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Genetic and Pharmacological Evidence of a Role for $GABA_B$ Receptors in the Modulation of Anxiety- and Antidepressant-Like Behavior

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Although there is much evidence for a role of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the pathophysiology of anxiety and depression, the role of $GABA_B$ receptors in behavioral processes related to these disorders has not yet been fully established. $GABA_B$ receptors are G-protein-coupled receptors, which act as functional heterodimers made up of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. Using recently generated $GABA_{B(1)}^{-/-}$ mice, which lack functional $GABA_B$ receptors, and pharmacological tools we assessed the role of $GABA_B$ receptors in anxiety- and antidepressant-related behaviors. In the light–dark box, $GABA_{B(1)}^{-/-}$ mice were more anxious than their wild-type littermates (less time spent in the light; reduced number of transitions). $GABA_{B(1)}^{-/-}$ mice were also more anxious in the staircase test. Conversely, acute and chronic treatment with GS39783, a novel $GABA_B$ receptor positive modulator, decreased anxiety in the light–dark box and elevated zero maze tests for anxiety. On the other hand, $GABA_{B(1)}^{-/-}$ mice had decreased immobility (antidepressant-like behavior) in the forced swim test (FST). These behavioral effects are unrelated to alterations in locomotor activity. In confirmation of the genetic data, acute and chronic treatment with CGP56433A, a selective $GABA_B$ receptor antagonist, also decreased immobility in the FST, whereas GS39783 did not alter this behavior. Taken together, these data suggest that positive modulation of the $GABA_B$ receptor may serve as a novel therapeutic strategy for the development of anxiolytics, whereas $GABA_B$ receptor antagonism may serve as a basis for the generation of novel antidepressants.
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INTRODUCTION

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and hence GABAergic neurotransmission regulates many physiological and psychological processes. There are two classes of GABA receptors: ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. The $GABA_B$ receptor is a heterodimer made up of two subunits, $GABA_{B(1)}$ and $GABA_{B(2)}$, both necessary for $GABA_B$ receptors to be functionally active (Calver *et al*, 2002). Clinical and preclinical evidence strongly implicates GABAergic dysfunction in anxiety (Millan, 2003) and depression (Brambilla *et al*, 2003; Krystal *et al*, 2002); however, evidence for a specific role for $GABA_B$ receptors is unclear. Although $GABA_B$ receptors were first proposed to play a role in psychiatric disorders such as depression and anxiety over 20 years ago (Pilc and Lloyd, 1984), further progress in the field has been largely hampered by the lack of appropriate tools. The prototypical $GABA_B$ receptor agonist baclofen, although highly selective and clinically available for over 30 years for the treatment of spasticity, produces severe sedation and muscle relaxation, which confounds its widespread use as a tool in behavioral paradigms related to anxiety and depression.

Two recent developments have added innovative new tools to the armamentarium of researchers. Firstly, mice that lack the $GABA_{B(1)}$ subunit (Prosser *et al*, 2001; Queva *et al*, 2003; Schuler *et al*, 2001) have been generated. Secondly, with positive allosteric modulators, novel pharmacological tools for $GABA_B$ receptors have been characterized (Urwyler *et al*, 2001; Urwyler *et al*, 2003). These molecules enhance the action of GABA at the $GABA_B$ receptor and have little or no intrinsic agonistic efficacy on their own (Urwyler *et al*, 2001; Urwyler *et al*, 2003). Application of $GABA_B$ receptor positive modulators in the presence of an agonist shifts the concentration–response curve to the left, as the modulators increase the potency of GABA. In addition, the maximal efficacy of GABA is increased. Allosteric positive modulation of metabotropic

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receptors is a recently identified phenomenon, providing novel means for the pharmacological manipulation of G-protein-coupled receptors acting at a site apart from the orthosteric binding region of the receptor protein (Soudijn *et al*, 2002). Such properties suggest that allosteric modulators may offer a number of potential pharmacological improvements over the use of conventional agonists as has been demonstrated for modulators acting at ligand-gated ion channels (Costa, 1989). In the case of $GABA_A$ receptors, such modulation has been therapeutically utilized with the benzodiazepines, which amplify the action of the endogenous neurotransmitter GABA. Therefore, we hypothesized that $GABA_B$ receptor positive modulators will be superior drugs, devoid of the side-effect profile associated with full agonists such as baclofen.

Therefore, we have novel tools, $GABA_{B(1)}$ knockout mice and positive modulators, to better examine the role of $GABA_B$ receptors in behavioral paradigms relevant to anxiety and depression. In these studies, we investigated the behavioral effects of mice lacking $GABA_{B(1)}$ receptor subunit in animal models of anxiety and depression and provide evidence for a role of $GABA_B$ receptors in the modulation of anxiety- and depression-like behavior. To further substantiate these observations in anxiety and depression paradigms, we investigated the behavioral effects of acute and chronic treatment of the selective $GABA_B$ receptor positive modulator GS39783 and the previously identified $GABA_B$ receptor antagonist CGP56433A (Brebner *et al*, 2002; Froestl *et al*, 1995).

MATERIALS AND METHODS

Animals

The $GABA_{B(1)}$ knockout mice were generated on a BALB/c background as described previously (Schuler *et al*, 2001). Age- and sex-matched mice were used at an age of 3–8 months. Both male and female animals were used in all experiments in approximately equal numbers, with the exception of animals used in the forced swim test (FST) and tail suspension test where only females were used. There was no effect of gender on behaviors observed. In order to minimize the influence of strain effects, all pharmacological studies were carried out in male BALB/c mice (23–26 g), which were obtained from Iffa Credo, France. In a number of initial studies, heterozygous mice ($GABA_B{}^{+/-}$) were also used. No gene dosage effect was found in any of the behaviors analyzed with heterozygotes behaving similarly to knockouts. Housing was at room temperature, in a 12 h light/dark cycle with lights on at 0600. Food pellets and tap water were available *ad libitum*. All behavioral experiments were conducted during the light cycle. All animals were experimentally naïve unless otherwise noted. Experiments were subject to institutional review and conducted in accordance with the Veterinary Authority of Basel-Stadt, Switzerland.

Light–Dark Box

The light–dark box test was carried out essentially as described previously (Cryan *et al*, 2003b; Holmes *et al*, 2002). The apparatus consisted of a clear plexiglass cage ($44 \times 21 \times 21$ cm) separated into two compartments by a partition, which had a small opening (12×5 cm) at the floor level. The open compartment was open topped made of transparent plexiglass and brightly illuminated by a 60 W desk lamp overhead (approximately 1000 Lux). The smaller compartment was 14 cm long and made from black plexiglass. It was covered on top also by black plexiglass. Mice were individually placed in the center of the brightly lit compartment, facing away from the partition and allowed to explore freely the apparatus for 10 min. The apparatus was cleaned thoroughly between subjects. The number of light–dark transitions, time spent in the light compartment, and latency to enter dark were recorded by a trained observer, with transitions being the most reliable indicator of anxiety-like behavior in the test (Crawley and Davis, 1982; Holmes, 2001). Two separate cohorts of $GABA_{B(1)}$ mice were used to confirm the phenotype.

Staircase Test

The test was carried out essentially as described earlier (Cryan *et al*, 2003b; Simiand *et al*, 1984) and consists of placing an experimentally naïve mouse in an enclosed staircase with five steps made of gray plastic. Each step was 2.5 cm in height, 7.5 cm in length, and 11 cm in width. The apparatus was 45 cm in length with one end 12 cm and the other 25 cm in height. The number of steps climbed and rearings made in a 3-min period were observed. The step-climbing count was increased every time the animal moved from one step to another in the ascending direction. The apparatus was briefly wiped with a wet paper towel and dried between animals. Animals were moved to the testing room at least 1 h prior to testing. The test has been validated using different anxiolytics (Simiand *et al*, 1984; Pick *et al*, 1996; Weizman *et al*, 1999) and has been used to examine anxiety-related phenotypes in genetically modified animals (Cryan *et al*, 2003b; Salas *et al*, 2003). The number of steps climbed and the rearing behavior of the mice are recorded as measures of anxiety-related behavior.

