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Epilepsy, Hyperalgesia, Impaired Memory, and Loss of Pre- and Postsynaptic $GABA_B$ Responses in Mice Lacking $GABA_{B(1)}$

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Summary

$GABA_B$ (γ-aminobutyric acid type B) receptors are important for keeping neuronal excitability under control. Cloned $GABA_B$ receptors do not show the expected pharmacological diversity of native receptors and it is unknown whether they contribute to pre- as well as postsynaptic functions. Here, we demonstrate that Balb/c mice lacking the $GABA_{B(1)}$ subunit are viable, exhibit spontaneous seizures, hyperalgesia, hyperlocomotor activity, and memory impairment. Upon $GABA_B$ agonist application, null mutant mice show neither the typical muscle relaxation, hypothermia, or delta EEG waves. These behavioral findings are paralleled by a loss of all biochemical and electrophysiological $GABA_B$ responses in null mutant mice. This demonstrates that $GABA_{B(1)}$ is an essential component of pre- and postsynaptic $GABA_B$ receptors and casts doubt on the existence of proposed receptor subtypes.

Introduction

$GABA_B$ receptors are the metabotropic receptors for the inhibitory neurotransmitter GABA. They activate second messenger systems and modulate potassium and calcium channel activity, thereby controlling presynaptic transmitter release and postsynaptic silencing of excitatory neurotransmission (Dutar and Nicoll, 1988; Bittiger et al., 1993; Lüscher et al., 1997; Marshall et al., 1999). On the basis of pharmacological differences, receptor heterogeneity between pre- and postsynaptic, as well as between auto- and heteroreceptors, on inhibitory and excitatory terminals, respectively, has been claimed. Some studies on neuronal $GABA_B$ receptors have predicted up to four distinct subtypes of $GABA_B$ receptors (Bonanno and Raiteri, 1993; Mott and Lewis, 1994) while others did not find any evidence for subtypes (Waldmeier et al., 1994). Two $GABA_B$ genes, each encoding several splice variants, have been identified (Marshall et al., 1999; Couve et al., 2000). Thus far, unique for a G protein-coupled receptor, $GABA_B$ function depends on dimerization of the two gene products (note: the NC-IUPHAR recommendation for nomenclature of $GABA_B$ receptors will be $GABA_{B(1x,2x)}$, where 1 and 2 refer to the subunits and x to the splice variants). The $GABA_{B(1)}$ subunit binds all known $GABA_B$ ligands whereas the $GABA_{B(2)}$ subunit appears important for surface trafficking and G protein coupling (Margeta-Mitrovic et al., 2000; Pagano et al., 2001; Calver et al., 2001). The expression pattern of the two cloned subunits matches the brain distribution of $GABA_B$ binding sites (Bischoff et al., 1999), and the heterodimeric $GABA_{B(1,2)}$ receptor was shown to activate all well-characterized $GABA_B$ effector pathways in transfected cells (Marshall et al., 1999). These findings, and the fact that cloning has failed to identify pharmacologically distinct receptor subtypes, led to speculations that the cloned subunits might be responsible for most, possibly all, $GABA_B$-mediated effects. Whether the pharmacological heterogeneity observed with native receptors relates to differences in the effector systems or to the existence of additional, as yet unidentified, $GABA_B$ receptor subtypes remains a key issue in the $GABA_B$ field. In that context, it is interesting to note that the $GABA_{B(2)}$ mRNA is exclusively expressed in neurons, while the $GABA_{B(1)}$ mRNA is localized to both neurons and glia (Clark et al., 2000). Moreover, while $GABA_{B(2)}$ mRNA is barely detectable in the rat caudate putamen, the mRNA for $GABA_{B(1)}$ is relatively abundant in this region. In support of a differential distribution of $GABA_{B(1)}$ and $GABA_{B(2)}$ protein, the pattern of immunoreactivity of the two proteins diverges in the rat striatum (Ng and Yung, 2001). These recent findings have further nourished speculations as to the existence of additional $GABA_B$ receptor proteins (Clark et al., 2000; Couve et al., 2000). It was hypothesized that $GABA_{B(1)}$ and $GABA_{B(2)}$ constitute functional receptors independent of each other, in association with proteins that are not necessarily part of the same gene family. An example for such an association are the calcitonin receptor-like receptors where coexpression of RAMPs, a family of single membrane spanning domain proteins, drastically alters the pharmacological profile of the G protein-coupled receptor (McLatchie et al., 1998). In heterologous cells, $GABA_{B(2)}$ couples to G proteins (Galvez et al., 2001) and trafficks to the cell surface in the absence of $GABA_{B(1)}$ (White et al., 1998). This suggested that in a neuronal context, $GABA_{B(2)}$ could form a receptor in its own right (Möhler and Fritschy, 1999). Given these spec-

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ulations on receptor subtypes, it is important to understand to which $GABA_B$ functions the cloned receptors contribute in vivo. To address this question, we generated $GABA_{B(1)}$-deficient mice and studied these in classical $GABA_B$ paradigms.

Results

Generation of $GABA_{B(1)}$-Deficient Balb/c Mice

$GABA_{B(1)}$-deficient ($GABA_{B(1)}^{-/-}$) mice were generated on the inbred Balb/c strain background. To this end, we carried out targeted mutagenesis of the $GABA_{B(1)}$ gene (Figures 1A and 1B) in Balb/c embryonic stem (ES) cells (Dinkel et al., 1999). $GABA_{B(1)}^{+/-}$ Balb/c ES cells were injected into C57BL/6 blastocysts and chimeric males were crossed with Balb/c females. This resulted in an F1 generation of inbred Balb/c $GABA_{B(1)}^{+/-}$ mutant mice, heterozygous only for the $GABA_{B(1)}$ mutant allele. The targeting vector contained a neomycin gene flanked by only 4.6 kb and 1.4 kb of 129Sv genomic sequences. Balb/c $GABA_{B(1)}^{+/-}$ mutant mice are therefore isogenic for all Balb/c genes, with the exception of 6 kb of the targeted $GABA_{B(1)}^{+/-}$ allele deriving from the 129Sv strain. In all the studies described below, we used age-matched $GABA_{B(1)}^{+/+}$, $GABA_{B(1)}^{+/-}$, and $GABA_{B(1)}^{-/-}$ mice from litters derived from Balb/c $GABA_{B(1)}^{+/-} \times$ Balb/c $GABA_{B(1)}^{+/-}$ breeding pairs. The behavioral, biochemical, and electrophysiological changes described below therefore reflect the consequences of ablating $GABA_{B(1)}$ in the Balb/c strain background. Mice lacking either one or both $GABA_{B(1)}$ alleles were viable and occurred at a Mendelian ratio. No $GABA_{B(1)}$ mRNA was detected in $GABA_{B(1)}^{-/-}$ mice by Northern blot (not shown) and in situ hybridization analysis (Figure 1C).

Loss of Detectable $GABA_B$ Binding Sites and GABA-Induced GTPγ[^{35}S] Binding in $GABA_{B(1)}^{-/-}$ Mice

[^{3}H]CGP54626 receptor autoradiography (Bischoff et al., 1999) and [^{125}I]CGP71872 photoaffinity labeling (Kaupmann et al., 1997) revealed a complete lack of antagonist binding sites in $GABA_{B(1)}^{-/-}$ brain, and obvious reduction of binding sites in $GABA_{B(1)}^{+/-}$ versus $GABA_{B(1)}^{+/+}$ animals (Figures 1C and 1D). Neither could we detect $GABA_{B(1)}$ proteins by immunoblot analysis of $GABA_{B(1)}^{-/-}$ brain extracts (Figure 1E). We have deleted exons 7–10 (T461-I621; Kaupmann et al., 1997) in the $GABA_{B(1)}^{-/-}$ animals, thereby removing part of the GABA binding domain (Galvez et al., 2000a) and the first transmembrane domain. Since we did not detect any truncated $GABA_{B(1)}$ protein using antibodies directed at extreme N- or C-terminal epitopes of $GABA_{B(1)}$, any dominant-negative effects mediated by a possible residual $GABA_B$ protein in the null mutant mice is unlikely. Saturation binding experiments with the antagonist [^{125}I]CGP64213 (Kaupmann et al., 1997) revealed a lack of binding sites in null mutant and a 18% decrease in $GABA_{B(1)}^{+/-}$ versus $GABA_{B(1)}^{+/+}$ animals (Figure 2A). A lack of binding sites was also apparent in agonist binding studies using [^{3}H]APPA (Kaupmann et al., 1997) and [^{3}H]baclofen (Figure 2B). In agreement with the results obtained with whole brain extracts, we did not detect any [^{125}I]CGP64213, [^{3}H]APPA, and [^{3}H]baclofen binding sites using cortex and cerebellum $GABA_{B(1)}^{-/-}$ tissue samples (data not shown).

