Variability in the Benzodiazepine Response of Serotonin 5-HT_{1A} Receptor Null Mice Displaying Anxiety-Like Phenotype: Evidence for Genetic Modifiers in the 5-HT-Mediated Regulation of GABA_A Receptors

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Benzodiazepines (BZs) acting as modulators of GABA_A receptors (GABA_ARs) are an important group of drugs for the treatment of anxiety disorders. However, a large inter-individual variation in BZ sensitivity occurs in the human population with some anxiety disorder patients exhibiting diminished sensitivity to BZ and reduced density of GABA_ARs. The mechanism underlying BZ treatment resistance is not known, and it is not possible to predict whether an anxiety patient will respond to BZ. 5-Hydroxytryptamine_{1A} receptor (5-HT_{1A}R) null mice (R^{-/-}) on the Swiss-Webster (SW) background reproduce several features of BZ-resistant anxiety; they exhibit anxiety-related behaviors, do not respond to BZ, have reduced BZ binding, and have decreased expression of the major GABA_AR subunits α 1 and α 2. Here, we show that R^{-/-} mice on the C57Bl6 (B6) background also have anxiety phenotype, but they respond to BZ and have normal GABA_AR subunit expression. This indicates that the 5-HT_{1A}R-mediated regulation of GABA_AR α subunit expression is subject to genetic modification. Hybrid SW/B6-R^{-/-} mice also exhibit BZ-resistant anxiety, suggesting that SW mice carry a genetic modifier, which mediates the effect of the 5-HT_{1A}R on the expression of GABA_AR α subunits. In addition, we show that this genetic interaction in SW mice operates early in postnatal life to influence the expression of GABA_AR α subunits at the transcriptional level. These data indicate that BZ-resistant anxiety results from a developmental arrest of GABA_AR expression in SW-R^{-/-} mice, and a similar mechanism may be responsible for the BZ insensitivity of some anxiety patients.

Key words: anxiety; benzodiazepine; GABA; serotonin; mouse; neurotransmitter

Introduction

A major goal in the postgenomic era is to predict the responsiveness of individuals to particular drugs and to understand the mechanisms underlying treatment resistance. Anxiety disorders, including panic disorder, generalized anxiety disorder, and post-traumatic stress disorder, are common with an estimated annual prevalence of 13–18% in the adult population (United States Department of Health and Human Services, 1999). Anxiolytic benzodiazepines (BZs) act on GABA_A receptors (GABA_ARs) to enhance the effects of endogenous GABA (Sigel and Buhr, 1997). However, sensitivity to BZs varies in the population (Domino et al., 1989; Gupta et al., 1997). It has been shown that subjects high in neuroticism (Glue et al., 1995) and panic disorder patients

(Roy-Byrne et al., 1990, 1996; Glue and Nutt, 1991) are less sensitive to BZ, with true BZ treatment resistance occurring in up to 24% of panic patients (Cowley et al., 1997). Because a reduced level of GABA_ARs, assessed by BZ binding, has been reported in panic disorder, generalized anxiety disorder, and post-traumatic stress disorder (Tiihonen et al., 1997; Malizia et al., 1998; Bremner et al., 2000a,b), it is possible that such deficit underlines or at least contributes to the BZ resistance.

Animal models of anxiety have reproduced some of these features of altered BZ responsiveness. A rat strain bred for heightened anxiety has a decreased BZ binding and reduced behavioral responses (Robertson et al., 1978; Commissaris et al., 1990). In a mouse strain specifically bred for its resistance to the BZ inverse agonist β -carboline, anxiety-related behaviors are enhanced, and BZ response is abolished (Lepicard et al., 2000; Rinaldi et al., 2000). Strain-specific differences in baseline anxiety-related behaviors are also evident in mice (van Gaalen and Steckler, 2000; Bouwknicht and Paylor, 2002). Although these models underscore the importance of genetic factors in GABA_AR regulation, the mechanism underlying BZ-resistant anxiety is still unknown.

The 5-hydroxytryptamine_{1A} receptor (5-HT_{1A}R) null (R^{-/-}) mouse has been shown to have an anxiety-like phenotype in various genetic backgrounds (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Groenink et al., 2003; Toth, 2003). We re-

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ported previously that Swiss-Webster (SW)-R^{-/-} mice not only have heightened anxiety but are also resistant to both the anxiolytic and sedative effects of BZ and that they have a reduced expression of GABA_A α 1 and α 2 subunits (Sibille et al., 2000). Here, we found that when the 5-HT_{1A} R null allele is transferred to the B6 background, animals are no longer resistant to the BZ diazepam. R^{-/-}-deficient mice on these two genetic backgrounds therefore represent a model of anxiety with variable BZ responsiveness. Here, we investigated the mechanism of how the genomic background can influence BZ responsiveness and GABA_A subunit expression in 5-HT_{1A} R^{-/-} mice. The importance of 5-HT_{1A} R deficits in human anxiety disorders has been demonstrated recently in panic disorder patients, which have reduced 5-HT_{1A} R binding (Neumeister et al., 2004).

Materials and Methods

Animals. As described by Parks et al. (1998), 5-HT_{1A} R^{-/-} mice were originally generated on the 129/Sv background and gradually backcrossed to the SW background (Taconic, Germantown, NY). For the work described here, the original mixed 129/SW 5-HT_{1A} R^{-/-} mice have been backcrossed seven times to SW mice (Taconic) and 12 times to B6 mice (C57BL/6Ntac; Taconic). Despite the extensive backcrossing, a small percentage of the 129 genome is still likely present in 5-HT_{1A} R^{-/-} mice on the SW and B6 backgrounds. R^{+/+} and R^{-/-} mice from these populations were crossed to generate F1 hybrid SW/B6 mice. Mice were maintained in groups of three to five in standard cages (22 × 16 × 14 cm) under 12 hr light/dark cycle conditions (lights on from 7:00 A.M. to 7:00 P.M.) with standard rodent food pellets and water freely available. Except for the developmental studies, 2- to 5-month-old male mice were used in all experiments.