Elevated Zero Maze

This test is similar to the more widely used elevated plus maze in that both tests rest upon similar naturalistic conflicts between the tendency to explore a novel environment and aversive properties of a novel brightly lit, open, and elevated area. However, whereas the elevated plus maze has a center area that is neither in the open or closed part of the arena, it can be difficult to interpret the level of anxiety of an animal if it stays in this central part. Indeed the $GABA_B$ agonist baclofen has been shown to promote time in the center of the plus maze (Dalvi and Rodgers, 1996). The zero maze has no central area, so the animal must be in either an open or a closed part of the arena. The apparatus was a 5.5-cm-wide circular track constructed of gray plexiglass with an inside diameter of 34 cm, a mid-track circumference of approximately 121 cm, and an elevation of 40 cm. It consisted of two open quadrants with a raised, 2 mm edge and two closed quadrants with walls 11 cm high. Mice were placed in one of the closed quadrants designated as the starting quadrant and were allowed to investigate the zero maze for a period of 5 min. During this time, an

observer scored mice on several anxiety-related variables as identified in previous studies (Shepherd *et al*, 1994; Tarantino *et al*, 2000). These included time spent in both open and closed quadrants, number of transitions between quadrants, latency to leave the dark quadrant, stretchings (elongated body posture with at least snout over open/closed divide) into open quadrant, rearings, grooming, head dips, and number of fecal boli in both open and closed areas.

Measurement of Locomotor Activity

Animals were placed in automated locomotor activity cages (31 cm length, 19 cm width, 16 cm height; TSE, Bad Homburg, Germany) and the distance traveled was measured by the number of horizontal beam-breaks as previously described (Spooren *et al*, 2000). Data were collected using a personal computer in 5 min intervals. In experiments involving $GABA_{B(1)}$ mice or chronic treatments, data were assessed in mice that were unhabituated to the apparatus. In order to detect any potential drug-induced hyperactivity, CGP56433A was administered to mice after 60 min habituation to the apparatus.

Forced Swim Test

FST was conducted as previously described (Cryan *et al*, 2001, 2003b). Briefly, mice were placed individually into plexiglass cylinders (24 cm tall × 21 cm in internal diameter) filled with water (23–25°C) to a depth of 15 cm. All test sessions were recorded by a video camera positioned directly above the cylinders. Videotapes were subsequently scored blind by a trained observer. The behavioral measure scored from videotape was the duration of immobility during the last 4 min of the 6 min test period as previously validated (Porsolt *et al*, 1978). A mouse was judged to be immobile when making only those movements necessary to keep its head above water.

Tail Suspension Test

The tail suspension test was carried out essentially as described previously (Cryan *et al*, 2003a, b; Steru *et al*, 1985), with the exception that an automated device was used to score immobility (BioSeb, Chaville, France). Mice were individually suspended by the tail to a metal hook (distance from floor = 18 cm) using adhesive tape (distance from tip of tail = 2 cm). Typically, mice demonstrated several escape-oriented behaviors interspersed with temporally increasing bouts of immobility. The computer recorded the number of seconds spent immobile over the entire 6 min period.

Drugs

Desipramine and chlordiazepoxide were obtained from Sigma (St Louis, MO). Fluoxetine, L-baclofen, GS39783 (*N*,*N*′-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4, 6-diamine), and CGP56433A (3-{1(*S*)-[3-(cyclohexyl-methyl)hydroxyphosphinyl)2(*S*)hydroxypropylamino]nethyl} benzoic acid) were synthesized in-house. All drugs were made up fresh prior to use and administered orally in a suspension of 0.5% methylcellulose at a concentration of 10 ml/kg. In the case of chronic studies, animals were injected in the afternoon (1400–1800) for 21 days and tested (either in light–dark box or in FST) on the morning following last injection. They were again injected immediately after the initial test and for the consecutive day, locomotor activity testing was carried out approximately 24 h following this last injection. Doses for chronic studies were selected from previous studies showing robust effects at these doses (Borsini *et al*, 2002) or the dose–response studies of acute administration of the compounds (data presented in these studies).

Statistics

All data were analyzed using the appropriate within-subject, and mixed-design ANOVAS or Student's *t*-test (in the case of comparisons between just two groups of animals) followed by, where appropriate, Fisher's *post hoc* tests. The level of significance was set at $P<0.05$.

RESULTS

Impact of Targeted $GABA_{B(1)}$ Receptor Subunit Deletion on Anxiety-Related Behavior

Light–dark box. Upon being placed in the light side of the apparatus, freezing behavior was observed in 30% of the $GABA_{B(1)}{}^{-/-}$ mice but none of the wild type. As shown in Figure 1, $GABA_{B(1)}{}^{-/-}$ mice displayed marked increases in anxiety-related behaviors in the light–dark box paradigm compared with wild-type ($GABA_{B(1)}{}^{+/+}$) or heterozygous ($GABA_{B(1)}{}^{-/+}$) mice. ANOVA revealed a significant effect of genotype on the time spent in the light compartments ($F(2,45)=11.02$, $P=0.001$) and on the number of transitions ($F(2,45)=4.39$, $P=0.018$). Further, there was a genotype influence on the latency to enter the dark compartment ($F(2,45)=4.86$, $P=0.012$.). *Post hoc* analysis revealed that $GABA_{B(1)}{}^{-/-}$ mice exhibited a decrease of the latency to enter the dark compartment compared to wild-type heterozygous mice. $GABA_{B(1)}{}^{-/-}$ mice showed a significant decrease in the time spent in the light compartment compared to heterozygote or wild-type mice (Figure 1b) and exhibited significantly fewer light–dark transitions (Figure 1a). This latter parameter was the most reliable indicator of anxiety in the light–dark box test. Heterozygote mice behaved in the same manner as wild-type mice in all parameters in this test. Altogether, these effects are indicative of an increased anxiety in $GABA_{B(1)}{}^{-/-}$ mice. In order to confirm the reliability of the phenotype, a second cohort of animals were tested in the light–dark box. These $GABA_{B(1)}{}^{-/-}$ mice had both qualitatively and quantitatively the same (anxious) phenotype (data not shown).

Staircase test. In the staircase test, another paradigm for assessing anxiety-related behaviors, $GABA_{B(1)}{}^{-/-}$ mice had lower number of rearings than wild-type and heterozygote mice ($F(2,45)=23.15$, $P=0.001$) (Figure 3b). In addition, the number of steps climbed by $GABA_{B(1)}{}^{-/-}$ mice was decreased compared to wild-type and heterozygote mice ($F(2,45)=52.61$, $P=0.001$) (Figure 3a). This lack of exploration in the test was associated with a substantial amount of freezing behavior.

