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ORIGINAL INVESTIGATION

Behavioral evaluation of mice deficient in $GABA_{B(1)}$ receptor isoforms in tests of unconditioned anxiety

Laura H. Jacobson · Bernhard Bettler · Klemens Kaupmann · John F. Cryan

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Abstract
Rationale Emerging data support a role for $GABA_B$ receptors in anxiety. $GABA_B$ receptors are comprised of a heterodimeric complex of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits. The predominant neuronal $GABA_{B1}$ receptor isoforms are $GABA_{B(1a)}$ and $GABA_{B(1b)}$. Recent findings indicate specific roles for these isoforms in conditioned fear responses, although their influence on behavior in tests of unconditioned anxiety is unknown.
Objective The aim of this study was to examine the role of the $GABA_{B(1)}$ isoforms in unconditioned anxiety.
Materials and methods Mice deficient in the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ receptor isoforms were examined in a battery of anxiety tests.
Results In most tests, genotype did not significantly affect anxious behavior, including the elevated plus maze, marble burying, and stress-induced hypothermia tests. Corticosterone and adrenocorticotropic hormone levels were similarly unaffected by genotype. Female, but not male, $GABA^{-/-}_{B(1a)}$ and $GABA^{-/-}_{B(1b)}$ mice showed increased anxiety relative to wild-type controls in the elevated zero maze. In the staircase test, male $GABA^{-/-}_{B(1b)}$ mice defecated more than male $GABA^{-/-}_{B(1a)}$ mice, although no other test parameter was influenced by genotype. In the light–dark box, female $GABA^{-/-}_{B(1a)}$ mice spent less time in the light compartment compared to the $GABA^{-/-}_{B(1b)}$ females, whereas male $GABA^{-/-}_{B(1b)}$ mice made fewer light–dark transitions than $GABA^{-/-}_{B(1a)}$ males.
Conclusions Specific roles for either $GABA_{B(1)}$ isoform in unconditioned anxiety were not explicit. This differs from their contribution in conditioned anxiety and from the anxious phenotype of $GABA_{B1}$ and $GABA_{B2}$ subunit knockout mice. The findings suggest that the $GABA_{B(1)}$ isoforms have specific relevance for anxiety with a cognitive component, rather than for innate anxiety per se.

Keywords Anxiety · Unconditioned · Point mutation · $GABA_{B(1)}$ receptor isoforms · Test battery

Introduction

G-protein coupled γ-aminobutyric acid (GABA) receptors, $GABA_B$ receptors, are heterodimers comprised of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. They are expressed both as presynaptic heteroreceptors and also postsynaptically, where they respectively modulate neuronal excitability. Heteroreceptors modulate the release of (excitatory) neurotransmitters, mainly via actions on presynaptic Ca^{+2} channels, and postsynaptic $GABA_B$ receptors activate slow inhibitory postsynaptic potentials via the activation of inwardly rectifying K^+ channels. $GABA_B$ receptors also function as autoreceptors on interneurons. Additionally, $GABA_B$ receptors are negatively coupled to adenylyl cyclase, through which they influence downstream molecular pathways (Bettler et al. 2004; Couve et al. 2000).

L. H. Jacobson · K. Kaupmann · J. F. Cryan
Novartis Institutes for BioMedical Research,
Novartis Pharma AG,
4002 Basel, Switzerland

B. Bettler
Department of Clinical-Biological Sciences, Pharmazentrum,
Institute of Physiology, University of Basel,
4056 Basel, Switzerland

Present address:
J. F. Cryan (✉)
Department of Pharmacology and Therapeutics,
School of Pharmacy, University College Cork,
Cork, Ireland
e-mail: j.cryan@ucc.ie

There is a growing body of evidence indicating that $GABA_B$ receptors play a critical role in anxiety (Cryan and Kaupmann 2005; Pilc and Nowak 2005). The classic $GABA_B$ receptor agonist, baclofen, has shown anxiolytic activity in some clinical settings. Baclofen reduced anxiety in posttraumatic stress disorder patients (Drake et al. 2003), in alcoholics after alcohol withdrawal (Addolorato et al. 2002, 2006; Ameisen 2005; Flannery et al. 2004), in panic disorder (Breslow et al. 1989), and in patients suffering from acute spinal trauma (Hinderer 1990). Baclofen has also demonstrated anxiolytic effects in several preclinical studies including ultrasonic vocalization in rat pups (Nastiti et al. 1991), punished drinking (Shephard et al. 1992), elevated plus maze (Andrews and File 1993; but see Dalvi and Rodgers 1996), and in the social interaction and elevated plus maze tests after withdrawal of dependent rats from either diazepam or alcohol (File et al. 1991a,b, 1992). $GABA_B$ receptor-positive modulators such as GS39783 also demonstrate an anxiolytic profile in both rats and mice in a wide range of tests (Cryan et al. 2004).

Perhaps the strongest preclinical evidence to date for a role of $GABA_B$ receptors in anxiety was demonstrated by the phenotype of $GABA_B$ receptor-deficient mice. Deletion of either the $GABA_{B(1)}$ or $GABA_{B(2)}$ receptor subunits results in a complete loss of typical $GABA_B$ functions and induces a highly anxious phenotype in mice in exploratory-based tests of anxiety (Mombereau et al. 2004a,b).