Behavioral studies. The elevated plus maze was performed using a cross maze with 12 × 2 inch arms at low light conditions (60 W bulb at 3 m height at 25% intensity). Animals were introduced to the middle portion of the maze facing an open arm. Entries into and time spent in the open and closed arms were measured by a video-tracking system (Noldus Information Technology, Wageningen, The Netherlands). The length of the test was extended from the customary 5–10 min, because receptor-deficient mice tend to freeze at the time of introduction, reducing the time they actively explore the maze. The open-field test used a 15 × 21 inch black box, divided into 12 even-sized (4 × 3 inch) rectangles. The total number of crosses in the open field was recorded at normal light conditions (60 W bulb at 3 m height at 100% intensity) for 10 min to measure the locomotor activity. The time spent in and number of entries into the two rectangles at the center of the field were recorded by the video-tracking system to evaluate anxiety. Response to BZs was tested in 10 × 15 inch plastic boxes, and total locomotor activity was measured as total distance traveled (in centimeters) in 1 hr. All testing was performed between 11 A.M. and 4 P.M. On test days, animals were transported to the dimly illuminated behavioral laboratory and left undisturbed for at least 2 hr before testing. Every animal was used only for a single test. Diazepam (Sigma, St. Louis, MO) was dissolved in ethanol and diluted in 0.9% physiological saline to appropriate concentrations each test day and was injected intraperitoneally 30 min before the test. Up to 1 mg/kg diazepam caused no detectable change in locomotor activity, and doses of 0.2 and 1 mg/kg were used in the elevated plus maze test. When the sedative effect of BZ was tested, up to 10 mg/kg diazepam was used. Diazepam above 10 mg/kg caused severe sedation. We reported previously that the genetic inactivation of the 5-HT_{1A} R does not influence drug disposition (Sibille et al., 2000).

Real-time reverse transcriptase-PCR. Total RNA was isolated from tissue punches using TRIZOL reagent (Invitrogen, San Diego, CA). Quantitative analysis of the abundance of GABA_A receptor α 1, α 2, and γ 2 subunit mRNAs was performed on the ABI PRISM 7700 Sequence Detector System (PerkinElmer Life Sciences, Foster City, CA). All reagents were obtained from Applied Biosystems (Foster City, CA), and the manufacturer protocols were followed. The reference gene used was 18S rRNA, detected using Taqman ribosomal RNA control reagents. Gene-

specific primers (*Gabra1*: 5'TGCGACCATAGAACCGAAAGA 3'; 5'CAGTCGGTTCGATTTTGCTGA 3'; *Gabra2*: 5'AAAAGAGGATGGGCTTGGA 3'; 5'CCGCATAGGCGTTGTTCTGT 3'; *Gabrg2*: 5'GGAGCCTGGAGACATGGGA 3'; 5'TGAACAAGCAAAAGGCGGTA 3'), and fluorescent Taqman probes (*Gabra1*: 5'TCAAGCCTGAGACAAACACCAGAACC 3'; *Gabra2*: 5'TGACAAAGAAAAGAGAAAGGCTCCGTCATG 3'; *Gabrg2*: 5'TTGCCAAAATGGACTCCTATGCTCGGAT 3') were designed using ABI software and GenBank sequences. All primer-probe combinations were optimized and validated for relative quantification of gene expression using the comparative threshold cycle method (Schmittgen et al., 2000) described in Applied Biosystems sequence detection system user bulletin number 2. Briefly, data for each target gene were normalized to the endogenous control gene (rRNA), and the fold change in target gene mRNA abundance was determined using the 2^{- $\Delta\Delta C_t$} method such that R^{-/-} levels are expressed relative to R^{+/+} level.

Semiquantitative Western blot analysis. Tissue punch samples were homogenized in 10 volumes of buffer containing 10 mM Tris, pH 7.4, 0.32 M sucrose, 5 mM EDTA, and protease inhibitor mixture (Complete Mini; Roche, Indianapolis, IN). The protein concentration of the supernatant was determined using BCA protein assay (Pierce, Rockford, IL). Aliquots of the crude membrane preparations were subjected to SDS-PAGE. For each sample, 10, 20, and 40 μ g of protein were separated on 10% acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Immunolabeling with GABA_A receptor subunit-specific antisera and quantitation procedures was performed as described previously (Sibille et al., 2000).

Antibodies. GABA_A receptor subunit-selective antisera for α 1 and α 2 subunits (Nusser et al., 1996) were diluted 1:500 (gift from Dr. D. Benke, Institute of Pharmacology, University of Zurich, Zurich, Switzerland) and detected with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:2500 (α 1) or horseradish peroxidase-conjugated goat anti-guinea pig IgG (α 2). Controls for the developmental studies were anti-PSD-95 (postsynaptic density-95) (Chemicon, Temecula, CA) and anti-synaptophysin (Calbiochem, La Jolla, CA). Western blots prepared from sucrose gradient fractionated samples were probed with affinity-purified anti-Jerky antibody (Liu et al., 2002) at a dilution of 1:1000 and with a 1:1000 dilution of a human autoimmune serum containing anti-P0 antibody (obtained from Keith Elkon, Hospital for Special Surgery, New York, NY).

In vitro actinomycin D treatment. Brains were dissected from postnatal day 28 (P28) R^{+/+} and R^{-/-} mice. Cortical slices (250 μ m) were prepared and maintained as described previously (Mathisen et al., 1997). After equilibration for 30 min, slices were incubated continuously in 5 μ g/ml actinomycin D (Calbiochem) for up to 18 hr with regular replacement of medium. Effective transcriptional blockade under these conditions was confirmed in control experiments. At various time points, slices were removed, weighed, snap frozen, and stored at -80°C for RNA isolation. RNA samples were subsequently subjected to real-time reverse transcriptase (RT)-PCR analysis.