To investigate whether $GABA_{B(1)}^{-/-}$ brains still expressed functional $GABA_B$ receptors, we used GTPγ[^{35}S] binding. This assay is able to detect any $GABA_B$ receptor coupled to $G\alpha_{i/o}$-type G proteins, the known effectors of native $GABA_B$ receptors (Galvez et al., 2000b). Regardless of whether the endogenous agonist GABA or baclofen was used for stimulation, whole brain (Figure 2C), cortex, and cerebellum (data not shown) $GABA_{B(1)}^{-/-}$ membranes showed no residual $GABA_B$ receptor activity. This demonstrates the absence of detectable functional $GABA_B$ receptors.

$GABA_{B(2)}$ Protein Is Downregulated in $GABA_{B(1)}^{-/-}$ Mice

Given the heterodimeric nature of functional $GABA_B$ receptors, we analyzed the expression of the $GABA_{B(2)}$ subunit. $GABA_{B(2)}$ mRNA expression levels and distribution in brain were unchanged in $GABA_{B(1)}^{-/-}$ mice (Figure 1C). However, $GABA_{B(2)}$ protein was almost undetectable when using different $GABA_{B(2)}$-specific antibodies (Figure 1E). This requirement of $GABA_{B(1)}$ for stable $GABA_{B(2)}$ expression supports that in wild-type mice, virtually all $GABA_{B(2)}$ protein is associated with $GABA_{B(1)}$, in agreement with previous biochemical studies (Benke et al., 1999). A similar crossregulation of protein expression was observed in knockout mice with other multisubunit proteins, e.g., the Kir3 channels (Slesinger et al., 1997) or the asialoglycoprotein receptor (Tozawa et al., 2001).

Loss of Pre- and Postsynaptic $GABA_B$ Responses in $GABA_{B(1)}^{-/-}$ Mice

Electrophysiological studies have established the presence of presynaptic $GABA_B$ hetero- and autoreceptors (Thompson et al., 1993; Wu and Saggau, 1997). First, we examined whether functional heteroreceptors were present in the hippocampus (Figure 3A). In CA1 pyramidal neurons from wild-type, but not in $GABA_{B(1)}^{-/-}$ mice, application of baclofen evoked the expected marked depression of excitatory postsynaptic currents (EPSCs) induced by stimulation in the Schaffer collateral-commissural area. Activation of A1 receptors by adenosine (Proctor and Dunwiddie, 1987; Thompson et al., 1992) reduced the EPSCs to the same extent in both genotypes, demonstrating that inhibition of glutamate release by a G protein-coupled receptor other than $GABA_B$ is still intact in $GABA_{B(1)}^{-/-}$ mice (Figure 3B). Next, we examined autoreceptor-mediated responses to baclofen in inhibitory interneurons. We recorded monosynaptic inhibitory postsynaptic currents (IPSCs) in CA1 pyramidal neurons in the presence of ionotropic glutamate receptor antagonists kynurenate or CNQX (Figure 3C). Baclofen was unable to inhibit IPSCs in $GABA_{B(1)}^{-/-}$ mice, showing that in contrast to wild-type mice, no autoreceptors were operational. This effect was specific to $GABA_B$ receptors since depression of IPSCs following application of the μ-opioid receptor agonist DAMGO (Cohen et al., 1992; Capogna et al., 1993) was equally effective in both genotypes. Postsynaptic $GABA_B$ receptors activate a potassium conductance underlying the late IPSP (Lüscher et al., 1997), which is partly or completely suppressed by $GABA_B$ antagonists (Bittiger et al., 1993). In $GABA_{B(1)}^{-/-}$

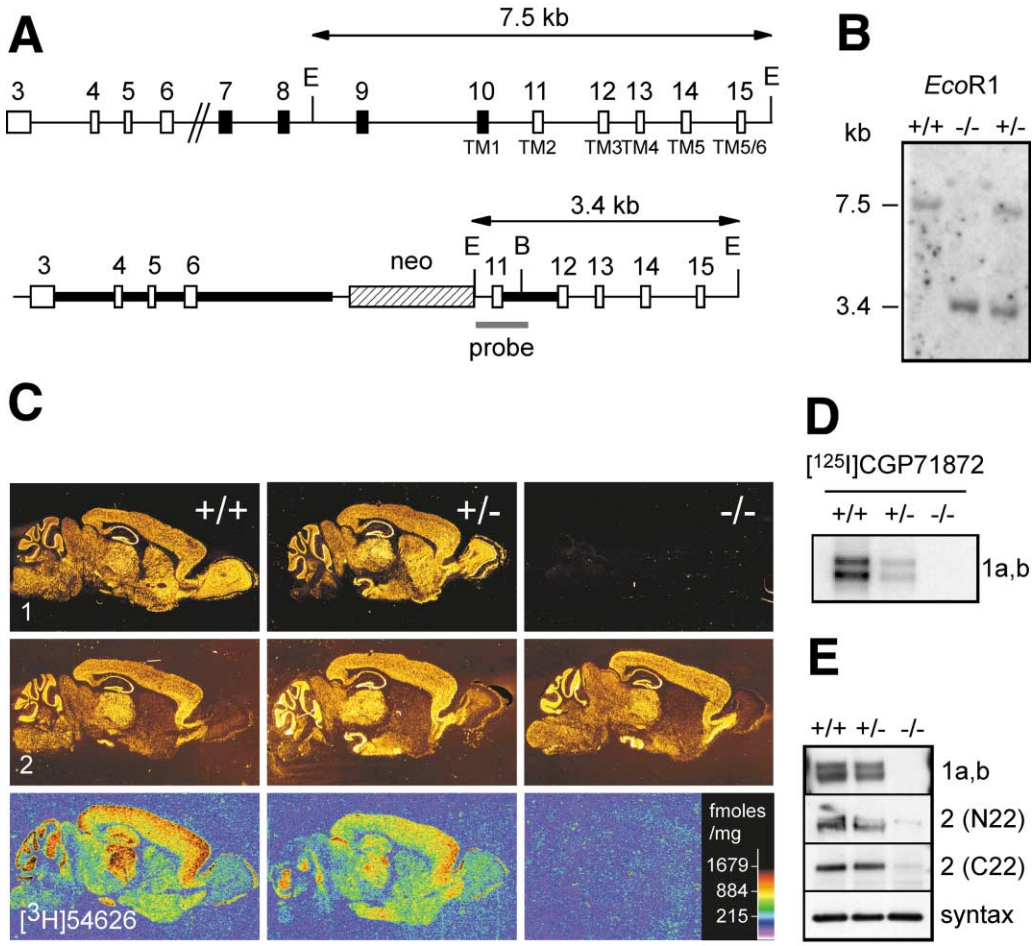

Figure 1. Generation and Characterization of $GABA_{B(1)}$-Deficient Mice

(A) Top line, $GABA_{B(1)}$ locus encompassing exons 3 to 15 (boxes), encoding part of the N-terminal and transmembrane (TM) domains 1–6. Bottom line, mutant allele after homologous recombination. The targeting vector contains a neomycin resistance marker (neo) flanked by 4.6 and 1.4 kb of 129Sv DNA (bold line). The 8.5 kb deletion removes exons 7, 8, 9, 10 (black boxes). The probe used in Southern blot analysis is indicated. E, EcoRI, B, BamHI. Balb/c embryonic stem cells were injected into C57BL/6 blastocysts. Several chimeric males yielded Balb/c ES cell-derived germline transmission after mating with Balb/c females, thereby insuring transmission and propagation of the mutant allele in the inbred Balb/c background.