The $GABA_{B(1)}$ subunit is predominantly expressed as one of two isoforms: $GABA_{B(1a)}$ or $GABA_{B(1b)}$, both of which are transcribed from different promoters in the *Gabbr1* gene (Steiger et al. 2004) and heterodimerize with the $GABA_{B(2)}$ subunit to form functional receptors (Bettler et al. 2004). The primary sequence of the $GABA_{B(1a)}$ isoform differs from that of the $GABA_{B(1b)}$ isoform by the inclusion of short consensus repeats (or "sushi domains") at the N terminus (Blein et al. 2004). Currently, there are no pharmacological tools available to dissect the physiological roles of these isoforms. Recently, however, the generation of mice deficient in either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms has facilitated the demonstration of specific and differential roles for the $GABA_{B(1)}$ isoforms. The two isoforms localize differentially: the $GABA_{B(1a)}$ isoform was predominantly expressed as a presynaptic heteroreceptor in the hippocampus (Vigot et al. 2006) and lateral amygdala (Shaban et al. 2006). In contrast, the $GABA_{B(1b)}$ isoform was predominantly located postsynaptically in these structures (Shaban et al. 2006; Vigot et al. 2006). Both the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms also fulfilled autoreceptor functions in the hippocampus and amgydala (Shaban et al. 2006; Vigot et al. 2006), although in the apical dendrites of layer 5 cortical neurons, the $GABA_{B(1a)}$ isoform was shown to be the predominant autoreceptor (Perez-Garci et al. 2006). The $GABA_{B(1)}$ isoforms also have differential impacts on neurophysiological processes, such as LTP in the hippocampus (Vigot et al. 2006) and amygdala (Shaban et al. 2006). Finally, deletion of either isoform demonstrates a divergence of behavioral phenotypes in conditioned aversive learning and memory paradigms (Jacobson et al. 2006b; Shaban et al. 2006). Specifically, in a conditioned fear paradigm $GABA^{-/-}_{B(1a)}$ mice showed generalized freezing to both paired and unpaired tones (Shaban et al. 2006). In a conditioned taste aversion paradigm (CTA), $GABA^{-/-}_{B(1a)}$ mice failed to acquire an aversion to a saccharin solution when paired with a lithium chloride-induced malaise, while $GABA^{-/-}_{B(1b)}$ mice acquired CTA, but demonstrated a profound failure in the extinction of this aversion (Jacobson et al. 2006b).

It is, therefore, well-established that $GABA_B$ receptors play a role in anxiety and that the $GABA_{B(1)}$ subunit isoforms are functionally relevant molecular variants of the $GABA_B$ receptor, which convey differential behavioral responses in tests of aversive learning and memory. However, the influence of the two $GABA_{B(1)}$ isoforms on behavior in tests of unconditioned anxiety is currently unknown. The aim of the present study, therefore, was to determine the influence of the different $GABA_{B(1)}$ subunit isoforms on innate anxiety-related behaviors, using mice deficient in either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms.

Materials and methods

Animals

The generation of wild-type, $GABA^{-/-}_{B(1a)}$, and $GABA^{-/-}_{B(1b)}$ mice as used in the present studies has been described previously (Vigot et al. 2006). Briefly, a knock-in point mutation strategy was adopted, whereby $GABA_{B(1a)}$ and $GABA_{B(1b)}$ initiation codons were converted to stop codons by targeted insertion of a floxed neo-cassette. All mutant and wild-type mice were maintained on a pure inbred BALB/c genetic background. $GABA^{-/-}_{B(1a)}$ and $GABA^{-/-}_{B(1b)}$ mice used in the present experiments were derived from homozygous breeding of siblings originating from the founding heterozygotic mice (F3–4). Homozygous wild-type controls for the $GABA_{B(1)}$ isoform mutant mice were derived from mating together wild-type siblings generated from $GABA^{+/-}_{B(1a)}$ and $GABA^{+/-}_{B(1b)}$ heterozygous breedings (F3–4). The breeding strategy was applied in accordance with the recommendations proposed by The Jackson Laboratory to obviate genetic drift and the formation of substrains (http://jaxmice.jax.org/geneticquality/guidelines.html).

Male and female mice were investigated in separate experiments for all tests performed, with the exception of the determination of corticosterone and adrenocorticotropic

hormone (ACTH) levels, in which only male mice were used. All mice were singly housed after achieving at least 8 weeks of age, with the exception of those used in the marble-burying test, that were housed in groups of two to four mice per cage. Mice were housed in macrolon cages with sawdust bedding, tissue paper nesting materials and one red, triangular, polycarbonate Mouse House® (Nalgene) per cage. Housing was at a constant room temperature of 22–24°C in a 12-h light:dark cycle with lights on at 6:00–6:30 a.m. Food pellets and tap water were available ad libitum (except during experimentation). Mice were allowed to settle for a minimum of 1 week after single housing before testing began. All mice were 8 weeks of age or older at testing. All mice were drug-naïve, and mice used in the stress-induced hyperthermia (SIH), light–dark box, marble-burying tests, and in the corticosterone and ACTH assessment were experimentally naïve. Mice used in the staircase test were the same as those from the SIH test, with an interval of approximately 3 and 5 weeks between tests for the male and female mice, respectively. Mice used in the marble-burying test were singly house immediately after the test and subsequently tested in the elevated plus maze after an interval of approximately 5 weeks and 1 week for male and female mice, respectively. Mice tested with the elevated zero maze had been previously exposed to a 5-min handling period approximately 1 week before testing, during which basic sensory and sensorimotor characteristics of the mutant mice and wild-type controls were examined (Jacobson et al. 2006a). All animal experiments were conducted during the light phase. All animal experiments were conducted in accordance with Swiss ethics guidelines and approved by the Veterinary Authority of Basel Stadt, Switzerland.