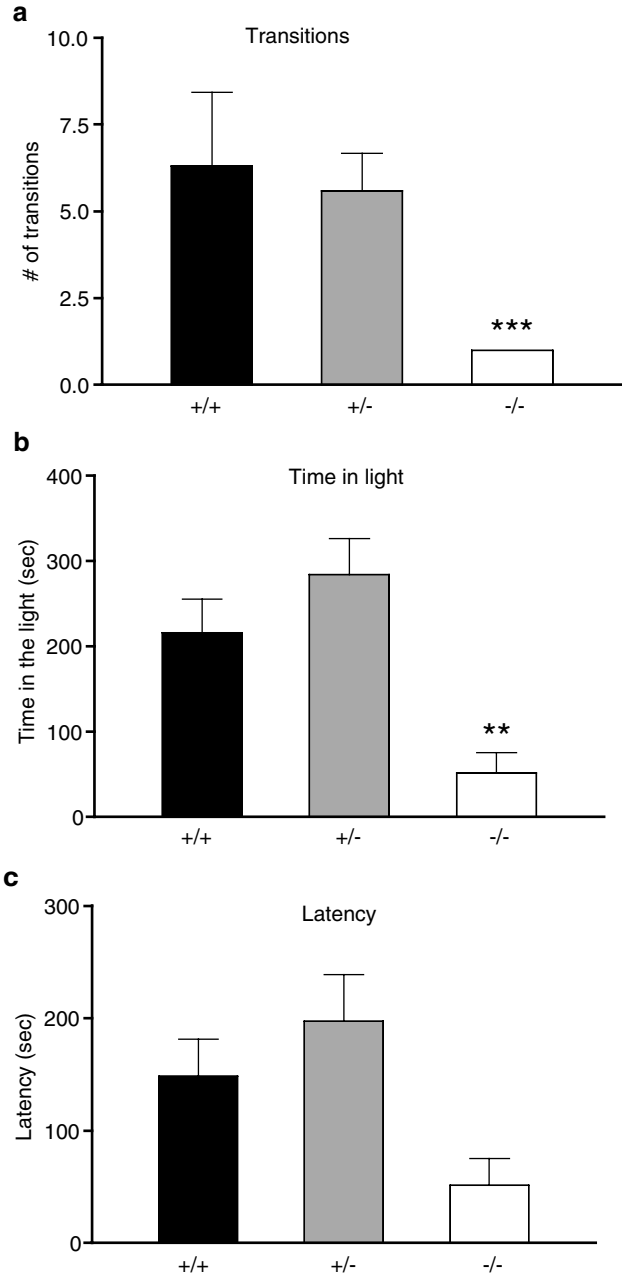

Figure 1 Increased anxiety in $GABA_{B(1)}$-deficient mice in the light–dark box. (a) $GABA_{B(1)}{}^{-/-}$ mice had a marked decrease of transitions between light and dark compartments compared with heterozygote or wild-type mice. (b) $GABA_{B(1)}{}^{-/-}$ mice spent less time in the light compartment in comparison to heterozygous or wild-type mice. (c) $GABA_{B(1)}{}^{-/-}$ mice ($n=16$) exhibited a decrease in latency to enter the dark compartment, compared to heterozygous ($n=16$), but not compared to wild-type mice ($n=16$). All bars represent mean values, with vertical lines indicating one SEM. *, **, ***Groups that differed significantly compared to wild-type mice ($P<0.05$, <0.01, and <0.001, respectively).

In summary, both the behavior in the light–dark test and staircase tests score demonstrate an increased level of anxiety in $GABA_{B(1)}{}^{-/-}$ mice.

Elevated zero maze. No functional data were obtained from examining the behavioral response of $GABA_{B(1)}{}^{-/-}$ mice in the elevated zero maze due to the fact that all of the $GABA_{B(1)}{}^{-/-}$ mice actively jumped off the maze. The

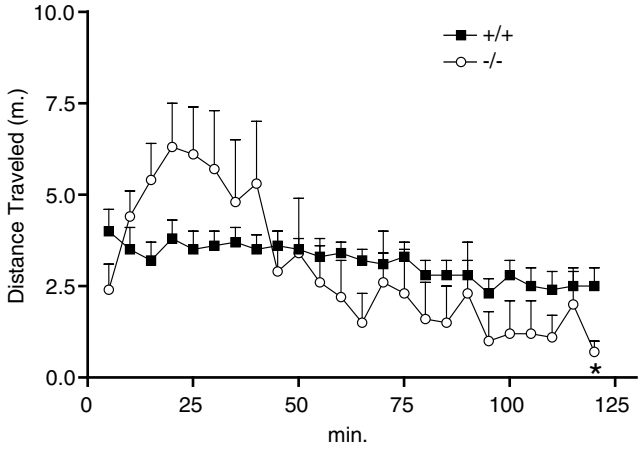

Figure 2 Effect of $GABA_{B(1)}$ deletion on locomotor activity in naïve mice. No significant effect of genotype was seen; however, three distinct phases of activity were observed in $GABA_{B(1)}{}^{-/-}$ mice compared with wild type: hypoactivity followed by a hyperactive response followed by rebound hypoactivity. $n=20$ per genotype group. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to wild-type mice ($P<0.05$).

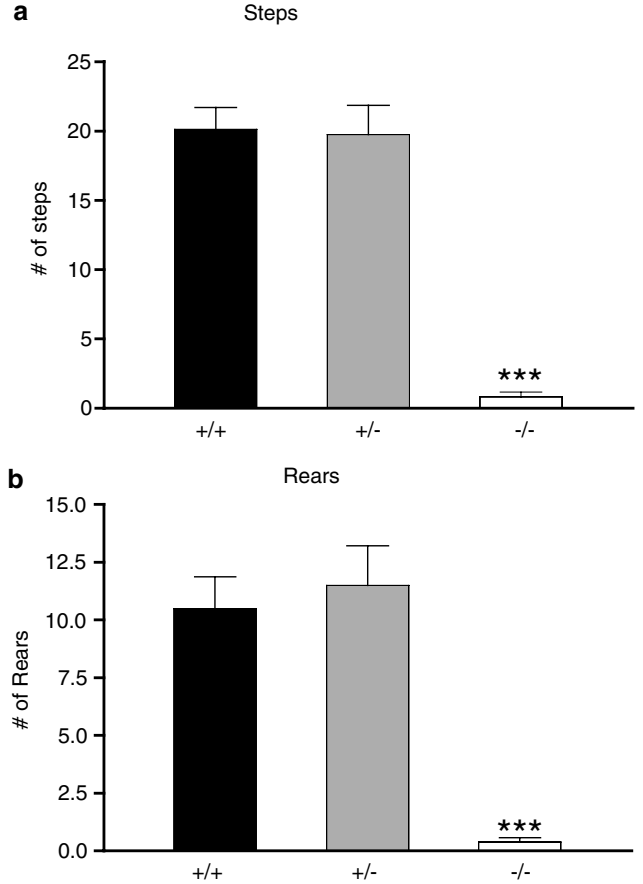

Figure 3 Increased anxiety in $GABA_{B(1)}{}^{-/-}$ mice in the staircase test. $GABA_{B(1)}{}^{-/-}$ mice ($n=16$) exhibited a decrease in the steps climbed compared to heterozygous ($n=16$) and wild-type ($n=16$) mice. (b) $GABA_{B(1)}{}^{-/-}$ mice had significantly less rearing events compared to heterozygous or wild-type mice. All bars represent mean values, with vertical lines indicating one SEM. ***Groups that differed significantly compared to wild-type mice ($P<0.001$).

reasons for this increased flight response are likely to reflect an increase in anxiety/panic-like behavior as opposed to lack of motor coordination as evidenced by absence of motor deficits in rotarod tests (Schuler *et al*, 2001; C Mombereau and JF Cryan unpublished observations). Further, similar flight reactions from an unstable elevated maze have been recently characterized as a novel model of panic/anxiety in rodents (Jones *et al*, 2002a, b; King, 1999a, b). Additionally, such an ethological response has also been demonstrated in the wild house mouse (*Mus musculus*) in the elevated plus maze (Holmes *et al*, 2000).

Locomotor activity tests in $GABA_{B(1)}{}^{-/-}$ mice. As shown in Figure 2, the locomotor activity of $GABA_{B(1)}{}^{-/-}$ mice is complex and can be divided into three parts: a short 'low-activity' pattern' (0–5 min), a 'rebound' pattern associated with a large increase of locomotor activity (10–45 min), and finally a pattern of hypoactivity (45–120 min). ANOVA revealed no effect of genotype on locomotor activity ($F(1,38) = 0.053$, $P = 0.819$), and there was a significant genotype × time interaction ($F(23,874) = 3.221$, $P = 0.001$).