(B) Southern blot of EcoR1 digested tail DNA from wild-type (+/+), null mutant (−/−), and heterozygous (+/−) mice.

(C) In situ hybridization analysis of $GABA_{B(1)}$ (1, top row) and $GABA_{B(2)}$ (2, middle row) transcripts. The $GABA_{B(1)}$ probe detects $GABA_{B(1a)}$ and $GABA_{B(1b)}$ variant transcripts. [^{3}H]CGP54626 receptor autoradiography (Bischoff et al., 1999) (bottom row) represents specific binding after subtraction of nonspecific binding in the presence of excess baclofen (10^{-5} M). Pseudocolors reflect densities of binding sites as defined (inset).

(D) SDS-PAGE and autoradiography of brain extracts with [^{125}I]CGP71872 photoaffinity-labeled $GABA_{B(1a)}$ (1a) and $GABA_{B(1b)}$ (1b) proteins (Kaupmann et al., 1997).

(E) Immunoblot of brain extracts using antibodies directed at N-terminal $GABA_{B(1)}$ (antibody 176; Kaupmann et al., 1998) and C- and N-terminal $GABA_{B(2)}$ (antibody C22, N22) (Kaupmann et al., 1998) epitopes. An antibody directed at a C-terminal epitope of $GABA_{B(1)}$ (antibody 174.1; Malitschek et al., 1998) does not recognize any full-length or truncated $GABA_{B(1)}$ protein in $GABA_{B(1)}^{-/-}$ mice either (data not shown). Equal loading of samples was controlled with anti-syntaxin antibodies (Sigma, St. Louis, MO).

CA1 pyramidal cells, baclofen had no effect on the holding current (nor on the input resistance, data not shown), indicating the absence of $GABA_B$ receptors (Figure 3D). In contrast, when applied for the same period of time to a wild-type cell, baclofen elicited an outward current (Figure 3D) and a concomitant drop of the input resistance (data not shown). Adenosine A1 receptor activation elicited outward currents that were statistically equal in magnitude when comparing $GABA_{B(1)}^{-/-}$ and wild-type mice (Figures 3D and 3E). These experiments were repeated several times for both baclofen and adenosine (Figure 3E). Baclofen-induced outward currents were never detected in CA1 pyramidal cells of $GABA_{B(1)}^{-/-}$ mice. In conclusion, electrophysiology in hippocampal slices shows the absence of $GABA_B$ auto- or heteroreceptors and postsynaptic $GABA_B$ receptors in $GABA_{B(1)}^{-/-}$ mice.

Spontaneous Epileptiform Activity in $GABA_{B(1)}^{-/-}$ Mice

The clinically effective muscle-relaxant baclofen is known to induce hypothermia, while $GABA_B$ antagonists

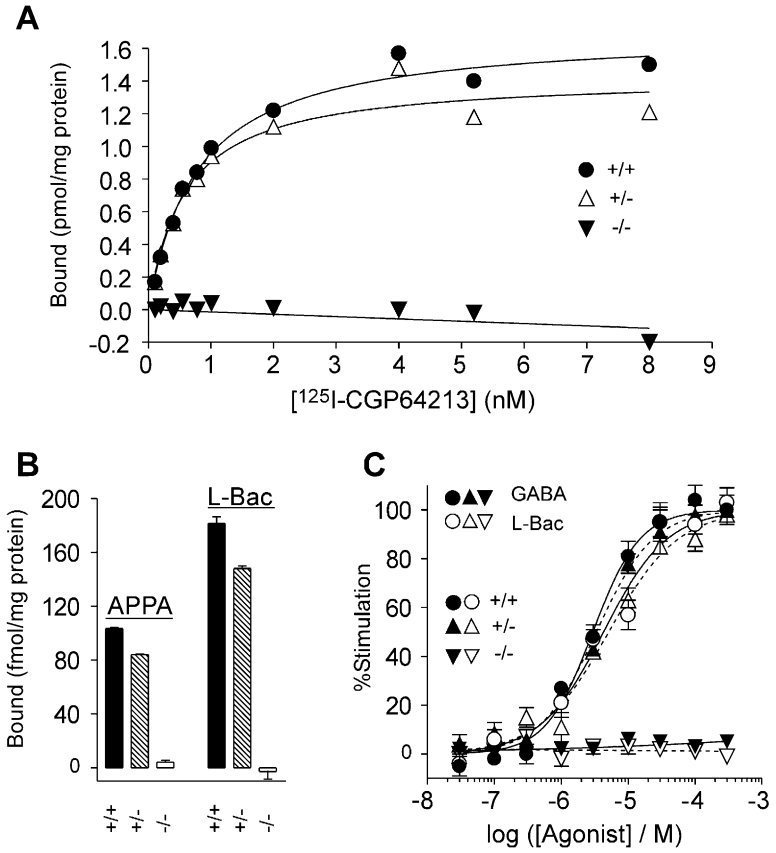

Figure 2. Pharmacological Analysis of Total Brain Membranes from $GABA_{B(1)}$-Deficient Mice

(A) Saturation curves of [^{125}I]CGP64213 antagonist binding. No specific binding is detected in $GABA_{B(1)}^{-/-}$ brain. The maximal numbers of binding sites (B_{max}) determined for $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{+/-}$ are 1.7 ± 0.02 and 1.2 ± 0.1 pmol/mg protein, K_d values are 0.84 ± 0.03 and 0.58 ± 0.06 nM, respectively (mean ± SEM, $n = 3$).

(B) No significant binding of the agonists [^{3}H]APPA (10 nM) and [^{3}H]baclofen (30 nM) is observed in $GABA_{B(1)}^{-/-}$ mice.

(C) No significant GABA (closed symbols, filled lines) or baclofen (open symbols, dotted lines) stimulated GTPγ[^{35}S] binding is detected in $GABA_{B(1)}^{-/-}$ membranes. Values were normalized to the maximal response obtained with wild-type mice. Error bars indicate SEM. Results from typical experiments performed in triplicate are shown.

at high doses can be proconvulsive and increase locomotion (Badran et al., 1997). In line with some of the effects of $GABA_B$ drugs, $GABA_{B(1)}^{-/-}$ mice exhibited overt and characteristic behavioral abnormalities. Adult $GABA_{B(1)}^{-/-}$ mice were hyperactive, displayed sporadic episodes of intensive running, and showed regularly spontaneous epileptiform activity. We continuously recorded the EEG in freely moving adult mice using implanted electrodes. During a two-week observation period, adult $GABA_{B(1)}^{-/-}$ mice generally displayed several episodes of spontaneous clonic seizures a day. These seizures lasted several seconds up to a minute. Occasionally, absence-type seizures that included phases of 3–5 Hz spike and wave discharges were observed (Figure 4A). Sporadically, tonic-clonic seizures occurred in $GABA_{B(1)}^{-/-}$ mice (Figure 4A). The analysis of five $GABA_{B(1)}^{-/-}$ animals over three randomly picked days (24 hr observation) shows that the mice had on average 5.0 (9/5/1), 0.3 (0/1/0), 3.6 (3/7/1), 5.0 (5/8/2), and 2.0 (6/0/0) seizures per day. Almost all seizures were of the clonic type. Absence-type seizures and spontaneous tonic-clonic seizures occurred rarely (< once daily) and not in every animal. Tonic-clonic seizures were reliably induced in most animals by audiogenic stimuli, e.g., rattling keys. Epileptiform activity was never observed in heterozygous or wild-type littermates. The occurrence of seizures in $GABA_{B(1)}^{-/-}$ mice may be explained by a loss of control over neuronal excitability, owing to the absence of tonic or phasic inhibition (missing late IPSP). Injection of baclofen induced an expected delta wave pattern (1–4 Hz) in the EEG of wild-type (Olpe et al., 1980) but not of $GABA_{B(1)}^{-/-}$ mice (Figure 4B), in agreement with a lack of $GABA_B$ activity.