Stress-induced hyperthermia

The SIH test is an ideal inclusion in an anxiety test battery as it is not unduly influenced by alterations in locomotor activity and is a translational model across strains and species (including mice and humans; see Bouwknecht et al. 2006). The test procedure for SIH was adapted from that reported by Van der Heyden et al. (1997) and was conducted essentially as described by Cryan et al. (2003), with the exception that mice in the present study were already singly housed before the time of SIH testing. Two SIH experiments were conducted, one with male and one with female mice. Age-matched (within gender), experimentally naive male and female wild-type, $GABA_{B(1a)}^{-/-}$, and $GABA_{B(1b)}^{-/-}$ mice (mean age: 10±0.4 weeks for males and 13±0.3 weeks for females, n=10 per sex and genotype) were rehoused in new cages overnight in the testing room. They had free access to water and food, but were without nesting materials or Mouse Houses®. The following day, rectal temperature was measured twice in each mouse i.e., at t=0 min (T_1) and t=+15 min (T_2). The first measurement of temperature serves as the stressor and results in a rapid hyperthermic response. The difference in temperature (T_2-T_1) was considered to reflect the SIH. Time points were based on previous experiments that showed that a T_2-T_1 interval of 15 min was optimal in terms of SIH (Spooren et al. 2002). Rectal temperature was measured to the nearest 0.1°C by an ELLAB instruments thermometer (Copenhagen, Denmark Model DM 852), by inserting a lubricated thermistor probe model PRA-22002-A (ELLAB), 2.2 mm in diameter, 20 mm into the rectum. The mouse was handheld at the base of the tail during this determination and the thermistor probe was left in place for 15 s.

Staircase test

The staircase test in mice (Simiand et al. 1984) allows the distinction between locomotor vs anxiolytic responses by comparing the ratio of steps climbed to rearings performed. Anxiolysis is thus interpreted when reductions in rearing are accompanied by an unchanged, or an increased, number of steps climbed. The test shows pharmacological selectivity for anxiolytics and, indeed, locomotor stimulants tend to decrease both parameters in the test (Simiand et al. 1984). Additionally, the number of fecal boli and urination spots made by the mice are easily quantifiable in this test and provide a further indicator of stress responses (Cryan et al. 2003; Gray and Lalljee 1974).

The test was carried out essentially as previously described (Cryan et al. 2003; Simiand et al. 1984). The apparatus comprised 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, such that the staircase rose to a height of 12.5 cm at the top step. The apparatus was 45 cm in length, and surrounded by walls 12.5 cm height at one end and 25 cm at the other. Mutant and wild-type mice utilized in the staircase test in the present study were the same animals as those for the SIH test, with the exception that of the wild-type female mice, only eight rather than ten mice were used. Experiments with male and female mice were conducted separately. Animals were moved in their home cages to the testing room at least 1 h before testing started. Mice were placed on the bottom level facing away from the stairs. The number of steps ascended and rearings made in a 3-min period were observed. The apparatus was wiped with a wet paper towel and dried between animals.

Light–dark box

The light–dark box test is a conflict-based anxiety test whereby the tendency to explore a novel environment contrasts against the natural aversion of mice to brightly lit

spaces (Crawley 2000). The test was included in the present study as the most reliable indictors of anxiety, such as light–dark transitions and time spent in the light (in that order; see Crawley and Davis 1982; Holmes et al. 2002), are expressed as passive avoidance behaviors, thus increasing the range of behaviors used in the present study to evaluate anxiety. The test was carried out similarly to that previously described (Cryan et al. 2003; Holmes et al. 2002; Mombereau et al. 2004a). The apparatus consisted of a Plexiglas enclosure (44×21×21 cm) divided into two compartments (one light and one dark) by a partition in which there was a small opening (12×5 cm) at the floor level. The light compartment was open-roofed, with walls of transparent Plexiglas and was brightly illuminated by a 60-W desk lamp overhead (approximately 1,000 lx). The smaller, dark compartment (14 cm width) was closed-roofed and was constructed of black Plexiglas. Male and female mice were investigated in separate experiments. Age-matched, experimentally naive wild-type, $GABA_{B(1a)}^{-/-}$, and $GABA_{B(1b)}^{-/-}$ mice (mean age: 12±0.4 weeks, n=10–11 per sex and genotype) were individually placed in the center of the light compartment, facing away from the partition and allowed to explore the apparatus freely 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 the dark were recorded by a trained observer.

Marble-burying test

The marble-burying test was similar to that previously described (Broekkamp et al. 1986; Spooren et al. 2000). This test was included, as animals that are more anxious must engage in active behaviors (defensive marble burying) as opposed to passive behaviors utilized to avoid anxiogenic stimuli in the light–dark box and elevated mazes. Male and female mice were investigated in separate experiments. Mice were group-housed, age-matched (within gender), experimentally naive male and female wild-type, $GABA_{B(1a)}^{-/-}$, and $GABA_{B(1b)}^{-/-}$ mice (males: mean age 15.2±0.4 weeks, wild-type n=14, $GABA_{B(1a)}^{-/-}$ n=19, $GABA_{B(1b)}^{-/-}$ n=26; females: mean age 20.6±0.6, wild-type n=23, $GABA_{B(1a)}^{-/-}$ n=16, $GABA_{B(1b)}^{-/-}$ n=35). Mice were placed individually in small cages (26×20×14 cm), in which ten marbles had been equally distributed on top of a 5-cm deep bed of sawdust, and a wire lid was placed on top of the cage. Mice were left undisturbed for 30 min, after which the number of buried marbles (i.e., those covered by sawdust three-quarters or more) was counted.