Sucrose density gradient fractionation of cytosol. Cerebral cortices from P28 R^{+/+} and R^{-/-} mice were homogenized in buffer containing 100 mM KCl, 20 mM Tris, pH 7.4, 5 mM MgCl₂, protease inhibitor mixture (Complete Mini; Roche), and 30 U of Rnasein (Promega, Madison, WI). Homogenates were incubated on ice for 10 min in the presence of 200 μ g/ml cycloheximide and then centrifuged. Supernatants were loaded onto 15–47% sucrose gradients followed by centrifugation as described previously (Liu et al., 2002). Twenty-three fractions of 500 μ l were collected from each gradient, and RNA and protein were extracted. A sample of RNA was electrophoresed in a 1% agarose gel containing ethidium bromide (50 μ g/ μ l) to visualize 18S and 28S rRNAs. For Western blot analysis, samples were loaded onto 10% acrylamide gels and transferred onto PVDF membranes for immunolabeling. Together, the RNA gel and Western blots allowed messenger ribonucleotide particle (mRNP), monosome, and polysome pools to be distinguished (see Fig. 6B). Subsequently, RNA samples from individual fractions were pooled and subjected to real-time RT-PCR analysis.

Statistics. When two means were compared, statistical significance of their difference was calculated using paired or nonpaired Student's *t* test. In multiple comparisons, data were analyzed by ANOVA or ANCOVA

Figure 1. A–D, Anxiety-related behavior in 5-HT_{1A}R^{-/-} mice in the elevated plus maze (A, B) and open field (C, D). Percentage of time spent in the open arm (A) and percentage of open arm entries (B) in the elevated plus maze as well as percentage of time spent in the center (C) and entries to the center (D) in the open field are shown (mean ± SD). R^{-/-} mice (■) on both the SW and B6 backgrounds exhibit increased anxiety-like behavior compared with their respective controls (R^{+/+}, □) (**p* < 0.05 and ***p* < 0.01; *n* = 6–10 per group). B6-R^{+/+} mice also displayed more anxiety-like behavior than SW-R^{+/+} mice in some measures ([†]*p* < 0.05).

with genotype and genetic background as independent variables and behavior as a dependent variable. In case of statistical significance, group data were analyzed by the Student–Newman–Keuls multiple comparisons test. The accepted level of significance for all tests was *p* < 0.05. Statistical analysis was performed by the program Statistica (StatSoft, Tulsa, OK).

Results

Anxiety-related behavior in 5-HT_{1A}R^{-/-} mice on the SW and B6 genetic background

Anxiety is likely determined by a complex interaction of genes that may vary from individual to individual (Wood and Toth, 2001; Clement et al., 2002). To evaluate whether the anxiety-like phenotype in 5-HT_{1A}R-deficient mice develops independently of the genetic background, the behavior of SW-R^{-/-} and B6-R^{-/-} mice was compared with corresponding R^{+/+} mice. B6 is an inbred strain providing a homogenous genetic background. Although not formally an inbred, the SW strain also has a very limited heterozygosity, because this line was derived from a few individuals (Beck et al., 2000). Indeed, the behavioral data obtained for SW and B6 mice showed similar Gaussian distributions (Kolmogorov and Smirnov test; *p* > 0.1) and equal SD (*p* > 0.05).

Anxiety-like behavior of R^{-/-} mice on both SW and B6 backgrounds was assessed in the elevated plus maze and open-field tests. ANOVA revealed a significant effect of genotype ($F_{(1,26)} = 8.1$; *p* = 0.0086) and background ($F_{(1,26)} = 14.1$; *p* = 0.0009) on time spent in the open arm of the elevated plus maze. Follow-up comparisons indicated that SW-R^{-/-} mice spent less time in the open arm (normalized to the total time spent in both arms) than SW-R^{+/+} mice, whereas there was a trend for less time spent in the open arm with B6-R^{-/-} mice (Fig. 1A). The difference was statistically significant when B6-R^{-/-} and -R^{+/+} mice were com-

pared directly (two-tailed *t* test; *p* = 0.0175). In addition, anxiety levels between the two strains were different, with B6-R^{+/+} mice spending less time in the open arm than SW-R^{+/+} mice (Fig. 1A). A higher anxiety-like behavior of B6 mice compared with SW was also observed by some (Griebel et al., 2000) but not other investigators (Rodgers et al., 2002). ANOVA also showed a significant effect of genotype ($F_{(1,26)} = 21.3$; *p* = 0.00009) and background ($F_{(1,26)} = 5.9$; *p* = 0.023) on open arm entries in the elevated plus maze. R^{-/-} mice on both SW and B6 backgrounds entered into the open arm significantly less frequently than SW-R^{+/+} and B6-R^{+/+} mice, respectively (Fig. 1B). Open arm entries were normalized to the total number of entries and shown in percentages, because the locomotor activity of R^{-/-} mice was reported to be reduced (Ramboz et al., 1998; Zhuang et al., 1999). When the number of closed arm entries, a more reliable index of general locomotor activity than total arm entries (Hogg, 1996), was compared in our experiments, no difference was found between B6-R^{-/-} and B6-R^{+/+} mice (22.5 ± 1.1 vs 25.7 ± 2.9 ; NS). In contrast, there was a significant reduction in the locomotor activity of SW-R^{-/-} compared with SW-R^{+/+} mice (15.6 ± 1.0 vs 27.0 ± 2.3 ; *p* < 0.01). Nevertheless, ANCOVA, using the number of closed arm entries as covariate, still showed a significant effect of genotype on open arm entries ($F_{(1,11)} = 20.1$; *p* = 0.0009), indicating that the reduction in open arm entries is independent of the change in locomotor activity in SW-R^{-/-} mice. A reduced locomotor activity in the elevated plus maze was also observed in 5-HT_{1A}R^{-/-} mice on the 129sv background (Zhuang et al., 1999). Based on factor analysis, these authors proposed that measures of behavior in anxiety-related tests reflect two main factors: anxiety related and general behavioral activation–exploration (Ramboz et al., 1998; Zhuang et al., 1999) and that 5-HT_{1A}R^{-/-} mice display increased anxiety-like behavior and reduced locomotor activity as separate traits.