Effects of a $GABA_B$ Receptor Positive Modulator on Anxiety-Related Behavior

Given the anxious phenotype of $GABA_B$ receptor knockout mice, we hypothesized that activation of the $GABA_B$ receptor would reduce anxiety. Hence we tested the effects of a novel $GABA_B$ receptor positive modulator GS39783 (Urwyler *et al*, 2003) in animal models of anxiety.

Light–dark box test. As shown in Figure 4, ANOVA indicated an effect of drug treatment on the number of transitions between dark and light compartments ($F(4,45) = 10.06$, $P = 0.001$). *Post hoc* analysis revealed that GS39783 (0.3–30 mg/kg, p.o.) and the benzodiazepine chlordiazepoxide (10 mg/kg, p.o.) increased the number of transitions. Treatment with GS39783 or chlordiazepoxide 1 h prior to testing failed to influence the latency to enter the dark chamber but increased the time spent in the light compartment ($F(4,45) = 9.30$, $P = 0.001$). *Post hoc* analysis indicated a significant effect of both chlordiazepoxide and GS39783 (only at the highest dose tested—30 mg/kg). These effects are not due to any confounding effect of GS39783 on locomotor activity as acute administration of GS39783 is devoid of any effects on locomotor activity (JF Cryan and WP Spooren, unpublished observations). It is of interest that the basal levels of anxiety in the light–dark test in Figure 4 are considerably different from those in Figure 1. The reason for this may lie in the fact that these mice are purchased from Iffa Credo and those in Figure 1 are wild-type BALB/c mice, which were housed with their more anxious littermates.

In an attempt to assess the effects of chronic administration of the positive modulator on anxiety-like behavior, we tested GS39783 in addition to CGP56433A (a selective $GABA_B$ receptor antagonist) and the antidepressants fluoxetine and desipramine in the light–dark box (20–24 h following last treatment). ANOVA revealed an effect of chronic drug treatment on the time spent in the light side of the arena ($F(4,55) = 2.573$, $P = 0.04$) and the number of

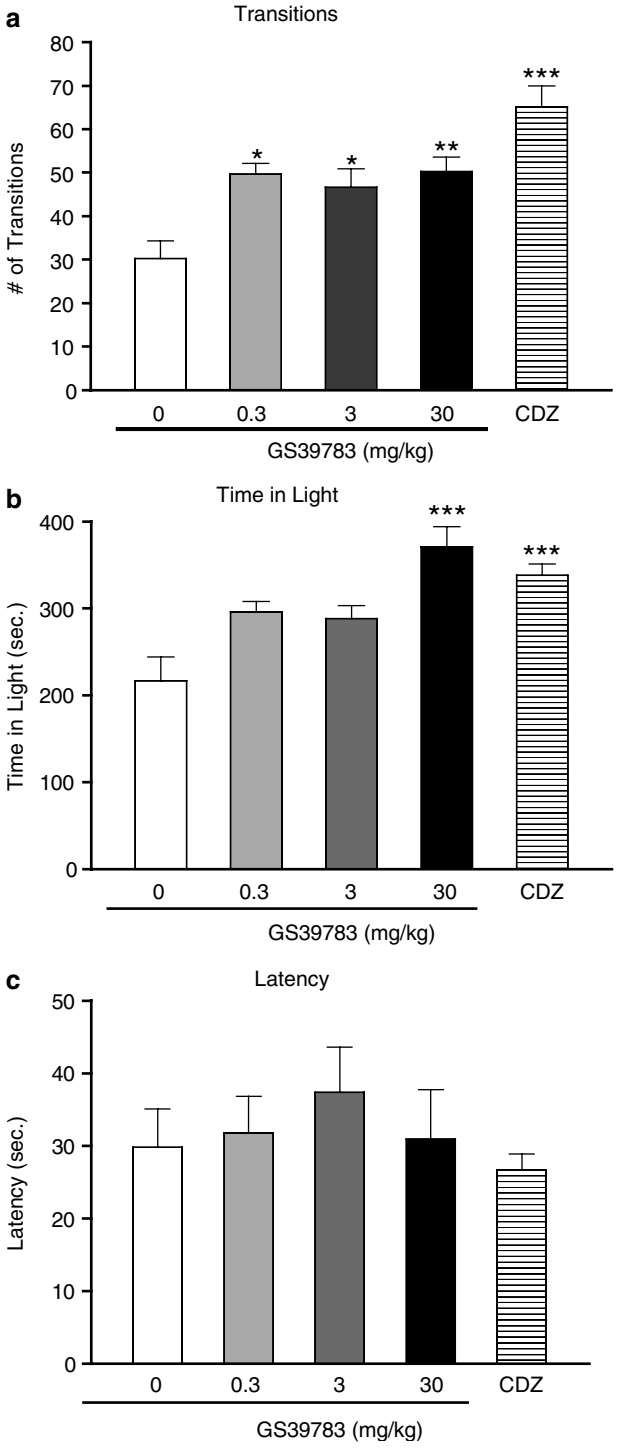

Figure 4 Anxiolytic effects of acute treatment with the $GABA_B$ receptor positive modulator GS39783 in the light–dark test. Effects of acute $GABA_B$ positive modulator treatment (doses: 0, 0.3, 3, or 30 mg/kg, p.o.) and chlordiazepoxide (CDZ, 10 mg/kg, p.o.) on (a) the number of transitions between light and dark compartments during the test, (b) the time spent in the light compartment, and (c) the latency to enter the dark compartment. $n = 10$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *,**,***Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$, < 0.01, and < 0.001, respectively).

transitions between the light and the dark sides ($F(4,55) = 2.637$, $P = 0.04$), but had no effect on the latency to enter the dark compartment (Figure 5a). *Post hoc* analysis revealed that GS39783 was the only compound tested to modify significantly the number of transitions (Figure 5a) and the time spent in the light side of the arena (data not shown). Taken together, these results indicate a potential anxiolytic effect of acute and chronic GS39783 treatment. As shown in Figure 5b, these effects are not due to any confounding effect of GS39783 on locomotor activity, as chronic administration of GS39783 did not affect locomotor activity ($F(4,53) = 0.9289$, $P = 0.4543$). It is of interest that the basal levels of anxiety in the light–dark test in Figure 5 are considerably different from those in Figure 4. The reason for this may lie in the fact that although all mice are purchased from Iffa Credo, those in Figure 5 have been handled and injected daily for 21 days and this stress has been shown to influence anxiety-like behavior in mice (Lapin 1995).