Elevated plus maze

The elevated plus maze is a widely used test for assessing anxiety behavior in mice (Crawley 2000; Holmes 2001; Rodgers 1997). Similar to the light–dark box, the elevated plus maze is based on the conflict between exploratory drive and avoidance of the innately aversive open arms and in the expression of passive behaviors that are employed to avoid the more anxiogenic areas of the mazes. However, the tests differ both in the nature of the anxiogenic stimuli (heights vs a brightly lit space) and in the starting place of the mice (choice point vs maximally anxiogenic area; Crawley 2000; Rodgers 1997). As such, results between the two tests can be expected to vary; therefore, the inclusion of both tests in the present study provides a more comprehensive test battery. Separate experiments were conducted with male and female mice. Mice were the same animals as those utilized in the marble-burying test, with the exception that of the female $GABA_{B(1b)}^{-/-}$ mice, only 34, rather than 35, mice were used. The elevated plus maze was carried out as described previously (Cryan et al. 2003; Rodgers et al. 1997). The apparatus comprised two open arms (30×5 cm) and two enclosed arms (30×5×15 cm), which extended from a common central platform (5×5 cm). The configuration formed the shape of a plus sign, with like arms arranged opposite one another, and the apparatus was elevated 60 cm above floor level on a central pedestal. The maze floor was made of black Plexiglas, while the side and end walls of the enclosed arms were made from clear Plexiglas. Grip on the edges of open arms was facilitated by inclusion of a small raised edge (0.25 cm) around their perimeter. Animals were transported from the holding room to the laboratory at least 1 h before testing. Mice were placed onto the central platform facing an enclosed arm. A 6-min trial was performed and, between subjects, the maze was thoroughly cleaned. Direct registrations were made by an observer sitting close to the maze using the following conventional parameters: number of open and closed arm entries (arm entry defined as all four paws entering an arm) and time spent on open arms (excluding the central platform).

Elevated zero maze

The elevated zero maze is an ideal complementary test to the elevated plus maze. It eliminates the central area of the plus maze and provides a continuous circular track to facilitate exploration, while maintaining end point parameters similar in construction to that of the elevated plus maze (Lee and Rodgers 1990; Shepherd et al. 1994). Furthermore, we have previously shown that mice lacking the $GABA_{B(1)}$ subunit jumped off the maze in a panic-like response (Mombereau et al. 2004a). It was therefore of interest to assess the influence of $GABA_{B(1)}$ receptor isoform deletion on mice in this test. The elevated zero maze test was conduced as previously described (Cryan et al. 2004). Male and female mice were

investigated in separate experiments. Age-matched (within gender), experimentally naive male and female wild-type, $GABA_{B(1a)}^{-/-}$, and $GABA_{B(1b)}^{-/-}$ mice (mean age: 21.2± 1.4 weeks for males and 23.8±0.8 weeks for females, n=11–12 for each sex and genotype) were used. Mice had been individually handled for about 5 min each 1 week before testing in the elevated zero maze. The apparatus was a 5.5-cm wide circular track constructed of gray Plexiglas with an inside diameter of 34 cm, a midtrack circumference of approximately 121 cm, and was elevated 40 cm above the floor. 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 (Cryan et al. 2004; 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 closed quadrant, stretch-attend postures (SAP, elongated body posture with at least snout over open/closed divide) into the open quadrant, rearing, and head dips over the sides of the open quadrants.

Corticosterone and ACTH

To investigate the effects of deletion of the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms on hypothalamic–pituitary–adrenal (HPA) axis activity, corticosterone and adrenocortico tropic hormone (ACTH) levels at 0700–0800 hours were measured in trunk blood of male, experimentally naïve wild-type (n=9), $GABA_{B(1a)}^{-/-}$ (n=9), and $GABA_{B(1b)}^{-/-}$ (n=6) mice [mean age (±SEM) 15.6±0.6 weeks]. Home cages for each individual mouse were removed from the housing room one at a time to another room where mice were decapitated immediately on arrival and trunk blood collected into EDTA-treated 1.5-ml tubes (Milian S.A., Meyrin, Switzerland). Blood samples were kept on ice for up to about 20 min until centrifugation at 10,000 rpm for 15 min in a refrigerated centrifuge (4°C). The plasma fraction was collected and stored at −80°C until subsequent analysis for corticosterone and ACTH. Plasma corticosterone and ACTH concentrations were measured using commercially available radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA, USA).

Statistical analyses

As studies which were carried out in both male and female mice were conducted in independent experiments, data for each sex were analyzed separately. Corticosterone levels the number of steps taken in the staircase test and head-dips and SAPs in the elevated zero maze were analyzed using one-way analysis of variance (ANOVA). Parameters measured within the SIH, other staircase test parameters, light–dark box, elevated plus maze, marble burying, the remaining elevated zero maze parameters, and ACTH data were analyzed using Kruskal–Wallis one-way ANOVA on ranks, followed by Dunn's Method post hoc comparisons, where indicated, by significant ANOVA factors.

Results

Stress-induced hyperthermia

SIH was not influenced significantly by genotype in either of the experiments using male or female mice (Fig. 1a,b; H=4.25, P=0.12 and H=1.64, P=0.44 for males and females, respectively). In the male mice, the first temperature recording, T_1, tended to be higher for the $GABA_{B(1a)}^{-/-}$ mice (Fig. 1a; H=5.27, P=0.072), although in the female mice this trend was not apparent (Fig. 1b; H=3.582, P= 0.17). The second temperature recording, T_2, was not significantly influenced by genotype in either the male or female mice (Fig. 1a,b; H=2.24, P=0.33 and H=3.40, P= 0.18 for male and female mice, respectively).