Increased anxiety-like behavior of 5-HT_{1A}R-deficient mice was also evident in the open field. ANOVA revealed a significant effect of genotype ($F_{(1,31)} = 12.9$; *p* = 0.0011) when time spent in the center of the open field was measured. R^{-/-} mice on both SW and B6 backgrounds spent significantly less time in the center than SW-R^{+/+} and B6-R^{+/+} mice, respectively (Fig. 1C). When the number of entries into the center was measured, ANOVA indicated a significant effect of background ($F_{(1,33)} = 15.9$; *p* = 0.00034). B6-R^{+/+} and B6-R^{-/-} mice entered into the center of the open field less frequently than SW-R^{+/+} and SW-R^{-/-} mice, respectively (Fig. 1D). Total locomotor activity was not different between SW-R^{-/-} and SW-R^{+/+} mice (176.7 ± 16.0 vs 250.3 ± 23.1 square crossings, NS), but there was a significant reduction in B6-R^{-/-} compared with B6-R^{+/+} mice (271.8 ± 20.1 vs 427.9 ± 31.5 ; *p* < 0.001). Together, these data indicate that, independently of the genetic background, deletion of the 5-HT_{1A}R results in increased anxiety-like phenotype. Also, some of the measures indicate a higher anxiety level in B6 than in SW mice. Therefore, based on the behavioral measures in both the elevated plus maze and open field, the anxiety-related behavior of these groups of animals may be described in a simplified manner as B6-R^{-/-} > B6-R^{+/+} = SW-R^{-/-} > SW-R^{+/+}.

BZ responsiveness of 5-HT_{1A}R^{-/-} mice is dependent on the genetic background

We reported previously that SW-R^{-/-} mice are insensitive to the anxiolytic effect of diazepam (Sibille et al., 2000). Here, the anxiolytic effect of the BZ diazepam (0, 0.2, and 1.0 mg/kg) was examined and compared in SW- and B6-R^{-/-} mice in the elevated plus maze. In these experiments, injection (presumably owing to injection-

associated stress) noticeably increased the anxiety of all groups (manifested as reduced open arm time and entry), including vehicle-injected controls, masking the difference in baseline anxiety seen in noninjected mice. Nevertheless, consistent with an anxiolytic effect, diazepam increased both the percentage of open arm time and percentage of entry of SW-R^{+/+} ($F_{(2,29)} = 4.9$, $p = 0.015$; $F_{(2,28)} = 4.8$, $p = 0.017$, respectively) and B6-R^{+/+} mice ($F_{(2,42)} = 16.2$, $p < 0.0001$; $F_{(2,38)} = 4.3$, $p = 0.022$). However, SW-R^{+/+} mice were more sensitive to the anxiolytic effect of diazepam because they responded to a dose of 0.2 mg/kg, whereas B6-R^{+/+} mice responded only to 1 mg/kg (Fig. 2*A,B,D,E*).

As expected, diazepam (up to 1 mg/kg) was without effect in SW-R^{-/-} mice ($F_{(2,21)} = 0.3$, $p = 0.74$, NS; $F_{(2,21)} = 0.6$, $p = 0.54$, NS for percentage of open arm time and entry, respectively) (Fig. 2*A,D*). Surprisingly, B6-R^{-/-} mice responded to the anxiolytic effect of BZ ($F_{(2,32)} = 3.7$, $p = 0.037$; $F_{(2,36)} = 3.5$, $p = 0.047$, for percentage of open arm time and entry, respectively) (Fig. 2*B,E*). A similarly normal BZ responsiveness was demonstrated in the 5-HT_{1A}R^{-/-} mouse on the 129/Sv background (Pattij et al., 2002). The effect of the 5-HT_{1A}R^{-/-} allele on BZ sensitivity was also assessed on the F1 SW/B6 hybrid background (50% SW/50% B6). Similarly to SW-R^{-/-} mice, hybrid-R^{-/-} mice failed to respond to the anxiolytic effect of 1 mg/kg diazepam ($p = 0.2251$, NS, and $p = 0.4498$, NS, for percentage of open arm time and entry, respectively) (Fig. 2*C,F*). Hybrid-R^{+/+} mice, as the other R^{+/+} mice, seemed to respond to diazepam ($p = 0.002$ for percentage of open arm time and $p = 0.0745$, trend but NS for percentage of open entries). We concluded that lack of the 5-HT_{1A}R in SW or in hybrid SW/B6 mice leads to BZ resistance that is not evident in B6-R^{-/-} mice. These results indicate that SW mice carry a genetic modifier that, depending on the expression of the 5-HT_{1A}R, influences BZ sensitivity.

Similar results were obtained when the sedative effect of diazepam was studied. This effect was examined by recording the total distance traveled in 1 hr. Diazepam (10 mg/kg) reduced the locomotor activity of SW-R^{+/+} mice ($F_{(3,23)} = 6.6$; $p = 0.0029$), whereas SW-R^{-/-} mice were resistant to the sedative effects of the drug ($F_{(3,23)} = 0.4$; $p = 0.77$, NS) (Fig. 2*G*). In contrast to the SW strain, B6-R^{+/+} and B6-R^{-/-} mice were similarly sensitive to the sedative effects of diazepam ($F_{(3,39)} = 4.3$, $p = 0.011$, and $F_{(3,46)} = 4.0$, $p = 0.0137$, respectively) (Fig. 2*H*). When vehicle-injected R^{+/+} and R^{-/-} mice were compared, a significant reduction in locomotor activity was observed in SW-R^{-/-} (Fig. 2*G*) and B6-R^{-/-} (Fig. 2*H*).