$GABA_{B(1)}^{-/-}$ Mice Exhibit Hyperlocomotor Activity

Locomotor activity of $GABA_{B(1)}^{-/-}$ and wild-type mice was quantified in a cylindrical chamber using the Ethovision recording system. During a 2 hr observation period, $GABA_{B(1)}^{-/-}$ mice moved with significantly increased velocity over longer distances, as compared with wild-type littermates, suggesting that $GABA_B$ receptors exert a tonic brake on locomotion (Figure 5A). In the rotarod test, null mutant mice showed no loss of motor coordination in response to baclofen (Figure 5B). This sharply contrasted with the expected muscle relaxation seen in control littermates. Finally, $GABA_{B(1)}^{-/-}$ mice exhibited no baclofen-induced hypothermia (Bittiger et al., 1993; Badran et al., 1997), further indicating the absence of $GABA_B$ receptors (Figure 5C).

$GABA_{B(1)}^{-/-}$ Mice Are Hyperalgesic

$GABA_B$ agonists are antinociceptive in models of acute and chronic pain (Sawynok, 1987; Patel et al., 2001). It is suggested that $GABA_B$-mediated mechanisms in the dorsal horn of the spinal cord exert a tonic control of nociceptive inputs from primary afferent fibers to spinothalamic tract neurons (Lin et al., 1996). We used the hot-plate, tail-flick, as well as the paw pressure techniques to characterize acute pain behaviors (Walker et al., 1999). The tail-flick is a reflex response to a noxious thermal stimulus applied to the tail and is generally taken to represent a spinal reflex response, while the hot-plate

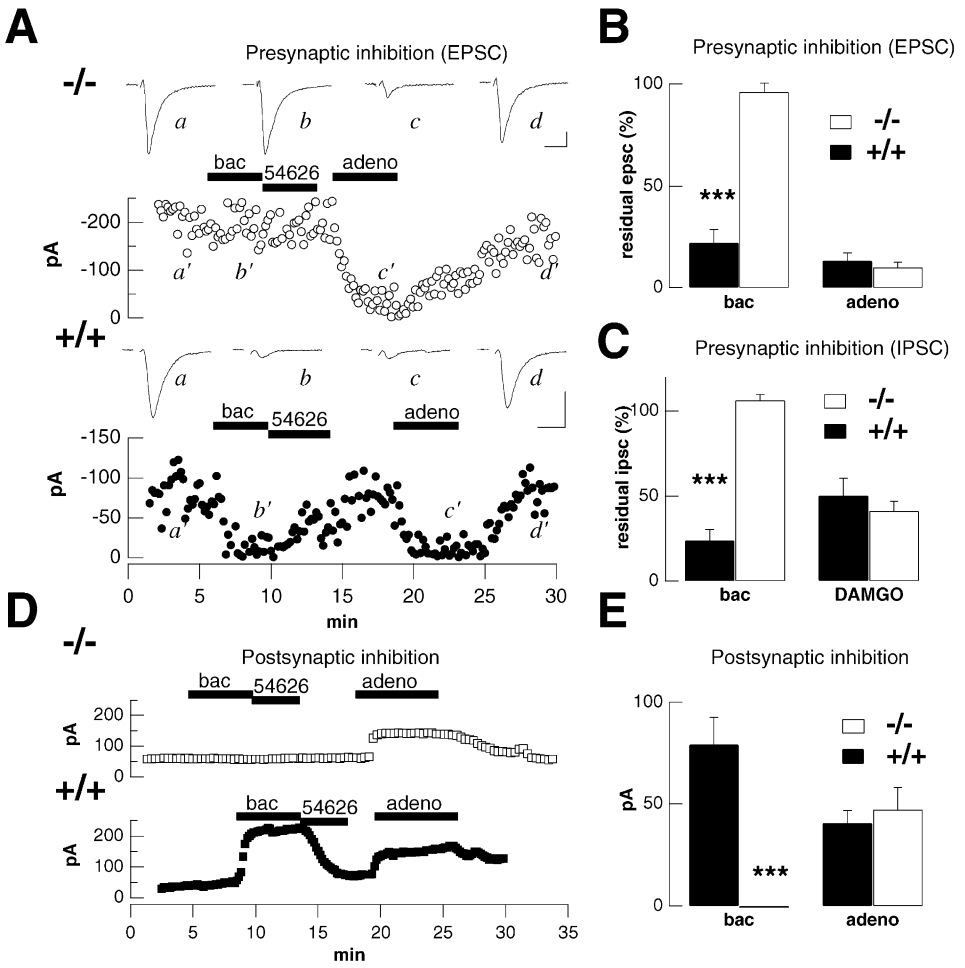

Figure 3. Absence of Baclofen-Induced Pre- and Postsynaptic Inhibition in CA1 Pyramidal Cells of $GABA_{B(1)}$-Deficient Mice

(A) EPSC peak amplitudes and representative traces from a $GABA_{B(1)}^{-/-}$ mouse (top, open circles) showing unchanged amplitudes during baclofen (50 μM) or CGP54626A (1 μM) application but substantial depression with adenosine (100 μM). In $GABA_{B(1)}^{+/+}$ mice (bottom, filled circles), both baclofen and adenosine depressed EPSCs. Current traces (*a-d*) show averages of ten synaptic responses at indicated time-points (scale bar 10 ms/20 pA).

(B) Summary bar graph. EPSC amplitude during baclofen application was 96 ± 3% of baseline ($p > 0.2$ for a hypothetical mean of 100%) in mutant mice, but significantly reduced in wild-types. No differences between genotypes were observed for adenosine.

(C) Summary graph of monosynaptic IPSC measurements. Comparison of the residual IPSC amplitude during the application of baclofen or the μ-opioid agonist [D-Ala^2, NMe-Phe^4, Gly^5-ol]-enkephalin (DAMGO, 1 μM). No significant depression was observed for baclofen in $GABA_{B(1)}^{-/-}$ mice. Depression induced by DAMGO was similar in both genotypes. Measurements were in the presence of kynurenate (2 mM) or CNQX (10 μM).

(D) Postsynaptic inhibition. Changes in the holding current in response to baclofen and adenosine of a representative CA1 pyramidal cell in voltage clamp ($V_h = -50$ mV). In the $GABA_{B(1)}^{-/-}$ mouse (top, open squares), neither baclofen nor CGP54626A induced an outward current, in contrast to adenosine. In a wild-type mouse (bottom, filled squares), both agonists reliably elicited outward currents.

(E) Summary graph of all cells examined. The amplitude of the outward current caused by the application of baclofen was absent in $GABA_{B(1)}^{-/-}$ mice (−1.5 ± 1.4 pA) while the amplitude of adenosine elicited currents did not differ significantly. (A–E) n = 5–10 for each agonist and genotype, mean ± SEM, $p^{***} < 0.0001$.

response to a noxious thermal stimulus to the plantar surface of the paws is thought to involve supraspinal sites. In these nociceptive tests, $GABA_{B(1)}^{-/-}$ mice showed pronounced hyperalgesia to noxious heat in the hot-plate (Figure 6A) and tail-flick (Figure 6B) tests and reduced paw withdrawal thresholds to mechanical pressure (Figure 6C). From these data, it is likely that $GABA_B$-mediated effects do indeed exert a tonic control of nociceptive processes in the naïve animal. The sites for this action are expected to be both spinal and supraspinal, although further experiments are needed to confirm this.

$GABA_{B(1)}^{-/-}$ Mice Are Impaired in Passive Avoidance Learning

In view of evidence that the effects of $GABA_B$ antagonists on memory processes may be facilitatory (Mondadori et al., 1996; Getova et al., 1997; Castellano et al., 1993) or inhibitory (Castellano et al., 1993; Brucato et al., 1996; Saha et al., 1993), it was of interest to investigate memory performance in the passive avoidance test (Venable and Kelly, 1990). The results shown in Figure 7 indicate that the genotype of $GABA_{B(1)}^{-/-}$ and $GABA_{B(1)}^{+/-}$ mice had a marked negative effect on memory performance.