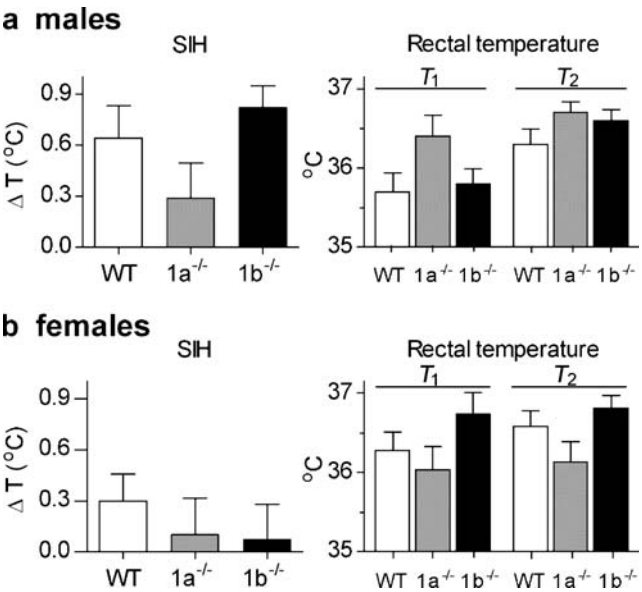

Fig. 1 Stress-induced hyperthermia (SIH). Genotype did not significantly influence SIH or rectal temperature at the two time points measured in experiments with **a** male (n=10 per genotype) or **b** female (n=10 per genotype) wild-type (*WT*), $GABA_{B(1a)}^{-/-}$(*1a*$^{-/-}$), or $GABA_{B(1b)}^{-/-}$ (*1b*$^{-/-}$) mice. *Bars* represent means+SEM. T_1 First rectal temperature recording, T_2 second rectal temperature recording taken 15 min later, ΔT temperature difference between T_1 and T_2

Staircase test

The number of steps taken, incidence of rearing, and the ratio of steps to rears in the staircase test were not significantly affected by genotype in either of the experiments using male or female mice (males: steps $F_{2,29}$=0.26, P=0.772; rears H=0.96, P=0.62; ratio H=0.88, P=0.64; females: steps $F_{2,27}$=0.996, P=0.38; rears H=3.61, P=0.17; ratio H=1.25, P=0.536; Fig. 2a,b).

With regard to physiological indicators of stress (Fig. 2a, b), genotype significantly influenced the number of fecal boli produced by male mice during the test (H=14.13, P<0.001). Post hoc comparisons demonstrated that the $GABA_{B(1b)}^{-/-}$ mice defecated more than $GABA_{B(1a)}^{-/-}$ mice (P<0.01), although neither mutant strain differed from the wild-types in this regard (P>0.05). Fecal boli production was not significantly affected by genotype in the female mice, as was also the case for the number of urine spots produced by male or female mice (females: fecal boli H=2.37, P=0.31; males urination score H=3.73, P=0.16; females urination score H=3.74, P=0.154).

Light–dark box

In the experiment with male mice, three wild-type mice, one $GABA_{B(1a)}^{-/-}$ and one $GABA_{B(1b)}^{-/-}$ mouse made no transitions during the test—all of their time in the test was spent in the light compartment in which they were initially placed, and the latency to enter the dark side was therefore greater than the 600-s duration of the test. As a consequence, absolute data values for latency for these mice were not determined. Furthermore, data from these mice may have introduced bias in other test measures such as time in the light compartment and light–dark transitions; therefore, these mice were excluded from statistical analysis.

Genotype influenced the number of light–dark transitions in male mice (H=6.48, P=0.039; Fig. 3a). Post hoc comparisons revealed that male $GABA_{B(1b)}^{-/-}$ mice conducted significantly less light–dark transitions than $GABA_{B(1a)}^{-/-}$ mice (P<0.05), although neither mutant differed from the wild-type controls in this regard. The latency to enter the dark compartment and the total time spent in the light compartment were not significantly influenced by genotype in the male mice (latency: H=1.55, P=0.46; time in light: H=2.26, P=0.32; Fig. 3a).

In the experiment with female mice, some mice also failed to enter the dark compartment (two wild-type, one $GABA_{B(1a)}^{-/-}$ and two $GABA_{B(1b)}^{-/-}$ mice) and were therefore excluded from statistical analysis. The number of light–dark transitions made by female mice was not significantly affected by genotype (H=1.99, P=0.37, Fig. 3b). However, $GABA_{B(1b)}^{-/-}$ female mice spent more time in the light compartment than $GABA_{B(1a)}^{-/-}$ female mice (P<0.05, Fig. 3b), although neither mutant genotype differed significantly from the wild-types (H=6.58, P=0.037; Fig. 3b). As for the experiment conducted with the male