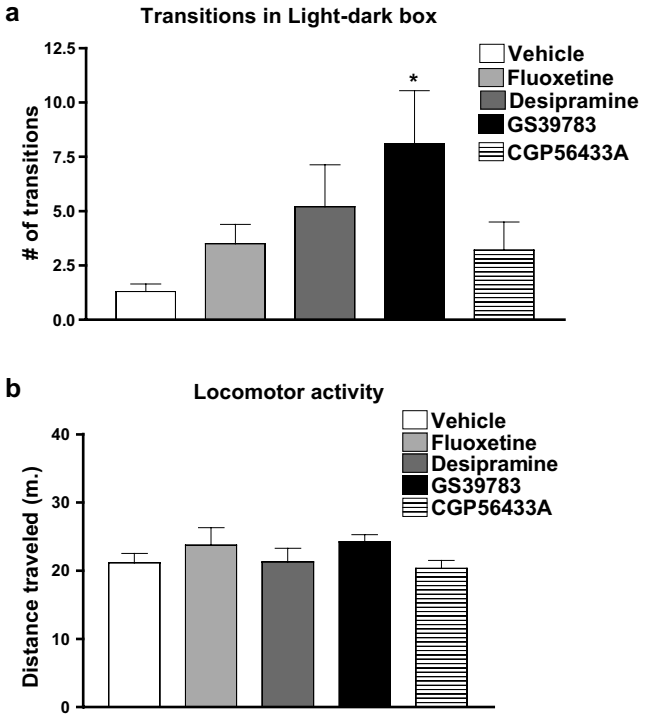

Figure 5 Chronic treatment with the $GABA_B$ receptor positive modulator reveals anxiolytic effects in the light–dark box test. Chronic treatment (21 days) with $GABA_B$ receptor positive modulator GS398783 (10 mg/kg, p.o., once daily) significantly increased (a) the number of transitions between light and dark compartments during the test, whereas fluoxetine (10 mg/kg, p.o., once daily), desipramine (15 mg/kg, p.o., once daily), and the $GABA_B$ receptor antagonist (3 mg/kg, p.o., once daily) were without effect. $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$). (b) Locomotor activity in a novel environment following chronic (23 days) administration of the $GABA_B$ receptor positive modulator (10 mg/kg, p.o.), fluoxetine (10 mg/kg, p.o.), desipramine (15 mg/kg, p.o.), and $GABA_B$ receptor antagonist (3 mg/kg, p.o.). Testing was carried out for 30 min 24 h following last dose in the same animals previously tested in the light–dark box. None of the treatments altered locomotor activity, indicating that the effects of GS39783 in the light–dark box are not due to any secondary stimulant effect. $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM.

Elevated zero maze. To further confirm the anxiolytic effects of GS39783, we tested it in comparison with chlordiazepoxide in the elevated zero maze in BALB/c mice, the background strain on to which $GABA_{B(1)}{}^{-/-}$ mice were generated. ANOVA revealed that drug treatment decreased the latency to enter the open sides of the maze ($F(4,55) = 3.192$, $P = 0.020$), the number of stretched-attend postures ($F(4,55) = 13.16$, $P < 0.0001$) and increased the time spent in the open side of maze ($F(4,55) = 3.932$, $P = 0.007$), increased the number of head dips ($F(4,55) = 6.995$, $P < 0.00001$), number of rearings ($F(4,55) = 8.233$, $P < 0.0001$), and the number of line crossings ($F(4,55) = 33.76$, $P < 0.0001$). *Post hoc* analysis revealed that chlordiazepoxide (10 mg/kg p.o.) significantly affected all parameters tested, whereas GS39783 treatment reduced the latency to enter the open side at the highest dose tested (30 mg/kg, p.o.; $P < 0.05$) (Figure 6a), and at doses of 3–30 mg/kg reduced the number of stretch-attend postures (Figure 6c) only. There was a trend toward GS39783 increasing the time in the open parts of arena, which failed to reach the level of significance (Figure 6b). GS39783 failed to affect the number of head dips, number of rearings, and the number of line crossings at any dose tested (data not shown). Taken together, these data further suggest an anxiolytic effect of GS39783, although the magnitude of the effects in this test are much less robust compared with that induced by benzodiazepine anxiolytics.

Impact of Targeted Deletion of $GABA_{B(1)}$ Receptor on Depressive-Related Behaviors

Forced swim test. The FST is the most widely used tool for assessing depression and antidepressant-related phenotypes in genetically altered mice (Cryan *et al*, 2002; Cryan and Mombereau, 2004; Porsolt, 2000); hence we examined the effects of mice with a targeted deletion of the $GABA_{B(1)}$ receptor subunit on behavior in this test. As shown in Figure 7a, there was a significant effect of genotype on immobility time in the FST (*t*-test, $P = 0.012$). $GABA_{B(1)}{}^{-/-}$ mice had a significantly lower immobility time as compared to wild-type control mice. The magnitude of reduced immobility of the $GABA_{B(1)}{}^{-/-}$ mice in this test is similar to that we and others have reported for a variety of antidepressants, including selective monoamine reuptake or oxidase inhibitors (Cryan *et al*, 2001; Lucki *et al*, 2001; Porsolt *et al*, 1978). It is noteworthy that there was no observable occurrence of seizures or altered motor patterns in animals subsequent to being submerged in water.

Tail suspension test. We also tested the animals in the tail suspension test, another well-validated model for assessing depression-related behavior in mice (Steru *et al*, 1985). Further confirming accumulating evidence, that both tests rely on different neurochemical substrates to mediate their behavioral effects, deletion of $GABA_{B(1)}$ receptor subunit failed to affect the immobility score in this test (*t*-test, $P = 0.710$) (Figure 7b). There was no observable occurrence of seizures or altered motor patterns in animals subsequent to being suspended by the tail. Further, no tail climbing was observed as has been reported with other background strains of mice (Mayorga and Lucki, 2001).

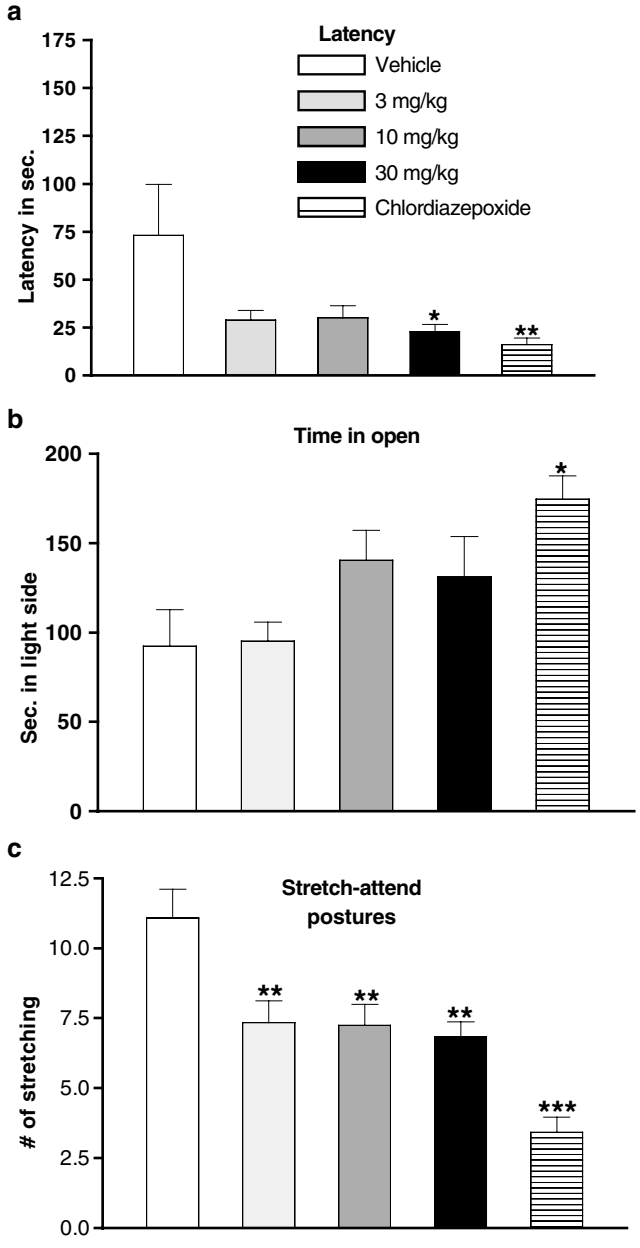

Figure 6 Effects of acute treatment with GS39783 on anxiety behavior in the elevated zero maze test. Both the acute $GABA_B$ positive modulator GS39783 and chlordiazepoxide (10 mg/kg, p.o.) affected (a) the latency to enter the open side of the maze and (c) the number of stretched-attend postures. However, only chlordiazepoxide significantly increased the time spent in the open quadrants of the maze (b). $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *·**·***Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$, < 0.01, and < 0.001, respectively).

