataxia, anxiolysis, hyperlocomotor activity, tolerance, and dependence are normal in $\gamma 2L^{-/-}$ mice

To establish that the $\gamma 2$ gene modification did not alter ethanol pharmacodynamics, we compared peak BEC and rate of clearance of ethanol from the systemic circulation following a single injection of ethanol. Neither of these parameters were found to differ between genotypes (Table 4), thus allowing valid comparisons of behavioral responses to ethanol.

The hypnotic effect of a single high dose of ethanol was compared using the sleep time assay. The duration of ethanol induced sleep was not different between $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice (55.9 ± 4.8, $n = 27$ vs. 62.6 ± 5.5 min $\pm$ SEM, $n = 24$, respectively).

The elevated plus maze has been validated for assessing anxiety as well as the anxiolytic and hyperlocomotor effects of a low dose of ethanol (Lister, 1987; Pellow et al., 1985). As shown in Table 3, basal locomotor activity as assessed by the total number of entries onto any arm of the maze did not significantly differ between genotypes. Anxiety, as assessed by both the percent of entries onto open arms and the percent of time spent on open arms was significantly influenced by genotype (Table 3). This modest but significant reduction in both parameters in the $\gamma 2L^{-/-}$ mice suggests that $\gamma 2L^{-/-}$ mice have higher levels of anxiety compared to $\gamma 2L^{+/+}$ mice. In $\gamma 2L^{+/+}$ and $\gamma 2L^{-/-}$ mice, ethanol significantly increased locomotor activity by $\sim 33\%$ (Table 3). The interaction of treatment and genotype was not significant indicating that mice of both genotypes responded equally to the stimulatory effects of ethanol on locomotor behavior. Ethanol also exerted a robust anx-

iolytic effect in the plus maze test increasing open arm entries and open arm time (Table 3). However, ethanol was equally effective in mice of both genotypes.

In the acute functional tolerance (AFT) test of Gallaher et al. (1982), the time to regain the ability to remain on a stationary rod following an initial injection of ethanol (t_1), BEC at t_1, time to regain the ability to remain on the rod following a second injection of ethanol (t_2), and AFT (defined as the difference in BEC at t_2 and BEC at t_1) did not differ between genotypes (Table 4).

Involvement of the γ2L splice variant on development of dependence to ethanol was assessed by comparing withdrawal hyperexcitability in $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ mice by scoring HIC following termination of chronic exposure to ethanol vapors. As shown in Table 5, BEC at the at the termination of the final ethanol exposure cycle did not differ between ethanol treated $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ mice. Initial body weight and percent body weight change also did not differ between groups (Table 5). Mean HIC curves for the 24 h period following the final treatment cycle are shown in Fig. 5A. Control treated mice of both genotypes had a low level of basal HIC activity that remained relatively constant during the period of observation. In contrast, ethanol treated mice of both genotypes displayed characteristic HIC that increased to a maximum at $\sim$6–8 h and subsequently returned to near baseline values by 24 h. Comparison of the area under the HIC curves (AUC) revealed a significant main effect of treatment (Fig. 5B); effect of genotype, and the interaction of genotype and treatment were not significant. Thus, the γ2L splice variant does not exert a significant influence on the development of dependence on ethanol as assessed by this assay.

4. Discussion

The gene knockout approach is providing fresh insights into the physiologic function of individual $GABA_A$-R subunits on development, behavior, epilepsy, and mechanisms of drug action (Gunther et al., 1995; Homanics et al., 1997b; Jones et al., 1997; Quinlan et al., 1998). In the present report the gene knockout approach was used to generate mice that completely and specifically lack the γ2L splice variant of the $GABA_A$-R. The amount of γ2S mRNA and protein appears to be present at an amount that is approximately equal to the combination of γ2S and γ2L that is present in $\gamma2L^{+/+}$ brain. This suggests that normal levels of receptors are being made unlike the $GABA_A$-R β3 knockout mouse (Homanics et al., 1997b). This also differs from the previously described γ2 (γ2L and γ2S) knockout where a substantial number of $GABA_A$-Rs lacking a γ subunit (i.e., $\alpha\beta$ receptors) are present (Gunther et al., 1995). The mice described here provide a unique opportunity to study the effects of deletion of the long splice variant of the γ2 subunit in a minimally perturbed system.

$GABA_A$-R function is known to modulate the emotional status of animals. Drugs such as diazepam that enhance GABA action are anxiolytic whereas drugs that inhibit GABA action are anxiogenic (Dalvi and Rodgers, 1996). $GABA_A$-R isoforms that contain the γ2 subunit are intimately involved in regulation of these behaviors; mice that have $\sim$50% reduction in the amounts of both γ2S and γ2L display signs of neophobia (Crestani et al., 1996). To test the effects of the γ2L gene knockout on anxiety, $\gamma2L^{-/-}$ mice were compared to $\gamma2L^{+/+}$ mice on the elevated plus-maze (Pellow et al., 1985Lister, 1987). Eventhough basal locomotor activity did not differ between genotypes, $\gamma2L^{-/-}$ mice demonstrated significantly increased levels of anxiety compared to $\gamma2L^{+/+}$ mice (Table 3). While this modest difference in anxiety is consistent with a change in $GABA_A$-R function, we cannot rule out the possibility that expression of the marker cassette that remains in the targeted locus influences the observed phenotype. Together, the results presented here and those of Crestani et al. (1996) strengthen the link between $GABA_A$-R isoforms containing the γ2 subunit and regulation of emotional status.

Considerable controversy has existed over the importance of the γ2L splice variant in mediating effects of ethanol (Wafford et al., 1991; Wafford and Whiting, 1992; Sigel et al., 1993; Marszalec et al., 1994; Harris et al., 1997). Nonetheless, since all prior investigations have utilized in vitro expression systems, it has not been possible to relate the results to in vivo measures of ethanol intoxication. Therefore, to test directly the role of the γ2L subunit in mediating the effects of ethanol, we compared $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ mice for ethanol induced responses at the cellular level employing electrophysiological techniques and at the whole animal level with several ethanol-induced behavioral endpoints. Electrophysiologically, ethanol at physiologically relevant concentrations (20–200 mM) potentiated GABA currents in neurons derived from both $\gamma2L^{+/+}$ and $\gamma2L^{-/-}$ animals to a similar degree. This was paralleled at the whole animal level, where behavioral analysis of ethanol induced sleep time, anxiolysis, acute functional tolerance, withdrawal hyperexcitability, and hyperlocomotor activity were all found to be unaffected by the γ2L knockout (Tables 3–5). These results argue against the γ2L subunit as a critical molecular site of action for ethanol potentiation of GABA action as well as whole animal sensitivity, tolerance, and dependence on ethanol.

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