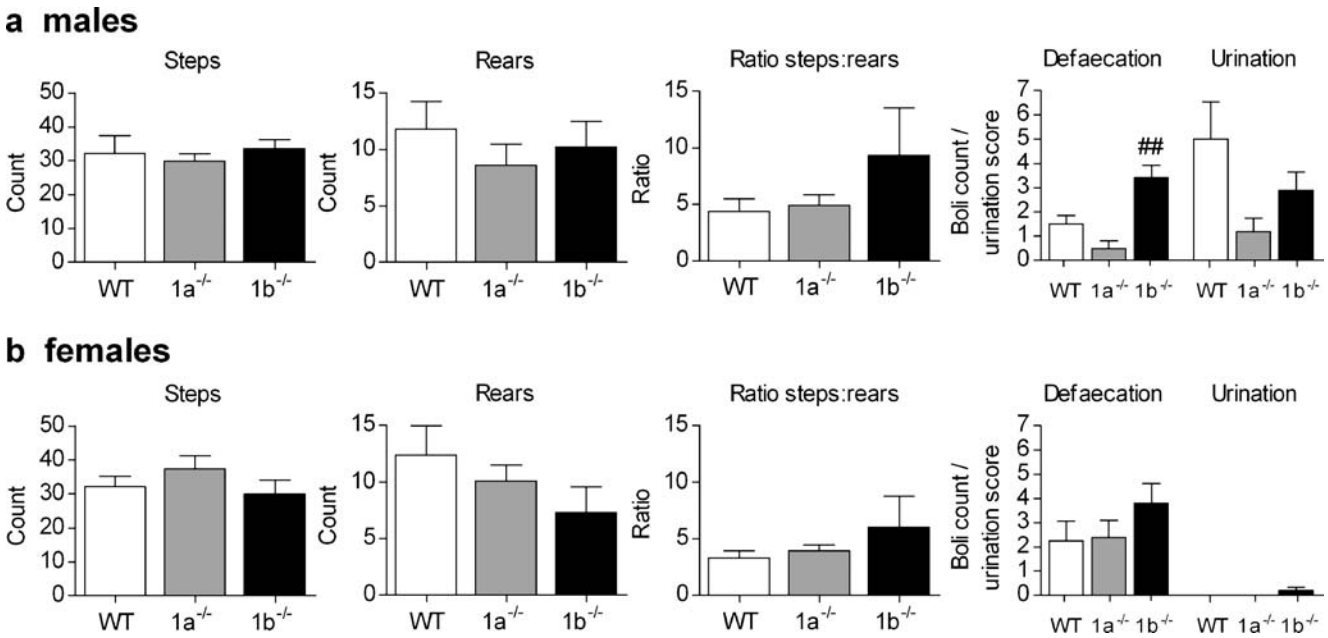

Fig. 2 Staircase test. Deletion of $GABA_{B(1a)}$ or $GABA_{B(1b)}$ receptor subunit isoforms did not influence the number of ascending steps taken (*Steps*), incidence of rearing (*Rears*), the ratio between these two parameters (*Ratio steps: rears*), or the amount of urination in the staircase test in experiments with **a** male (n=10 per genotype) and **b** female (n=10 per genotype) mice, although male $GABA_{B(1b)}^{-/-}$ mice defecated more than $GABA_{B(1a)}^{-/-}$ male mice. *Bars* represent means +SEM. The symbol ## is used to denote P<0.01 for $GABA_{B(1a)}^{-/-}$ vs $GABA_{B(1b)}^{-/-}$. *WT* Wild-type, $1a^{-/-}GABA_{B(1a)}^{-/-}$, $1b^{-/-}GABA_{B(1b)}^{-/-}$