GABA_AR subunit mRNA expression correlates with BZ responsiveness in the different strains of 5-HT_{1A}R^{-/-} mice

Recent reports suggest that the anxiolytic effect of BZ is mediated by α_2 subunit-containing GABA_ARs, whereas the sedative effect is mediated by α_1 subunit-containing GABA_ARs (Rudolph et al.,

Figure 2. Anxiolytic effect of diazepam in the elevated plus maze. *A–F*, Mean ($\pm$ SD) percentage of open arm time and entries for SW (*A*, *D*), B6 (*B*, *E*), and hybrid SW/B6 (*C*, *F*) R^{+/+} and R^{-/-} mice. The anxiolytic effect of the BZ diazepam (0.2, 1 mg/kg), compared with vehicle-injected controls (V), was evident in all R^{+/+} mice and in B6-R^{-/-} mice (* $p < 0.05$; ** $p < 0.01$; $n = 12$ –16 per group). SW-R^{-/-} (*A*, *D*) and SW/B6-R^{-/-} (*C*, *F*) mice were not responsive. *G*, *H*, The sedative effect of the BZ diazepam (1, 3, 10 mg/kg) detected as a decrease in locomotor activity (distance traveled times 10^3 cm/hr) is also displayed. Diazepam (10 mg/kg) (BZ) resulted in significant reduction in locomotor activity in SW-R^{+/+} (*G*), B6-R^{+/+}, and B6-R^{-/-} (*H*) but not in SW-R^{-/-} (*G*) mice (* $p < 0.05$; ** $p < 0.01$; $n = 8$ –12 per group). Decreased locomotor activity of SW-R^{-/-} (*G*, lane V) and B6-R^{-/-} (*H*, lane V) compared with their background-matched controls was also detected (††† $p < 0.01$; †††† $p < 0.001$).

1999; Low et al., 2000; McKernan et al., 2000). As described previously (Sibille et al., 2000), SW-R^{-/-} mice that were insensitive to both the anxiolytic and sedative effects of diazepam (Fig. 2) expressed up to 50% less α_1 and α_2 subunit mRNAs in frontal cortex and amygdala when compared with mRNA levels in BZ-sensitive SW-R^{+/+} mice (Fig. 3*A,B*). The downregulation of the α_2 subunit in the amygdala is especially noteworthy, because this region has been shown to be important in the anxiolytic action of BZs (Shibata et al., 1989; Menard and Treit, 1999). As in SW-R^{-/-} mice, the expression of both GABA_A α_1 and α_2 subunit mRNAs in hybrid SW/B6-R^{-/-} mice was reduced when compared with R^{+/+} mice of the same genetic background. In contrast, no such downregulation of α_1 and α_2 subunit mRNAs was evident in BZ-responsive B6-R^{-/-} mice (Fig. 3*A,B*). Rather, the level of the α_2 subunit mRNA was upregulated in the amygdala of B6-R^{-/-} mice (Fig. 3*B*). We reported previously that there is no change in the pattern of GABA_A subunit expression in the BZ-responsive 129/Sv-R^{-/-} mice either (Bailey et al., 2002). The observation that γ_2 subunit mRNA levels are not affected by the inactivation of the 5-HT_{1A}R, in either brain region examined and in all strain backgrounds, indicates that the downregulation in R^{-/-} mice is specific for α subunits of the GABA_AR. We concluded that resistance to the anxiolytic and sedative action of BZ

Figure 3. GABA_A subunit mRNA expression in 5-HT_{1A}^{+/+} and R^{-/-} mice. *A–D*, GABA_A α_1 , α_2 , and γ_2 subunit mRNA abundance in frontal cortex (*A*, *C*) and amygdala (*B*, *D*) was measured by real-time RT-PCR. Levels in R^{-/-} for each gene are expressed relative to the corresponding strain background R^{+/+} levels (*A*, *B*). In *C* and *D*, expression levels in R^{+/+} mice on different backgrounds are directly compared with each other. Levels in B6- and hybrid SW/B6-R^{+/+} are expressed relative to SW R^{+/+} level (dotted line, 1). Significant changes in expression are indicated (* p < 0.05; mean $\pm$ SE; n = 3).

is correlated with the downregulation of the α_2 and α_1 GABA_A subunit mRNAs in the different strains of R^{+/+}/R^{-/-} mice.

Because SW-R^{+/+} mice were more sensitive to the anxiolytic effect of diazepam than B6-R^{+/+} mice (responded to 0.2 and 1 mg/kg, respectively) (Fig. 2*A,B,D,E*), we tested whether this difference is reflected in the expression level of the α_2 subunit mRNA. Indeed, real-time RT-PCR measurements indicated a reduced α_2 subunit mRNA level in B6 mice in both the cortex and amygdala (Fig. 3*C,D*). In contrast, the α_2 subunit mRNA level was not reduced in the amygdala of the BZ-insensitive F1 SW/B6-R^{+/+} mice (Fig. 3*B*), and there was no significant difference in α_2 subunit mRNA level between the moderately BZ-sensitive B6-R^{+/+} or B6-R^{-/-} and the insensitive SW-R^{-/-} mice (data not shown). These data indicate that there is no correlation between BZ sensitivity and α_2 subunit expression in amygdala when mice with different genetic backgrounds are compared. This finding may not be surprising, considering the numerous genetic differences between the strains that can modulate BZ responsiveness.