Locomotor activity tests in $GABA_{B(1)}{}^{-/-}$ mice. In order to address the issue of whether the behavioral effects of $GABA_{B(1)}{}^{-/-}$ mice seen in the FST are related to potential hyperactivity, we analyzed the locomotor pattern. In a novel environment, the locomotor activity of the same mice that had previously undergone the FST was recorded over a period of 30 min. Repeated measures ANOVA revealed a clear impact of the targeted deletion of $GABA_{B(1)}$ receptor subunit on locomotor activity ($F(1,29) = 9.9$, $P = 0.001$). As

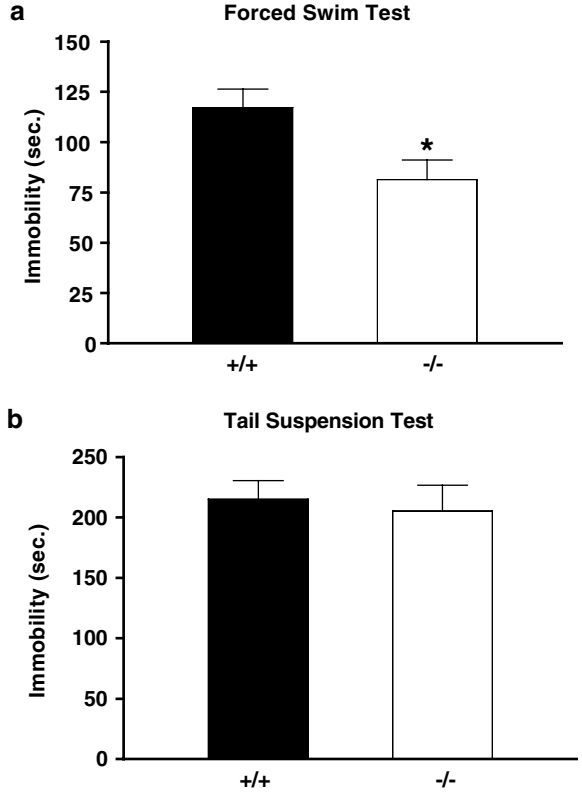

Figure 7 Antidepressant-like behavior in $GABA_{B(1)}{}^{-/-}$ mice. (a) $GABA_{B(1)}{}^{-/-}$ mice ($n = 16$) had a much lower immobility score than wild type ($n = 16$) in the mouse FST, which indicates an antidepressant-like effect. (b) $GABA_{B(1)}{}^{-/-}$ mice ($n = 15$) exhibited no difference in immobility compared to wild-type mice ($n = 16$) in the mouse tail suspension test. All bars represent mean values, with vertical lines indicating one SEM. *Groups that differed significantly compared to wild-type mice ($P < 0.05$).

shown in Figure 8a, $GABA_{B(1)}{}^{-/-}$ mice exhibited a lower horizontal activity compared to wild-type mice during the first 20 min of the trial. This reduction of locomotor activity during the first minutes of trial could translate into a deficit in habituation to a novel environment in $GABA_{B(1)}{}^{-/-}$ mice and/or to an increased freezing behavior.

Correlations were also made between activity in the FST and the first 10 min in the novel locomotor activity chambers. Similar correlations were made with data obtained in the tail suspension test. As shown in Figure 8b, there was no correlation between locomotor activity (distance traveled) and immobility in FST in wild-type mice ($R = 0.349$, $P = $ NS) as well as in $GABA_{B(1)}{}^{-/-}$ mice ($R = 0.008$, $P = $ NS). These results suggest an absence of a stimulant effect as a result of $GABA_{B(1)}$ deletion. Additionally, no correlation was observed between immobility in the tail suspension test and locomotor activity in a novel environment (data not shown).

Effect of a $GABA_B$ Receptor Antagonist on Depressive-Related Behavior

Acute studies with CGP56433A. To test whether the antidepressant-like effect due to genetic deletion of the $GABA_{B(1)}$ receptor subunit could be recapitulated following pharmacological antagonism, we tested the highly selective and potent $GABA_B$ receptor antagonist CGP56433A in the

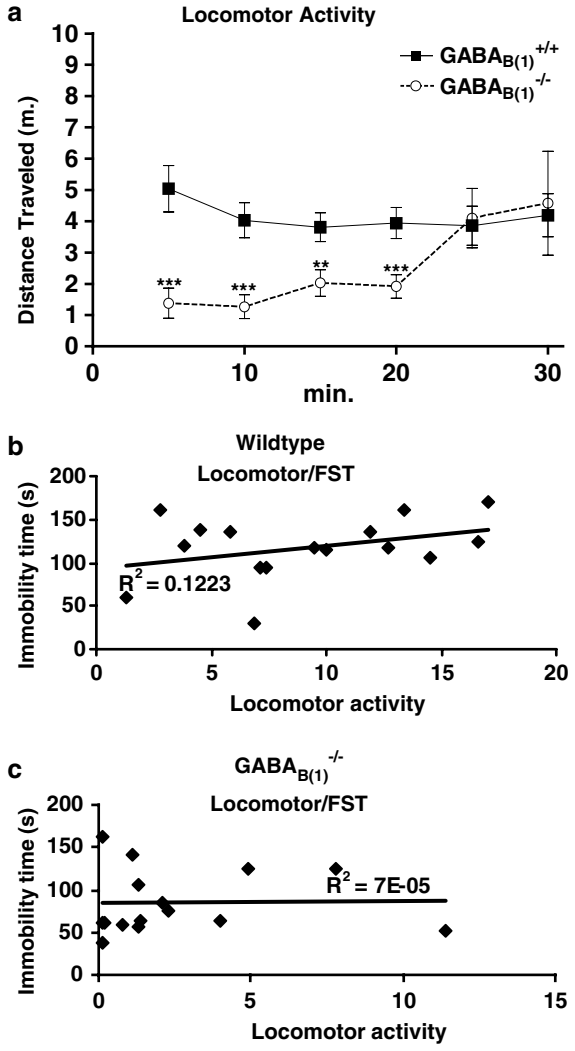

Figure 8 Effect of $GABA_{B(1)}$ deletion on locomotor activity in mice pretested with FST: deficits in habituation and lack of correlation with FST. (a) $GABA_{B(1)}{}^{-/-}$ mice ($n=15$) had a much lower locomotor activity score than wild-type mice ($n=16$) during the first 20 min of the 30 min trial. There was no consistent correlations between immobility score in the FST and locomotor activity score in wild-type mice (b) and $GABA_{B(1)}{}^{-/-}$ mice (c). All bars represent mean values, with vertical lines indicating one SEM. **, ***Groups that differed significantly compared to wild-type mice ($P<0.01$ and <0.001, respectively).