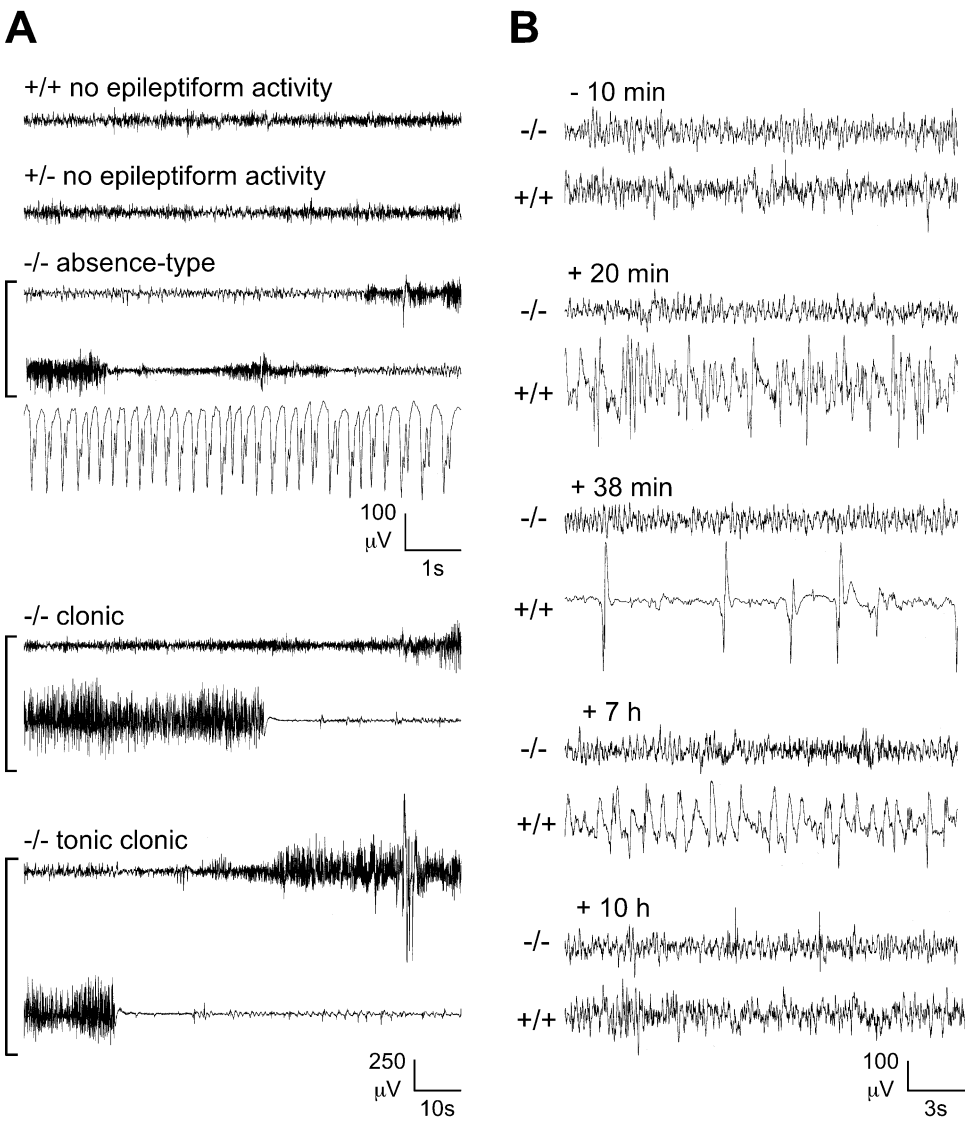

Figure 4. Spontaneous Epileptiform Activity in $GABA_{B(1)}$-Deficient Mice

(A) Traces from continuous EEG recorded for 2 weeks in groups of freely moving mice. Top two traces are EEGs recorded from a wild-type $GABA_{B(1)}^{+/+}$ and a heterozygous $GABA_{B(1)}^{+/-}$ mouse without epileptiform activity, lower traces from a $GABA_{B(1)}^{-/-}$ mouse during absence-type, clonic, and tonic-clonic seizures. Generally, absence-type seizures included 3–5 Hz spike-wave (see enlarged separate trace) and slow-wave EEG patterns. During absence-type seizures, the mice were motionless but sometimes exhibited slight head nodding. The length of the observed seizures and the EEG pattern is indicative of "atypical" rather than "typical" absence seizures. Clonic seizures were characterized by rearing and bilateral clonus of the forelimbs. During tonic-clonic seizures, typically lasting 10–20 s, the mice exhibited a tonic extension of the fore- and hindlimbs, followed by synchronous clonic twitches of the hindlimbs. In some animals, tonic-clonic seizures were preceded by wild running or jumping. The main features of the tonic-clonic seizures observed in $GABA_{B(1)}^{-/-}$ mice are comparable to the type of seizure seen in mice after maximal electroshock. Tonic-clonic seizures occurred rarely spontaneously. The tonic-clonic seizure shown was provoked by audiogenic stimulation (rattling keys). The experimental groups were composed of mixed sex (wild-type, 4 female/2 male; heterozygote, 6 female/2 male; null mutant, 4 female/1 male). Brackets delineate continuous EEG traces.

(B) After the 2 weeks of recording, $GABA_{B(1)}^{-/-}$ and $GABA_{B(1)}^{+/+}$ mice were given baclofen (6 mg/kg, i.p.). The EEG of wild-type and $GABA_{B(1)}^{-/-}$ mice was comparable 10 min prior to baclofen application (−10 min). However, 20 min after baclofen application, delta waves appeared in the EEG of wild-type but not in $GABA_{B(1)}^{-/-}$ mice (+20 min). 30–90 min after baclofen application, wild-type mice typically showed body jerks and single spikes in the EEG (+38 min). Later on, delta waves reappeared in the EEG and lasted for several hours (+7 hr). Ten hours after baclofen administration, the EEG traces of wild-type and $GABA_{B(1)}^{-/-}$ mice are again similar (+10 hr).

Two-factor (sex, genotype) analysis of variance (ANOVA) on latency to enter the dark (shock) compartment on the training trial showed that the only significant effect was that of sex ($F(2,48) = 6.5$, $p < 0.05$), the female animals entering this compartment with a slightly longer latency. On retention test latencies, the index of memory performance, there was a significant effect of genotype ($F(2,37) = 30.7$, $p < 0.001$), but no effect of sex and no sex/genotype interaction (both $p > 0.5$). Therefore, to perform multiple comparisons between wild-type, heterozygote, and homozygote mice, one-factor (genotype) ANOVA on retention latencies was performed. This con-