In agreement with the similar sensitivity of SW and B6 mice to the sedative effect of diazepam, these strains did not significantly differ in the expression of the α_1 subunit (Fig. 3*C,D*). SW/B6 mice resembled SW mice, except that they expressed more α_1 subunit mRNA in the amygdala. The expression of the γ_2 subunit mRNA was not different between SW and B6 mice, but again hybrid mice expressed a higher level of this mRNA in both cortex and amygdala.

Figure 4. GABA_A subunit protein expression in 5-HT_{1A}^{-/-} mice. Protein levels were estimated by semiquantitative Western blot analysis. *A*, Representative blots showing increasing amounts of total protein (10, 20, and 40 μ g) from frontal cortex and amygdala samples were probed with specific antibodies against the α_1 and α_2 subunits. *B*, Examples of quantification of the intensity of α_1 immunolabeling in the cortex of SW, B6, and hybrid SW/B6 mice (R^{+/+}, $\circ$; R^{-/-}, $\bullet$). *C*, *D*, Relative subunit expression level is displayed as the ratio of R^{-/-} to R^{+/+} labeling intensity for each strain (* p < 0.05; mean $\pm$ SE; n = 2 two independent blots).

GABA_A subunit expression correlates with the BZ responsiveness of 5-HT_{1A}^{-/-} mice

Changes in GABA_A subunit protein expression were also found in R^{-/-} mice. Semiquantitative Western blotting revealed that both the α_1 and α_2 subunits were downregulated to 45–50% of R^{+/+} levels in SW and hybrid SW/B6-R^{-/-} mice but not in B6-R^{-/-} mice in frontal cortex and amygdala (Fig. 4*C,D*). Interestingly, the elevated α_2 subunit mRNA level seen in B6-R^{-/-} amygdala (Fig. 3*D*) was not reflected at the protein level (Fig. 4*D*), suggesting that there is compensation at the translational or post-translational level. Together, these results show that the downregulation of the α_1 and α_2 subunit mRNAs and proteins in SW-R^{-/-} and SW/B6-R^{-/-}, but not in B6-R^{-/-} mice, correlates with the BZ resistance (SW and hybrid SW/B6) and sensitivity (B6) of these mice.

Increase of α_1 and α_2 subunit expression during development is disrupted in the BZ-resistant SW 5-HT_{1A}^{-/-} mice

During normal postnatal development, a substantial increase is seen in the expression of *Gabra1* and, to a lesser extent, *Gabra2* genes encoding the α_1 and α_2 subunits, respectively (Laurie et al., 1992; Roberts and Kellogg, 2000) (Fig. 5*A*). Because 5-HT_{1A}Rs have been reported to be involved in neuronal development (Azmitia, 2001), we examined whether the downregulation of α_1 and α_2 subunit mRNAs seen in the adult cortex of SW-R^{-/-} mice occurred during postnatal development. At P7, there was no difference between R^{+/+} and R^{-/-} mice (Fig. 5*B*). However, from

Figure 5. Developmental arrest of GABA_A α_1 and α_2 subunit expression in frontal cortex of SW-R^{-/-} mice. *A*, Developmental time course of subunit mRNA accumulation in SW-R^{+/+} mice measured by real-time RT-PCR. For each gene, expression at each stage is relative to P28 value (mean $\pm$ SE; $n = 3$). *B*, GABA_A subunit mRNA abundance in R^{-/-} at each postnatal stage is expressed relative to R^{+/+} level (* $p < 0.05$; t test; $\pm$ SE; $n = 6$). *C*, Semiquantitative Western blot analysis of GABA_A subunit levels expressed as the ratio of R^{-/-}:R^{+/+} mice (* $p < 0.05$; mean $\pm$ SE; $n = 6$). *D*, Developmental expression of the presynaptic marker synaptophysin and the postsynaptic PSD-95 are not altered in SW-R^{-/-} compared with SW-R^{+/+} mice. Mean ($\pm$ SE) relative intensity of immunoblot labeling is expressed relative to P28 ($n = 3$).

P14 to P28, a deficit in the expression of the α_1 and α_2 subunit mRNAs was evident in R^{-/-} mice. Throughout this period, there was no change in the developmental expression of the GABA_A γ_2 subunit in R^{-/-} mice, indicating that, as in the adult, the expression deficit is specific for the α subunits (Fig. 5*B*). Also, Western blot analysis revealed that by P14, a significant reduction in the level of α_1 and α_2 subunit proteins was also evident in SW-R^{-/-} mice (Fig. 5*C*). A gross abnormality of synaptogenesis that could cause changes in GABA receptor expression after inactivation of the 5-HT_{1A} R was not apparent, because the expression of the presynaptic marker synaptophysin and the postsynaptic density protein PSD-95 was not altered in SW-R^{-/-} mice (Fig. 5*D*).

Putative mechanism leading to reduced expression of GABA_A subunits

A reduced steady-state level of GABA_A α_1 and α_2 subunit mRNAs in SW-R^{-/-} mice could arise from a reduced transcription or an accelerated rate of decay. Although the direct measurement of transcriptional rate in cortical slices was not feasible because of the low transcription rate of *Gabra1* and *Gabra2*, it was possible to determine the half-life of the $\alpha_{1/2}$ subunit mRNAs. The long-term viability of cortical slices was indicated by the 95 and 90% level of the $\alpha_{1/2}$ subunit mRNAs after a 6 and 12 hr incubation, respectively (data not shown). The rate of decay of the α_1 , α_2 , and γ_2 subunit mRNAs in SW-R^{+/+} and R^{-/-} mice (Fig. 6*A*) was not significantly different (α_1 : 3.0 ± 0.2 vs $2.8 \pm$

Figure 6. Altered mRNA stability does not account for the downregulation of the GABA_A α_1 and α_2 subunit expression in the cortex of P28 SW-R^{-/-} mice. *A*, In actinomycin-D-treated samples, the half-lives of the α_1 , α_2 , and γ_2 subunit mRNAs are similar in R^{+/+} and R^{-/-} mice. *B*, Identification of mRNP, monosome (mono), and polysome (poly light) pools separated by sucrose density gradient sedimentation. Distribution of ribosomal RNAs (top panel) and of Jerky and P0 proteins (bottom panel) from fractions 1–12 is shown. Fractions 13–23 were divided equally to poly intermediate and poly heavy pools. *C*, Association of $\alpha_{1/2}$ subunit mRNAs with mRNP, monosome, and polysome pools detected by real-time RT-PCR. For each pooled sample, abundance in both R^{-/-} and R^{+/+} mice is expressed relative to the R^{+/+} mRNA pool (mean $\pm$ SE, $n = 3$; * indicates a significant difference between genotypes; $p < 0.01$; Fisher post hoc test).