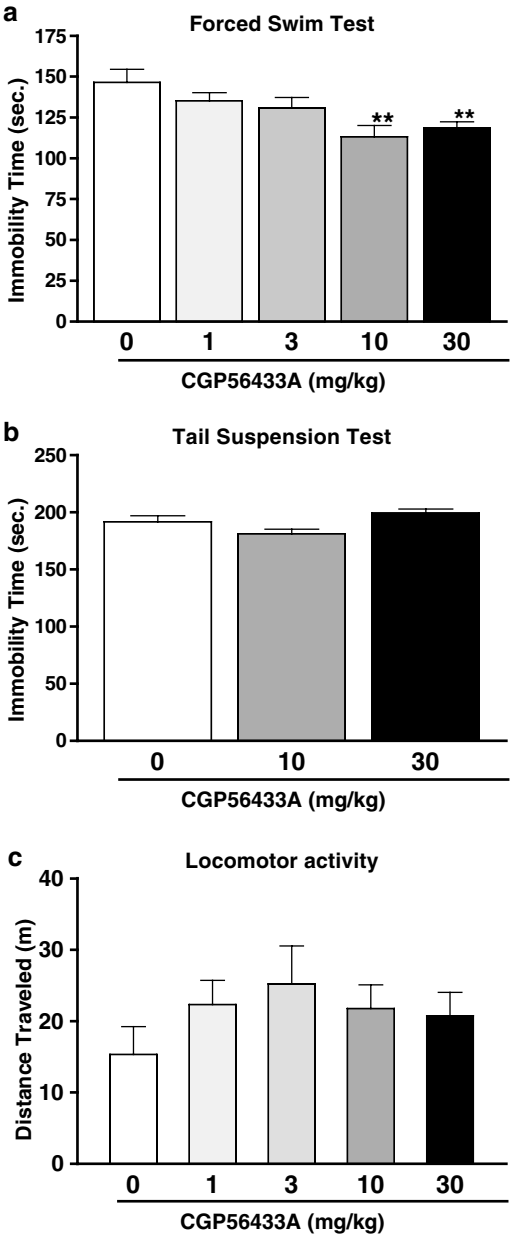

Figure 9 Acute treatment with CGP56433A reduces immobility in FST but not TST. (a) Effect of CGP56433A treatment (doses: 1, 3, 10, and 30 mg/kg, p.o.) on immobility time in FST. $n=10$–12 per treatment group. (b) Effect of CGP56433A treatment (doses: 0, 10, and 30 mg/kg, p.o.) on immobility time in the tail suspension test. $n=10$ per treatment group. (c) Effect of CGP56433A treatment (doses: 1, 3, 10, and 30 mg/kg, p.o.) on locomotor activity (60 min) in mice that were habituated (for 60 min) to the novel environment. $n=12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. **Groups that differed significantly compared to vehicle-treated mice ($P<0.01$).

FST. As shown in Figure 9a, acute administration of CGP56433A affected immobility time in the FST ($F(4,53)=4.56$, $P=0.003$). *Post hoc* analysis revealed that CGP56433A (10 and 30 mg/kg) produced a significant decrease in immobility.

Further, we tested CGP56433A in the TST also. As shown in Figure 9b, CGP56433A failed to alter immobility in the test ($F(2,27)=0.24$, $P=0.791$), thus replicating the profile of genetic antagonism. Of note, CGP56433A failed to influence locomotor activity in habituated mice significantly (Figure 9b). These data exclude any potential stimulant effect of CGP56433A contributing to behavior in the FST.

Chronic studies. As shown in Figure 10, animals administrated chronically (21 days) with both CGP56433A (3 mg/kg, p.o., once daily) and desipramine (10 mg/kg, p.o., once daily) reduced immobility times in the FST whereas GS39783 was without any effect ($F(3,44)=7.966$, $P=0.001$).

DISCUSSION

In these studies, we sought to combine pharmacological and genetic approaches to obtain converging information on the

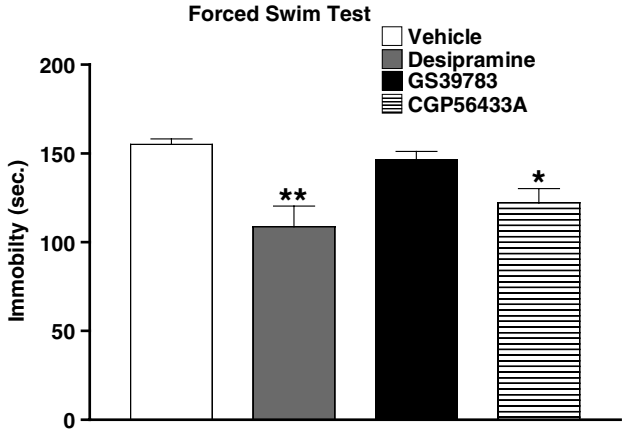

Figure 10 Chronic treatment with CGP56433A and desipramine reduces immobility in the FST. Effects of chronic treatment (21 days) with the $GABA_B$ antagonist CGP56433A (3 mg/kg, p.o.), desipramine (15 mg/kg, p.o.), and the $GABA_B$ positive modulator GS39783 (10 mg/kg, p.o.) on immobility time in the FST. $n = 12$ per treatment group. All bars represent mean values, with vertical lines indicating one SEM. *,**Groups that differed significantly compared to vehicle-treated mice ($P < 0.05$ and < 0.01, respectively).

function of $GABA_B$ receptors in behavioral processes. Using this dual approach, we demonstrate that through differential pharmacological manipulation of $GABA_B$ receptors, one can modify behaviors relevant to anxiety and depression. Deletion of $GABA_{B(1)}$ receptor subunit results in a more anxious phenotype in mice and an increased resistance to stress-induced behavioral despair. Congruent with these data, activation of $GABA_B$ receptors results in anxiolysis, whereas treatment with a $GABA_B$ receptor antagonist results in antidepressant-like effects in animal models. Given the complex overt behavioral phenotype of $GABA_{B(1)}{}^{-/-}$ mice, which includes a high propensity for spontaneous epileptic seizures, hyperalgesia, and amnesia (Schuler *et al*, 2001), it was important to combine both genetic and pharmacological approaches. Together, these studies clearly demonstrate that $GABA_B$ receptors play a role in the modulation of behaviors relevant to anxiety and depression.

Using the light–dark box, one of the most widely used tests for assessing anxiety-related behavior in rodents (Holmes, 2001), we clearly show that $GABA_{B(1)}{}^{-/-}$ mice are more anxious than their wild-type counterparts (Figure 1). Complimentary data were also found in the staircase anxiety test, where $GABA_{B(1)}{}^{-/-}$ mice had a substantial increase in freezing behavior and failed to explore the elevated platform compared to wild-type animals (Figure 3). It should be noted that this increase in anxiety-related behaviors is robust and not masked by the already high anxiety of the parental strain. In a variety of paradigms, it has been shown that BALB/c mice exhibit increased anxiety-related behaviors compared to other inbred strains of mice (Belzung and Griebel, 2001). The use of mice on this background strain was essential for the generation of $GABA_B$-related knockout animals, as mice on other background strains died very prematurely (Prosser *et al*, 2001; Queva *et al*, 2003). Interestingly, unlike genetic deletion, chronic pharmacological antagonism of $GABA_B$ receptors with CGP56433A failed to alter anxiety-related behavior in the light–dark box (Figure 5). This indicates that loss of the receptor during development may be critical for the increased anxiety phenotype to be unveiled; indeed using conditional knockout technology, such an assertion has recently been ascertained for the $5\text{-}HT_{1A}$ receptor (Gross *et al*, 2002). It is unlikely that the increased anxiety-like behavior is due to motor failure in the animals. Although $GABA_{B(1)}{}^{-/-}$ mice have less activity in locomotor chambers, their activity increases over time as they habituate to the environment (see Figures 2 and 8).

Given that $GABA_{B(1)}{}^{-/-}$ mice have elevated anxiety-like behavior, we hypothesized that by activating $GABA_B$ receptors we would be able to decrease anxiousness in normal animals placed in an aversive environment. Following acute administration of the recently identified $GABA_B$ receptor positive modulator GS37983 (Urwyler *et al*, 2003), animals displayed reduced anxiety in the light–dark box test (Figure 4) and elevated zero maze (Figure 6). Further, the anxiolytic effects of GS39783 were also observed following chronic treatment (Figure 5). Being a positive modulator, GS37983 is potentially advantageous over full $GABA_B$ agonists, which potentially engenders it more amenable for use *in vivo*. The major side effects associated with full agonists include sedation, muscle relaxation, hypothermia, and cognitive impairing effects.

Previous data investigating $GABA_B$ mechanisms in anxiety are limited and rather variable. This is largely because investigators relied on using the prototypical full $GABA_B$ receptor agonist baclofen for such analysis. Baclofen has a narrow efficacy window before confounding side effects are manifested in anxiety paradigms (Dalvi and Rodgers, 1996). That said, baclofen has demonstrated anxiolytic-like effects in a number of tests. It reduced separation induced calling by mouse pups (Nastiti *et al*, 1991) and enhanced punished drinking in rats (Ketelaars *et al*, 1988; Shephard *et al*, 1992) and had an anxiolytic-like response to novelty in a T-Maze (Quintero *et al*, 1985). Further, baclofen also reversed the anxiogenic response induced by withdrawal from chronic diazepam or alcohol treatment (Andrews and File, 1993; File *et al*, 1991; File *et al*, 1992). Clinically, baclofen reversed the anxiety associated with alcohol withdrawal (Addolorato *et al*, 2002) and post-traumatic stress (Drake *et al*, 2003). Thus our data suggest that $GABA_B$ receptor positive modulators may be a novel class of anxiolytic agents devoid of side effects associated with baclofen or benzodiazepines.