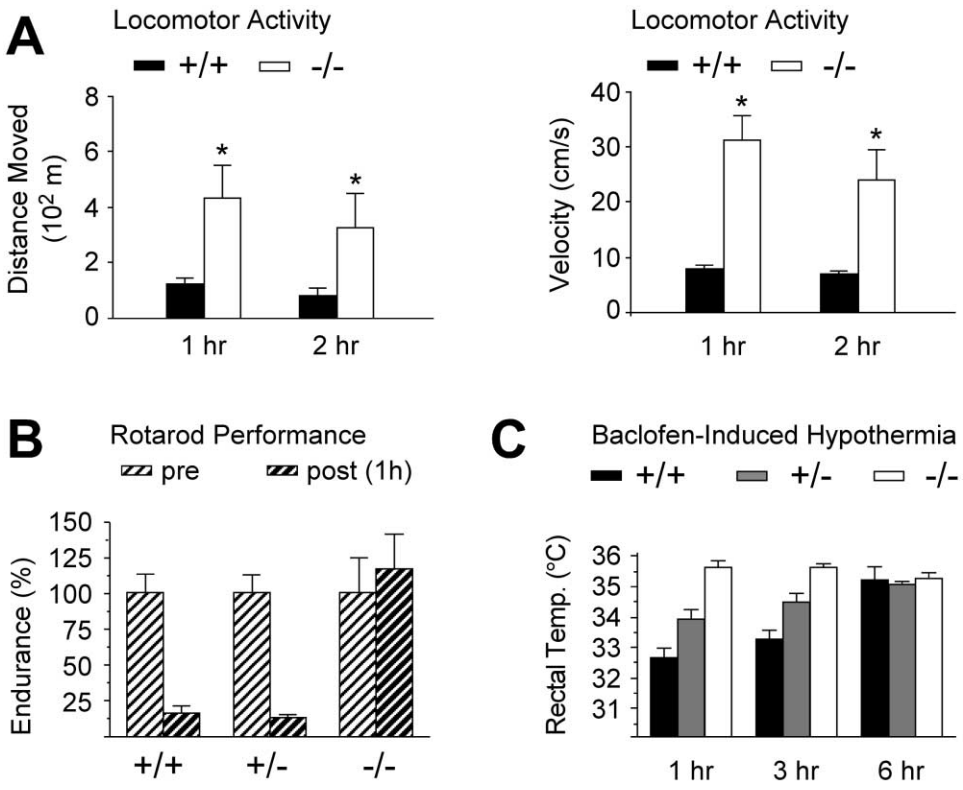

Figure 5. Behavioral Analysis of $GABA_{B(1)}$-Deficient Mice

(A) Hyperlocomotor activity in $GABA_{B(1)}^{-/-}$ mice measured with Ethovision. *P $\leq$ 0.05 t test (mean $\pm$ SEM).

(B) Rotarod (12 rpm) endurance performance (mean $\pm$ SEM) before (pre) and 1 hr after (post) injection of baclofen (12.5 mg/kg, p.o.).

(C) No baclofen-induced hypothermia in $GABA_{B(1)}^{-/-}$ mice. Rectal body temperature (mean $\pm$ SEM) 1, 3, and 6 hr after baclofen application (12.5 mg/kg, p.o.).

firmed the effect of genotype ($F(2,40) = 33.4$, $p < 0.001$) and showed that $GABA_{B(1)}^{-/-}$ differed from both $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{+/-}$ mice (Tukey's test, $p < 0.001$, in both cases), and that $GABA_{B(1)}^{+/+}$ and $GABA_{B(1)}^{+/-}$ also differed significantly from each other (Tukey's test, $p < 0.05$). Our data indicate that these passive avoidance deficits are a reflection of impaired memory processes and not merely due to some other behavioral change such as locomotor hyperactivity. Thus, on the training day, there was no evidence from the ANOVA that in the passive avoidance apparatus, the $GABA_{B(1)}^{-/-}$ and $GABA_{B(1)}^{+/-}$ animals had faster latencies to enter the dark compartment than wild-type animals. To increase the chance of detecting such a difference, multiple t tests were performed without employing any correction for multiple comparisons. These all showed no difference to wild-type animals (t tests: $GABA_{B(1)}^{-/-}$ versus $GABA_{B(1)}^{+/+}$ female, $p > 0.5$; male, $p > 0.5$; pooled sexes, $p > 0.8$; $GABA_{B(1)}^{+/-}$ versus $GABA_{B(1)}^{+/+}$ female, $p > 0.3$; male, $p > 0.1$; pooled sexes, $p > 0.9$). To be sure that this was also true for the animals that took part in the retention test, we also compared training latencies of only those animals whose retention latencies were measured (i.e., excluding animals that exceeded the 150 s cutoff criterion on the training trial). Two-factor (sex, genotype) ANOVA in this case showed no significant effects (genotypes $p > 0.5$; sex $p > 0.1$; interaction $p > 0.7$). Again, comparisons of individual groups to wild-type animals showed no differences in latencies (t tests: $GABA_{B(1)}^{-/-}$ versus $GABA_{B(1)}^{+/+}$ female, 74.6 $\pm$ 17.8 versus 80.0 $\pm$ 21.5 s, $p > 0.8$; male, 44.4 $\pm$ 22.8 versus 46.4 $\pm$ 12.5 s, $p > 0.9$; pooled sexes, 64.0 $\pm$ 14.1 versus 61.3 $\pm$ 12.1 s, $p > 0.8$; $GABA_{B(1)}^{+/-}$ versus $GABA_{B(1)}^{+/+}$ female, 83.7 $\pm$ 19.1 versus 80.0 $\pm$ 21.5 s, $p > 0.8$; male, 38.9 $\pm$ 5.1 versus 46.4 $\pm$ 12.5 s, $p > 0.5$; pooled sexes, 62.5 $\pm$ 11.4 versus 61.3 $\pm$ 12.1 s, $p > 0.9$).

Discussion

The overt phenotype of the $GABA_{B(1)}^{-/-}$ mice includes spontaneous epileptic seizures. Given that $GABA_B$ antagonists are effective anti-absence drugs in a strain of rats (GAERS) with genetic absence epilepsy (Marescaux et al., 1992), the appearance of absence-type seizures in the $GABA_{B(1)}^{-/-}$ mice is somewhat of a surprise. However, the absence-type seizures seen in the $GABA_{B(1)}^{-/-}$ mice are not comparable to the "typical" absence seizures observed in the GAERS. The seizures in the GAERS are characterized by frequent and short EEG bursts, while the ones seen in the $GABA_{B(1)}^{-/-}$ mice are rare, of much longer duration, and indicative of "atypical" absence seizures. The experimental $GABA_{B(1)}^{-/-}$ mice were from two litters that were born five days apart. The animals were tested starting 19–20 weeks after birth. We did not yet record EEGs from younger or older animals. It will

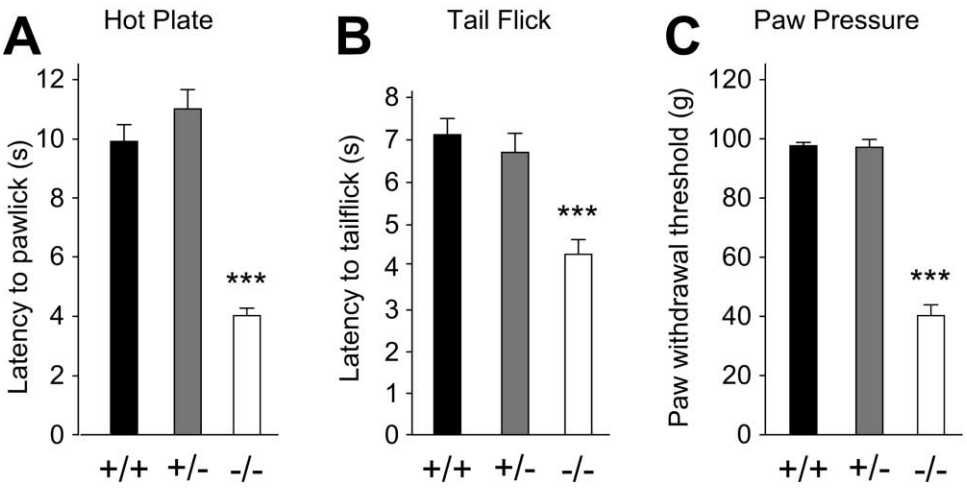

Figure 6. Assessment of Nociceptive Behavior in Wild-Type and Mutant Mice

(A) Hot-plate test (supraspinal thermal nociception). $GABA_{B(1)}{}^{-/-}$ versus $GABA_{B(1)}{}^{+/+}$ or $GABA_{B(1)}{}^{+/-}$ mice show significant difference in latency (*** $p < 0.001$). Response latencies for paw lick were assessed at 55°C.

(B) Tail-flick test (spinal thermal nociception). $GABA_{B(1)}{}^{-/-}$ versus $GABA_{B(1)}{}^{+/+}$ or $GABA_{B(1)}{}^{+/-}$ mice showed significantly reduced tail-flick latency (*** $p < 0.001$). Response latencies were assessed at infrared intensity 14 (see Experimental Procedures).