0.1; α_2 : 5.1 ± 0.2 vs 5.4 ± 0.3 ; γ_2 : 2.3 ± 0.1 vs 2.2 ± 0.1 ; mean $t_{50\%}$ (hour) $\pm$ SD; $n = 3$; $p > 0.05$). These data rule out the possibility that GABA_A mRNA downregulation in SW-R^{-/-} mice involves decreased mRNA stability and indicate that a transcriptional problem is the most likely mechanism.

Reduced transcription of the $\alpha_{1/2}$ subunit mRNAs may lead to a compensatory increase in their translation. Association of mRNAs with polyribosomes has been proposed to be an indicator of translational efficiency (Feng et al., 1997a,b). Cytoplasmic extracts from P28 frontal cortex of SW-R^{+/+} and R^{-/-} mice were fractionated in sucrose density gradients to translationally inactive mRNPs as well as to monosomes and polysomes. These fractions were identified by the 62 kDa mRNP associated protein Jerky (Liu et al., 2002), the 34 kDa monosome associated ribosomal protein P0 (Remacha et al., 1995), and the 18S and 28S ribosomal RNAs (Fig. 6*B*). The polysomal fractions were divided to “light,” “intermediate,” and “heavy” polysomes according to their sedimentation. Fractions were pooled, RNA extracted, and subjected to real-time RT-PCR analysis (Fig. 6*C*). Repeated measures ANOVA revealed a significant effect of the distribution of subunit mRNAs among the fractions because the majority of both the α_1 and α_2 mRNAs were present in the mRNP fraction (α_1 : $F_{(4,16)} = 332$, $p < 0.0001$; α_2 : $F_{(4,16)} = 164$, $p < 0.0001$). ANOVA also revealed an interaction between genotype and mRNA distribution (α_1 : $F_{(4,16)} = 60$, $p < 0.0001$; α_2 : $F_{(4,16)} = 44$, $p < 0.0001$). In agreement with previous data, subunit mRNA levels in the mRNP pool were lower in R^{-/-} than in R^{+/+} sam-

ples for both α_1 and α_2 mRNAs ($p < 0.01$; Fisher *post hoc* test). Although there was a difference between genotype in the mRNA pool, the level of subunit mRNAs was comparable between $R^{-/-}$ and $R^{+/+}$ samples in the monosomal and polysomal pools. This indicates that despite the overall decrease in subunit mRNAs, the translationally competent mRNA pool was not reduced in $R^{-/-}$ mice. However, this mechanism is apparently not sufficient or effective to normalize the abnormally low GABA_AR subunit levels in SW- $R^{-/-}$ mice.

Discussion

Reduced sensitivity to BZ and reduced GABA_AR density both have been reported in panic disorder patients (Roy-Byrne et al., 1990, 1996; Glue and Nutt, 1991; Cowley et al., 1997; Tiihonen et al., 1997; Malizia et al., 1998; Bremner et al., 2000a,b). Interestingly, a lower 5-HT_{1A}R binding has also been found in panic disorder patients (Neumeister et al., 2004), implicating a connection between the 5-HT and GABA systems in the pathogenesis of anxiety. 5-HT_{1A} $R^{-/-}$ mice on different genetic backgrounds recapitulate features of BZ-resistant and -sensitive anxiety seen in patients. Here, we show that 5-HT_{1A}R-deficient mice on the SW genetic background do not respond to the anxiolytic drug diazepam and have a reduced expression of the main GABA_AR α subunits. In contrast, receptor-deficient mice on the B6 genetic background exhibit a normal anxiolytic response to diazepam and have normal levels of α subunits. Hybrid SW/B6- $R^{-/-}$ mice have a phenotype similar to that of SW- $R^{-/-}$ mice. We have shown previously that the reduction in GABA_AR subunit message and protein correlates with a reduction in functional GABA_A receptor expression in 5-HT_{1A}R $R^{-/-}$ mice (Sibille et al., 2000). This is consistent with the observation that α subunit availability is an important factor in GABA_AR assembly (Fritschy et al., 1997; Bollan et al., 2003). These data indicate a correlation between sensitivity to BZ and expression of GABA_AR within strains. Also, these data imply that BZ insensitivity in some anxiety patients could involve reduced signaling via 5-HT_{1A}R, leading to a diminished GABA_AR expression. Diminished 5-HT_{1A}R signaling could be attributable to lower expression but also to structural receptor polymorphism. At least four structural variants of the 5-HT_{1A} receptor have been described that affect various domains of the receptor, including the first transmembrane domain, second intracellular loop, and third intracellular loop that is involved in G-protein binding (Rotondo et al., 1997; Kawanishi et al., 1998).