The mouse FST is the most widely used experimental paradigm for detecting antidepressant activity and to assess alterations in depression-like behavior in genetically modified animals (Borsini and Meli, 1988; Cryan *et al*, 2002; Cryan and Mombereau, 2004). The behavioral responses in the FST are thought to comprise a coping strategy (Thierry *et al*, 1984) in which immobility behaviors represent the psychological concept of 'entrapment' described in clinical depression (Dixon, 1998; Gilbert and Allan, 1998; Lucki, 2001). Here we demonstrate that $GABA_{B(1)}{}^{-/-}$ mice have an antidepressant-like effect in the FST as indicated by significantly lower immobility than their wild-type controls. This effect is not due to hyperactivity *per se,* as a reduced locomotor response was observed in the very same mice after being placed in a novel locomotor activity chamber, with activity increasing over

time. This is compatible with the anxious phenotype of $GABA_{B(1)}{}^{-/-}$ mice and suggests that they are more fearful upon being placed in a novel environment. In opposition to normal habituation responses in a novel environment, locomotor activity in $GABA_{B(1)}{}^{-/-}$ mice slowly increased with time, indicating a disinhibition of their initial anxiety. Further, there was no correlation between activity in the FST and that in the locomotor activity apparatus (Figure 8). This initial hypoactivity was unrelated to prior exposure to swim stress or age, as it was also evident (although not as pronounced) in experimentally naïve mice (Figure 2). However, at later time points, these animals became somewhat more active than wild-type controls, which is in accordance with previous data (Schuler *et al*, 2001).

Interestingly, $GABA_{B(1)}{}^{-/-}$ mice behave similarly to their wild-type controls in the tail suspension test. The tail suspension test is another well-characterized test for assessing depression- and antidepressant-like activity (Cryan *et al*, 2001, 2002, 2003b; Porsolt, 2000). Although this test is similar to the FST in the constructs that it purports to assess (immobility) and for its ability to detect a broad spectrum of antidepressants (Steru *et al*, 1985), it is becoming clear that both tests are probably different from each other in terms of the biological substrates that underlie their observed behaviors (Bai *et al*, 2001; Cryan and Mombereau, 2004; Renard *et al*, 2003). Accordingly, it is believed that using both paradigms can give complementary and/or converging information on activities of novel potential antidepressants or molecular pathways including those altered in genetically modified animals (Bai *et al*, 2001; Conti *et al*, 2002; Cryan *et al*, 2003b; Porsolt, 2000). The current data are among the first to show differential effects of a genetic modification in the FST and the tail suspension test, and confirm the assertion of a differential neurochemical underpinning to each test.

In order to confirm the antidepressant-like phenotype of the $GABA_{B(1)}{}^{-/-}$ mice pharmacologically, we assessed the effects of the $GABA_B$ receptor antagonist CGP56433A in the FST. Our data demonstrate that this $GABA_B$ receptor antagonist when administered acutely also decreases immobility in the FST without having any significant change in locomotor activity (Figure 9). Chronic administration of CGP56433A also produced an antidepressant-like effect similar to that of the antidepressant desipramine (Figure 10). Although accumulating evidence implicates GABAergic dysfunction in depression (Brambilla *et al*, 2003; Krystal *et al*, 2002), evidence for a specific role for $GABA_B$ receptors in depression and in the mechanism of action of antidepressants is limited and controversial, with rival hypotheses being purported that both positive and negative modulation of this receptor may be a useful antidepressant therapy (Lloyd *et al*, 1987; Nakagawa *et al*, 1999). Of late, more emphasis has been placed on $GABA_B$ receptor antagonism as a potential therapeutic strategy for depression (Bowery *et al*, 2002). In support of this, antidepressant-like effects were reported after chronic treatment with the $GABA_B$ receptor antagonist CGP51176 in the chronic mild stress model of depression in rats and in the rat FST (Bittiger *et al*, 1996). Further, using the learned helplessness model, it has been shown that the $GABA_B$ receptor antagonist CGP36742 had an antidepressant-like response (Nakagawa *et al*, 1999), whereas baclofen increased susceptibility to helplessness and attenuated the effects of antidepressants (Nakagawa *et al*, 1996a, b). Furthermore, baclofen also reduced the efficacy of antidepressants in the FST (Nakagawa *et al*, 1996c). Of note, $GABA_B$ receptor antagonists (including CGP56433A) increase BDNF expression in the hippocampus and cortex (Heese *et al*, 2000), which may contribute to their antidepressant-like effects (Conti *et al*, 2002; Shirayama *et al*, 2002). Taken together, our current data support the contention that antagonism of $GABA_B$ receptors may be a suitable target for the development of antidepressant agents.

Superficially at least, it may seem counterintuitive that modulation of a given receptor may induce a differential effect on anxiety- and depression-like behaviors, given the extensive comorbidity of such disorders clinically (Moller, 2002). However, $GABA_B$ receptors are localized both pre- and postsynaptically, and the elucidation of the relative contribution of these individual receptor populations to behavioral phenotypes is currently not possible. Interestingly, mice lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65), which plays an essential role in GABA synthesis, have a similar phenotype to $GABA_{B(1)}{}^{-/-}$ mice (increased anxiety and decreased depression-related behavior; Stork *et al*, 2000, 2003). $GAD65^{-/-}$ mice have a deficit in the temporal increase in GABA synthesis, which occurs postnatally in wild-type animals. It is tempting to speculate that the phenotype of these mice may be in part related to insufficient agonist occupancy at $GABA_B$ receptors especially during critical postnatal periods. Also of note is the fact that such a behavioral pattern is also observed in mice lacking the $5\text{-}HT_{1A}$ receptor (Ramboz *et al*, 1998) and in mice overexpressing CRF (van Gaalen *et al*, 2002). $GABA_B$ receptors are densely localized on, and intricately interact with, serotonergic neurons in the dorsal raphe nucleus (DRN) (Abellan *et al*, 2000a, b; Burman *et al*, 2003; Serrats *et al*, 2003; Tao *et al*, 1996). Given that serotonin can modulate anxiety and depression in opposite manners, with high serotonergic activity being associated with anxiety and low activity with depression (Cryan and Leonard, 2000; Graeff *et al*, 1996), it is plausible that differential interaction of $GABA_B$ receptors on 5-HT neuronal firing at the level of the DRN may be in part responsible for the behavioral effects subsequent to genetic and pharmacological manipulations of $GABA_B$. However, future studies are needed to understand the functional interactions of $GABA_B$ receptors with 5-HT and with other neurotransmitter systems and how these may contribute to the manifestation of differential anxiolytic- and antidepressant-like effects of $GABA_B$ receptor positive allosteric modulators and antagonists, respectively.

In conclusion, the current results demonstrate that $GABA_B$ receptors are important regulators of emotional behavior. However, we acknowledge both the inherent difficulties and the caution needed in the interpretation of behavioral analysis of genetically modified mice such as the $GABA_{B(1)}{}^{-/-}$ mice, which have overt behavioral disturbances, in more defined tests relevant to psychopathology. Nonetheless, the current data show that even such mice can still be utilized to give important indicators of the role of a given protein, in this case the $GABA_B$ receptor, in a molecular pathway relevant for the manifestation of anxiety or depression. These assertions can then be confirmed more

parametrically using appropriate pharmacological activators and antagonists as we have done using novel $GABA_B$ receptor positive modulators or antagonists.

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