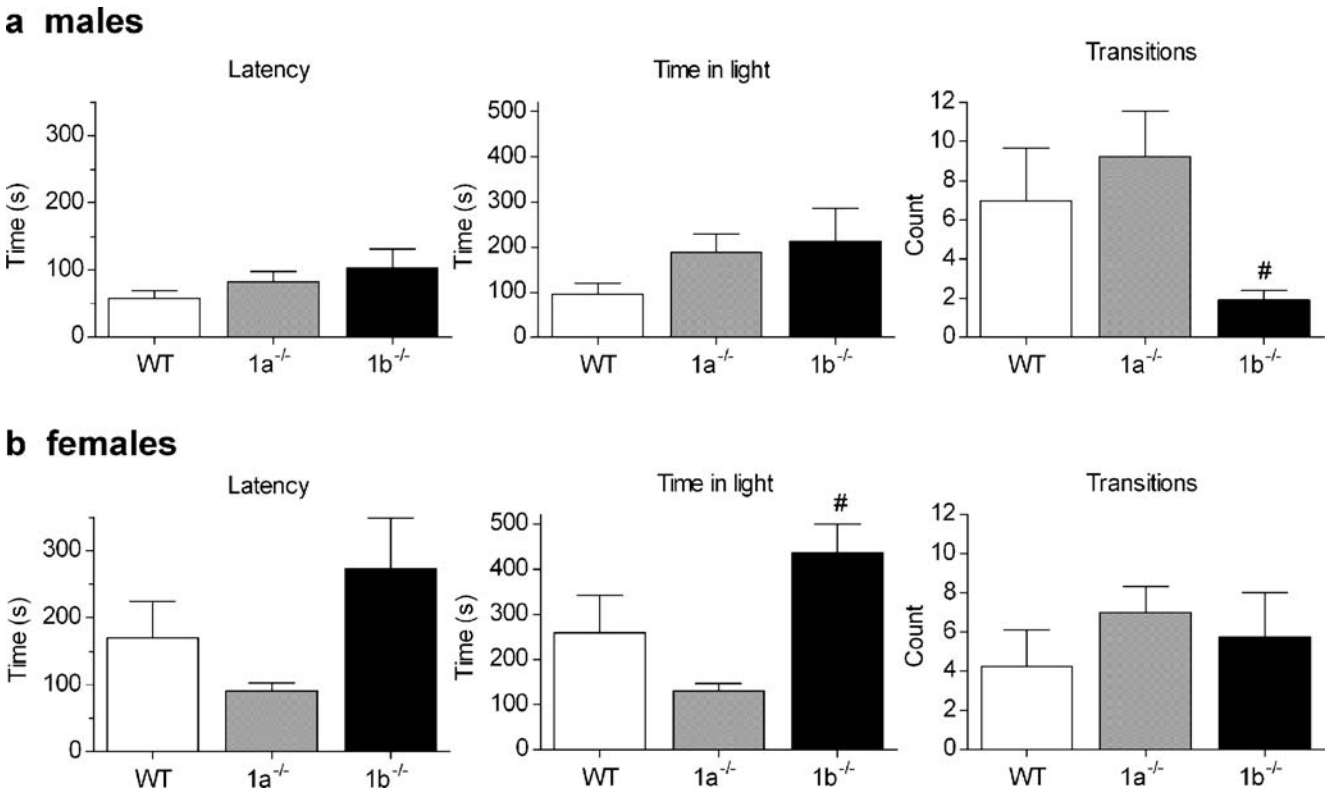

Fig. 3 Light–dark box. In male mice (**a**), $GABA_{B(1b)}^{-/-}$ (*1b*$^{-/-}$) mice made fewer transitions between the light and dark compartments (*Transitions*) relative to the $GABA_{B(1a)}^{-/-}$ (*1a*$^{-/-}$) mice (n=10 per genotype). In a separate experiment with female mice (**b**), $GABA_{B(1b)}^{-/-}$ mice spent more time in the light compartment (*Time in light*) than $GABA_{B(1a)}^{-/-}$ mice (n=10–11 per genotype). Genotype did not influence the time take for mice of either sex to initially enter the dark compartment (*Latency*). *Bars* represent means+SEM. The symbol # denotes $P<0.05$ for $GABA_{B(1a)}^{-/-}$ vs $GABA_{B(1b)}^{-/-}$. *WT* Wild-type

mice, latency to enter the dark compartment was not significantly affected by genotype in the female mice (H=0.71, P=0.70).

Marble-burying test

The number of marbles buried in the experiments with either male or female mice were not significantly affected by genotype (H=0.33, P=0.85 and H=2.49, P=0.29 for males and females, respectively; Table 1).

Elevated plus maze

Genotype did not significantly influence any of the parameters measured either in the elevated plus maze in the experiments involving the male or the female mice. The number of open arm entries for male (H=0.71, P=0.70) or female mice (H=2.62, P=0.27) was not affected by genotype, nor were closed arm entries for male or female mice (H=0.91, P=0.63; H=0.71, P=0.70 for males and females respectively), nor the total number of arm entries (H=0.87, P=0.65; H=1.67, P=0.44 for males and females, respectively). With regard to total arm entries, some mice made only one or no arm entries during the experiment. In female mice, this was the case for 4 of the 23 wild-type, 1 of the 16 $GABA_{B(1a)}^{-/-}$, and 9 of the 35 $GABA_{B(1b)}^{-/-}$ mice. In the experiment with male mice, all of the wild-type and $GABA_{B(1a)}^{-/-}$ mice made more than one arm entry, while 2 of the 26 $GABA_{B(1b)}^{-/-}$ males made one or no arm entries. The ratio of the number of open to closed arm entries was not affected by genotype in the experiments with either sex (H=2.16, P=0.34 and H=0.87, P=0.65 for males and females, respectively). Finally, the amount of time spent on the open arms by the mice in each respective experiment was not significantly affected by genotype (H=2.35, P=0.31 and H=2.05, P=0.36 for males and females, respectively; Table 2).

Table 1 Mean (±SEM) number of marbles buried by male (wild-type n=14, $GABA_{B(1a)}^{-/-}$ n=19, $GABA_{B(1b)}^{-/-}$ n=26) and female (wild-type n=23, $GABA_{B(1a)}^{-/-}$ n=16, $GABA_{B(1b)}^{-/-}$ n=35) wild-type and $GABA_{B(1)}$ isoform-deficient mice

	Wild-type	$GABA_{B(1a)}^{-/-}$	$GABA_{B(1b)}^{-/-}$
Males	7.6 (±0.6)	7.4 (±0.6)	7.3 (±0.4)
Females	6.7 (±0.5)	6.6 (±0.6)	7.5 (±0.3)

Table 2 Parameters measured in the elevated plus maze were not significantly influenced by deletion of the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms (data are means±SEM)

	Males			Females		
	Wild-type	$GABA^{-/-}_{B(1a)}$	$GABA^{-/-}_{B(1b)}$	Wild-type	$GABA^{-/-}_{B(1a)}$	$GABA^{-/-}_{B(1b)}$
n	14	19	26	23	16	34
Open arm entries	5.0 (±1.0)	4.8 (±0.6)	4.3 (±0.6)	3.3 (±0.5)	3.3 (±0.7)	2.3 (±0.3)
Closed arm entries	7.9 (±1.4)	6.4 (±1.0)	5.8 (±0.8)	3.6 (±0.7)	4.0 (±0.9)	3.4 (±0.6)
Ratio open to closed entries	0.39 (±0.03)	0.48 (±0.05)	0.44 (±0.04)	0.49 (±0.06)	0.52 (±0.06)	0.49 (±0.1)
Total arm entries	12.9 (±2.3)	11.2 (±1.4)	10.1 (±1.3)	6.9 (±1.1)	7.3 (±1.4)	5.7 (±0.8)
Time open (s)	109.1 (±13.3)	153.3 (±20.3)	135.8 (±16.8)	103.0 (±18.2)	137.9 (±25.8)	110.1 (±20.7)

Elevated zero maze

In the experiment with male mice, the latency to enter the open side, time spent on the open side, the number of head dips over the side of the open areas, and number of rears were not significantly affected by genotype (H=1.98, P=0.371; H=0.62, P=0.733; $F_{2,34}$=1.75, P=0.19 and H=1.18, P=0.55, respectively). There was a tendency for genotype to influence the distance travelled in the maze (line crossings: H=5.50, P=0.064) and the number of SAPs performed by the mice ($F_{2,34}$=2.577, P=0.092; Fig. 4a).