(C) Paw pressure test (mechanical nociception). $GABA_{B(1)}{}^{-/-}$ mice in comparison to $GABA_{B(1)}{}^{+/+}$ or $GABA_{B(1)}{}^{+/-}$ mice showed significantly reduced paw withdrawal latency (*** $p < 0.001$). Withdrawal thresholds of the left hind paw were assessed. All experimental groups were composed of mixed sex (wild-type, 8 female/10 male; heterozygote, 10 female/9 male; null mutant, 8 female/6 male). Nociception tests were analyzed with Tukey's HSD test. In all tests, there were no significant differences in the behavior of wild-type and heterozygous animals or between males and females. During nociceptive tests, two animals had seizures and three others vocalized in the $GABA_{B(1)}{}^{-/-}$ group.

be important to determine the exact postnatal time-of-onset of the different seizure types observed in $GABA_{B(1)}{}^{-/-}$ mice, and to see whether their appearance is coincidental or sequential. It is also of interest to see whether continuous spontaneous seizure activity in $GABA_{B(1)}$-deficient mice results in morphological and/or gene regulatory changes that may counteract or aggravate the epileptic phenotype.

The fact that the $GABA_{B(1)}{}^{-/-}$ mice are hyperalgesic suggests the presence of a $GABA_B$-related tone in wild-type mice. This is possibly of importance for the management of pain disorders. For example, positive modulators acting at $GABA_B$ receptors may be therapeutically more useful than $GABA_B$ agonists because they act synergistically with endogenous GABA. Modulators discriminate between activated and nonactivated receptors and therefore may be devoid of the unwanted side effects seen with $GABA_B$ agonists while retaining the therapeutic effects. The interpretation of the results from pain tests is, however, somewhat complicated by the hyperlocomotion observed in knockout animals. It could be argued that the response to painful stimuli reflects an increased reactivity, irrespective of the stimulus. However, it is well established that, e.g., morphine, the best known analgesic drug, produces hyperactivity and that this hyperactivity does not interfere with the effects of morphine in tail-flick, paw-lick, and mechanical pressure paradigms (Babbini and Davis, 1972). Moreover, the fact that $GABA_{B(1)}{}^{-/-}$ animals are hyperalgesic in paradigms that involve distinct motor responses makes a broad confounding effect through hyperactivity less likely. For example, paw-licking is a behavior where the response to the painful stimuli is easily observable. Two $GABA_{B(1)}{}^{-/-}$ animals, but no heterozygous or wild-type animal, vocalized when placed on the hot plate. $GABA_{B(1)}{}^{-/-}$ or wild-type animals do not lick their paw

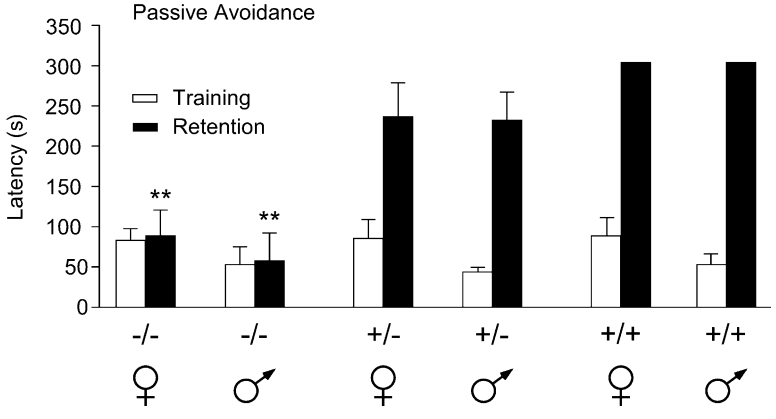

Figure 7. Stepthrough Passive Avoidance Learning in Male and Female, Wild-Type and Mutant Mice

Ordinate represents latency in seconds (mean ± SEM) to step into the dark (shock) compartment on the training trial and retention test of learned behavior. Increase in latency to enter the dark compartment is taken as an index of memory of the initial experience. ** $p < 0.01$ versus same sex (+/+) animals (One-way ANOVA of each sex, $p < 0.001$ in both cases, followed by Tukey's test). The experimental groups were composed as follows: wild-type (8 female/10 male), heterozygote (10 female/9 male), null mutant (11 female/6 male).

once placed on a plate at room temperature, further arguing against an increased reactivity in $GABA_{B(1)}^{-/-}$ mice independent of the stimulus. Vocalization is also observed in the paw withdrawal test, in addition to paw withdrawal to noxious pressure. Again, we do not observe paw withdrawal or vocalization in restrained $GABA_{B(1)}^{-/-}$ animals without the application of pressure to the paw. Changes in the noxious thermal and mechanical threshold suggest that there is a loss of central $GABA_B$-related tone in the nociceptive system of the $GABA_{B(1)}^{-/-}$ animals. In the spinal cord, $GABA_B$ receptors have a major presynaptic inhibitory influence on nociceptive transmission in the dorsal horn (Malcangio and Bowery, 1996). This inhibitory system strongly modulates the polysynaptic nocifensive reflex that is used in the nociceptive assays. In addition, to a lesser extent, $GABA_B$ receptor activation can influence the monosynaptic spinal reflex in the spinal cord. Taken these considerations together, it is likely that the loss of $GABA_{B(1)}$ receptors in the $GABA_{B(1)}^{-/-}$ animals results in a strong alteration of the nocifensive reflex and suggests increased central hyperexcitability of the spinal nociceptive pathway. Although hyperlocomotion does not imply that knockout animals have a lower threshold to engage motor programs, the definite separation of the nociceptive and direct motor components of the decreased nociceptive threshold requires detailed electrophysiological investigation of mono- and polysynaptic transmission in the spinal cord.

In the present studies, a clear impairment of passive avoidance performance was observed which was related to gene dosage. Our data indicate that these passive avoidance deficits are a reflection of impaired memory processes rather than some other behavioral change such as locomotor hyperactivity. Thus, even though in this experiment training latencies were relatively long, with a potential to be reduced, they were not reduced in $GABA_{B(1)}^{-/-}$ or $GABA_{B(1)}^{+/-}$ animals. This suggests either that hyperactivity is not present in the passive avoidance apparatus or that if present, it can be expressed in the part of the apparatus where the animal prefers to be without influencing stepthrough latency. It is established that $GABA_B$ receptor function influences cognitive performance in the mammalian brain (Mondadori et al., 1996). High doses of the clinically used $GABA_B$ agonist baclofen are documented to induce amnesia (Sandyk and Gillman, 1985). Similarly, the lasting anterograde amnesia that occurs under the influence of the "date-rape drug" γ-hydroxy butyrate (GHB) (Schwartz et al., 2000) is likely related to the agonistic action of GHB at $GABA_B$ receptors (Lingenhoehl et al., 1999). Baclofen, like GHB, induces EEG slow waves that have been associated with reduced memory performance (Figure 4B; Jones-Gotman et al., 1994). $GABA_B$ receptor antagonists exhibit a wide range of memory-enhancing effects in a variety of learning situations. This includes learning of passive avoidance in mice, active and passive avoidance in aged rats, radial maze in rats, social recognition in rats, and color-place concept tasks in Rhesus monkeys (for reference see Mondadori et al., 1996). It was proposed that $GABA_B$ receptor antagonists facilitate cholinergic transmission, given the known memory-enhancing effects of cholinergic substances in animals (Mondadori et al., 1996). Likewise, it was stressed that the known modulatory effects of $GABA_B$ antagonists on glutamatergic synapses could produce similar effects (Mondadori et al., 1996). On the other hand, memory-impairing effects of $GABA_B$ antagonists are also reported (Castellano et al., 1993; Brucato et al., 1996), as well as memory-improving effects of baclofen (Saha et al., 1993; Castellano et al., 1993). The diversity of these memory effects makes it difficult to identify a common mechanism. The widespread distribution of $GABA_B$ receptors in the brain and the numerous modulatory effects on various synapses leave ample room for speculations. The lack of presynaptic and postsynaptic $GABA_B$ receptors (Figure 3) in null mutant mice leads to a loss of control over both excitatory and inhibitory neurotransmission. The experiments with null mutant and heterozygous mice show that they are impaired in passive avoidance learning, more in line with the observed memory-impairing effects of $GABA_B$ antagonists (Castellano et al., 1993; Brucato et al., 1996).