Deletion of the 5-HT_{1A}R could be predicted to have a developmental impact, because the receptor has been implicated in neuronal maturation, synaptogenesis, and dendritic spine development (Borella et al., 1997; Yan et al., 1997). Although 5-HT raphe neurons appear before birth, the arrival of 5-HT axons in projection areas is temporally related to neuronal differentiation (Lauder et al., 1983). In addition, 5-HT_{1A}R expression normally increases to adult levels between P7 and P14 (Miquel et al., 1994). Although we have not observed any gross abnormality in the neuronal architecture of 5-HT_{1A}R $R^{-/-}$ mice, we cannot exclude the possibility that there are more subtle morphological alterations. The importance of this early postnatal period has also been demonstrated in the development of the anxiety-phenotype in 5-HT_{1A}R $R^{-/-}$ using a conditional rescue strategy in $R^{-/-}$ (Gross et al., 2002). These authors demonstrated that expression of the 5-HT_{1A}R early in postnatal development was necessary to establish normal adult anxiety-related behavior. Together, these data suggest that the 5-HT_{1A}R is essential for the normal expression of GABA_ARs during postnatal development in SW mice.

The mechanism underlying the reduced expression of

Figure 7. Summary of the interaction between 5-HT_{1A}R and GABA_AR α subunits in the amygdala of SW and B6 mice. In SW, the 5-HT_{1A}R has a positive effect (yellow arrows) on α subunit transcriptional activation. In B6, the 5-HT_{1A}R has no effect or an inhibitory action (yellow line) on α subunit mRNA expression. At the translational or post-translational level (blue arrows), there is also positive feedback regulation in B6 (blue line) but not in SW mice. Inactivation of the 5-HT_{1A}R in SW mice therefore results in a downregulation of $\alpha_{1/2}$ subunits, altered assembly of functional GABA_ARs (green lines), which are not responsive to BZ. The expression of GABA_AR α subunits is unchanged in B6- $R^{-/-}$ mice. Gray shading represents changes in subunit expression (the same level as background represents no change) and the diameter of circles scales with the magnitude of the change. For simplicity, genetic background-dependent differences in subunit expression are not taken into account.

GABA_AR subunit mRNAs in SW mice likely involves a transcriptional problem. GABA_AR subunit gene expression is known to be sensitive to regulation by depolarization, cAMP, brain derived neurotrophic factor, and mitogen-activated protein kinases (MAPK) (Bulleit and Hsieh, 2000; Thompson et al., 2000; Ives et al., 2002; Obrietan et al., 2002). The 5-HT_{1A}R couples to multiple $G_{i/o}$ -protein-signaling pathways including the inhibition of adenylyl cyclase, activation of nuclear factor- κ B, and the MAPK/ERK (extracellular signal-regulated kinase) pathway (Raymond et al., 1999). We are currently investigating which of these gene regulatory pathways is involved in mediating the downregulation of GABA_AR transcription seen in the SW 5-HT_{1A}R $R^{-/-}$ mouse.

The effect of strain background on the molecular phenotype of the 5-HT_{1A}R $R^{-/-}$ mouse is summarized in Figure 7. On the SW background, the presence of the 5-HT_{1A}R is necessary for the expression of the $\alpha_{1/2}$ GABA_AR subunit mRNAs, whereas on the B6 background, the 5-HT_{1A}R is either not required for the expression of the α subunit genes or is actually inhibiting (in the amygdala) the expression of the α_2 subunit gene. The downregulation of the subunit mRNAs in SW- $R^{-/-}$ mice is not compensated, whereas the upregulation of the α_2 subunit mRNA in B6- $R^{-/-}$ mice is fully compensated at the protein level. Clearly, the 5-HT_{1A}R is required for the optimal expression of *Gabra1* and *Gabra2* in SW and SW/B6 hybrid mice but not in B6 mice. This suggests the presence of a dominant modifier gene in the SW strain.

Genetic modification occurs when the expression of one gene alters the expression of another gene. Examples of genetic modifiers are frequent in mouse models and are increasingly recognized as a cause of phenotypic variability in humans (Nadeau, 2003). For example, a dominant modifier influences the appearance of hearing loss caused by the mutation of DFNB-26 (reces-

sive form of isolated deafness) (Riazuddin et al., 2000), the expression of HbF modifies the severity of β -thalassaemia (Weatherall, 2001), and mutations in two genes are necessary to elicit the complex heterogeneous disorder Bardet-Biedl syndrome (Katsanis et al., 2001). In our model, the molecular mediator of this regulation in SW mice and why it is not functional in B6 mice are not known. The modifier effect may be simply based on a polymorphism in the regulatory regions controlling *Gabra1* and 2 genes. Alternatively, the modifier may operate at an upstream target in the transcriptional regulatory cascade of these subunit genes.

Can the levels of the GABA_A α_1 and α_2 subunits, besides correlating with BZ sensitivity, be associated with anxiety-like phenotype in mice? As mentioned above, diminished GABA_A binding has been found in anxiety disorder patients, suggesting a link between a deficit of GABA_ARs and anxiety. In animal models, even a partial reduction of the γ_2 subunit that is a common component of most α_1 or α_2 subunit containing receptors results in anxiety in mice (Crestani et al., 1999). Interestingly, downregulation of the GABA_A α_1 subunit alone does not result in an anxiety phenotype. Although α_1 subunit knock-out mice exhibit altered sensitivity to BZ, these mice do not display significantly enhanced anxiety-related behaviors (Kralic et al., 2002). Compensatory increases in the GABA_A $\alpha_{2/3}$ subunits have been demonstrated in the GABA_A α_1 subunit knock-out mouse (Sur et al., 2001; Kralic et al., 2002), which may account for the lack of anxiety phenotype seen or may simply reflect the fact that these mutations affect a smaller fraction of the GABA_AR pool. Therefore, it is possible that in SW-R^{-/-} mice, as in BZ-resistant anxiety disorder patients, the lower level of α_1 or α_2 subunits could contribute to the anxiety phenotype. In contrast, in the B6-R^{-/-} mice, which have no alterations in GABA_A α subunit expression, another mechanism is responsible for their increased anxiety-like behavior. The molecular pathways implicated in anxiety are numerous and may include other changes in the GABAergic system or alterations in other transmitter systems linked to anxiety (Wood and Toth, 2001).

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