For a more specific intervention in nervous system disorders, the existence of distinct $GABA_B$ subtypes would be desirable (Bittiger et al., 1993). Many studies suggest that such subtypes do exist (Bonanno and Raiteri, 1993; Mott and Lewis, 1994). However, cloned $GABA_B$ receptors do not reproduce the pharmacological diversity of native receptors (Marshall et al., 1999). Whether the heterogeneity of native receptors is explained by the existence of unidentified $GABA_B$ receptor subunits remains one of the key issues in the $GABA_B$ field. $GABA_{B(1)}^{-/-}$ animals now demonstrate a lack of detectable $GABA_B$ responses in all biochemical, electrophysiological, and behavioral paradigms studied. This indicates that most, probably all, brain $GABA_B$ receptors incorporate the $GABA_{B(1)}$ subunit. It has been speculated that in a neuronal context, the $GABA_{B(2)}$ subunit could act as a receptor in its own right (Möhler and Fritschy, 1999). From the data herein, it follows that $GABA_{B(2)}$ likely does not function as an autonomous receptor. While our results are in line with previous work that did not find evidence for pharmacologically distinct $GABA_B$ receptor subtypes (Waldmeier et al., 1994), there remains the possibility that unidentified splice variants or $GABA_{B(1)}$-associated proteins generate diversity. For example, promiscuity amongst G protein-coupled receptors (Ginés et al., 2000; AbdAlla et al., 2000) may have some bearing on $GABA_B$ receptor pharmacology. Given the results presented herein, it is, however, important to keep in mind that the differences in the potency of a particular drug in functional studies could relate to differences in the neuronal effector systems and do not necessarily need to reflect subtypes. Further insight will certainly derive from the study of $GABA_{B(2)}^{-/-}$ mice.

Experimental Procedures

Generation and Analysis of Null Mutant Mice

Null mutant mice were generated using Balb/c embryonic stem cells (Dinkel et al., 1999) as outlined in Figure 1. In situ hybridization and Northern blot analysis was performed as described (Kaupmann et al., 1997; Bischoff et al., 1999), using probes derived from residues 442–599 of the $GABA_{B(1a)}$ (Kaupmann et al., 1997) and residues 83 to 324 of the $GABA_{B(2)}$ (Kaupmann et al., 1998) cDNAs. Photoaffinity labeling, radioligand binding experiments, and immunochemistry were carried out as described elsewhere (Kaupmann et al., 1997, 1998; Bischoff et al., 1999; Galvez et al., 2000b). All ligands used

in binding studies were synthesized in house. [^{125}I]CGP64213 and [^{125}I]CGP71872 were labeled to a specific radioactivity of >2000 Ci/mmol (ANAWA AG, Wangen, Switzerland). All animal experiments were subject to institutional review and conducted in accordance with Swiss guidelines.

Electrophysiology

Horizontal slices containing the hippocampus (300 μm thick) were prepared from P12 to P21 mice using standard procedures. Visualized whole-cell voltage-clamp recording techniques were used to measure holding currents and synaptic responses of CA1 pyramidal cells. To evoke synaptic potentials, we delivered stimuli (0.2 ms duration) at 0.1 Hz (0.05 Hz for IPSCs) through bipolar stainless steel electrodes. The recording chamber was superfused (2 ml/min) with an external solution containing (in mM) NaCl 119, KCl 2.5, $MgCl_2$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, and Glucose 11. To dampen polysynaptic activity, 4 mM Ca^{2+}, 4 mM Mg^{2+}, and 200 nM CNQX were added in EPSC recordings. IPSC recordings were in the presence of 2 mM kynurenate or 10 μM CNQX. All experiments were carried out at 32°C–34°C. The internal solution contained (in mM): K-Gluconate 140, HEPES 5, $MgCl_2$ 2, EGTA 1.1, Na_2^+ATP 2, creatine-phosphate 5, and Na^+_3GTP 0.6. Recordings (sweeps of 500 ms every 10 s) were amplified with an Axopatch 1D (Axon Instruments, Foster City, CA) or Visual patch 500 (Bio-logic, France), filtered at 2 kHz and digitized at 10 kHz (National Instruments Board PCI-MIO-16E4, NI-DAQ Igor Software, Wave Metrics) and stored on a hard disk. Data are expressed as means ± SEM. Drugs were from CNQX (Tocris), kynurenic acid (RBI), baclofen (RBI), adenosine (Sigma), CGP54626A (Novartis), DAMGO (Sigma).

EEG Measurements

A three-pole socket was implanted over the cortex and embedded in dental cement under anesthesia (Hypnorm/diazepam, buprenorphin-hydrochloride). Bipolar leads from the mice were recorded via cables connected to a slip-ring system. The behavior of the animals, which were housed singly in wooden observation cages, was observed over a closed-circuit TV system, starting 21 days after the operation. The EEGs were amplified (Spectralab EEG-2104), recorded on a thermo recorder (MTK95, Astromed), and collected on a personal computer.

Measurement of Locomotor Activity and Baclofen-Induced Hypothermia

For measuring locomotor activity, a color video camera (DXC-107AP, Sony) surveyed the open field. The camera signal was analyzed using EthoVision® 1.90 software (Noldus Information Technology, Netherlands). For rotarod performance, mice were first trained twice and the next day tested for endurance performance (cutoff time, 300 s). Rectal temperature was measured to the nearest 0.1°C by an Ellab instruments thermometer (Copenhagen, Denmark) via a lubricated thermistor probe (2 mm diameter) inserted 20 mm in the rectum while the mouse was held near the base of the tail.

Nociceptive Tests

The hot-plate (Eddy and Leimbach, 1953), tail-flick (D'Amour and Smith, 1941), and paw pressure (Randall and Selitto, 1957) techniques are well-established models of acute pain (Walker et al., 1999). Antinociceptive tests in the mouse were performed essentially as originally described and as outlined below. In the hot-plate test, the latency to a front paw lick response was measured for mice placed onto an aluminium plate (Socrel) preheated to 55°C and confined within a clear Perspex cylinder. When the threshold of pain is reached, the mice lick their paws according to an easily identifiable stereotypic behavior. The cutoff time for nonresponding mice was 30 s. In the tail-flick test, mice were placed inside a cotton pouch and the tail exposed to a focused beam of radiant heat at a point 2 cm from the tip using a tail-flick unit (Ugo Basile, Italy). Infrared intensities of 14 were used. Tail-flick latencies were defined as the interval between the onset of the thermal stimulus and the flick of the tail. To prevent tissue damage, a 15 s cutoff was employed. In the paw pressure test, nociceptive thresholds to a mechanical stimulus were determined using an analgesymeter (Ugo Basile, Italy) where the hindpaw is placed under a blunt cone-shaped probe with increasing pressures, using a cutoff threshold of 150 g. The paw withdrawal thresholds (PWT) were determined as the first sign of a pain response, normally exhibited as a paw withdrawal, in the animal.

Passive Avoidance

Before passive avoidance training, mice were housed singly on a 12 hr light-dark cycle (lights on at 6:15) with lab chow and water available ad libitum. One trial stepthrough passive avoidance training was performed as previously described in detail (Venable and Kelly, 1990). The Veterinary Authority of the state of Basel-Stadt approved the studies. In brief, on the training trial, each mouse was placed singly into the light side of a two-compartment trough-shaped apparatus. The door to the dark compartment was opened and, simultaneously, timing by a computer was initiated. When the mouse broke a photocell beam located 10.5 cm into the dark compartment, the latency from opening the door to the animal breaking the beam (stepthrough latency) was automatically recorded and a Campden Instruments 521 C Shock Source was automatically activated. This resulted in the application of a footshock (0.5 mA rectangular current waves) between the stainless steel plates, which comprised the dark compartment. The footshock lasted a maximum of 5 s or until the animal escaped back to the light compartment. In the present experiments, all animals escaped back to the light compartment within 5 s. Animals which did not enter the dark compartment within 150 s on the training trial were given a training latency of 150 s, received no footshock, and were excluded from the memory retention test. The memory retention test was performed on the day following the training trial and was identical to it except that no footshock was administered. Maximum latency in the retention test was 300 s. Statistical analysis was done using SYSTAT (SPSS Inc. Version 8.0).

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