In the experiment with female mice, genotype significantly influenced the majority of parameters measured in the elevated zero maze. The latency to enter the open-sided area of the maze (H=11.39, P<0.01) was significantly longer in both $GABA^{-/-}_{B(1a)}$ and $GABA^{-/-}_{B(1b)}$ mice than in the wild-type mice. Both mutant strains also performed significantly less head dips over the maze open sides ($F_{2,35}$=21.07, P<0.001) and rearings (H=13.52, P=0.001) than the wild type mice. Genotype also influenced the time spent on the open-sided areas (H=11.60, P<0.01) and the number of line crossings (H=9.09, P<0.05), and post hoc comparisons revealed a significant difference between $GABA^{-/-}_{B(1b)}$ mice and wild-types in these parameters. The mutants did not differ significantly from each other in post hoc comparisons of the aforementioned parameters. The only parameter measured in the elevated zero maze that was not significantly influenced by genotype in the female mice was the number of SAPs ($F_{2,35}$=0.80, P=0.46; Fig. 4b).

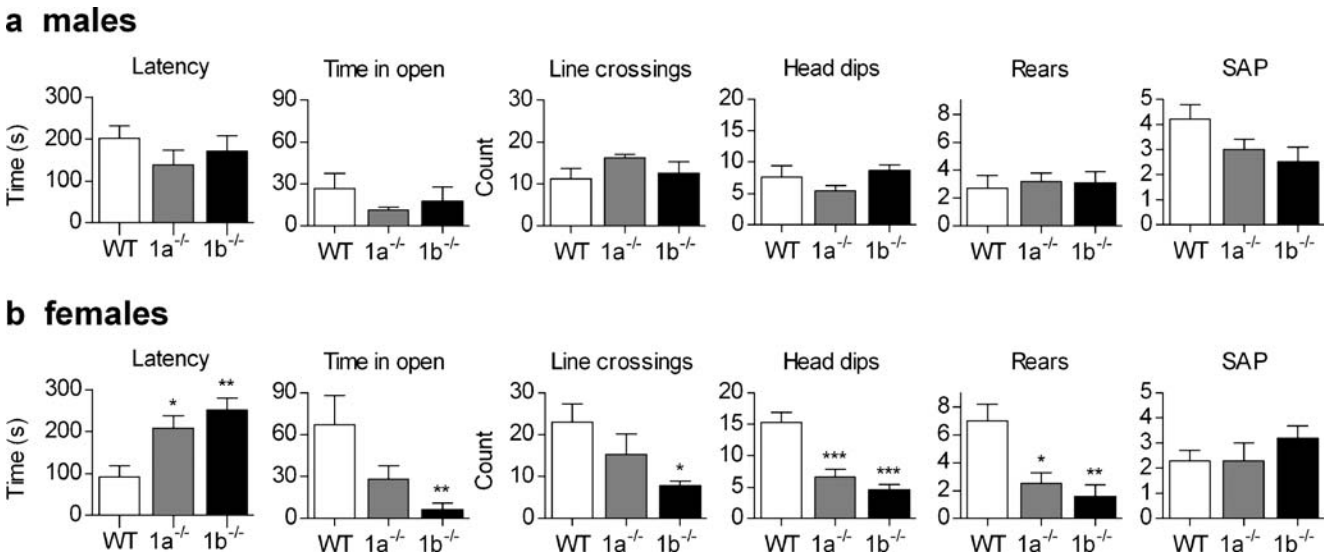

Fig. 4 Elevated zero maze. In male mice (**a**), deletion of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms did not significantly influence the time taken to enter an open-sided quadrant (*Latency*), time spent in the open-sided quadrants (*Time in open*), ambulation in the maze (*Line crossings*), or the ethological parameters: incidence of dipping the head over the open sides (*Head dip*), rearing (*Rears*), and the number of stretch-attend postures (*SAP*) performed into the open quadrants (WT n=12, $1a^{-/-}$ n=11, $1b^{-/-}$ n=12). In a separate experiment with female mice (**b**), genotype influenced all elevated zero maze parameters measured with the exception of SAPs (WT n=12, $1a^{-/-}$ n=12, $1b^{-/-}$ n=12). *Bars* represent means+SEM. A *single asterisk* denotes P<0.05, while *two asterisks* denote P<0.01, and *three asterisks* denotes P<0.01 vs wild-type (*WT*). $1a^{-/-}GABA^{-/-}_{B(1a)}$, $1b^{-/-}GABA^{-/-}_{B(1b)}$

Basal corticosterone and ACTH

Mean plasma corticosterone appeared to be slightly elevated in the $GABA_{B(1a)}^{-/-}$ mice compared to the $GABA_{B(1b)}^{-/-}$ mice (Fig. 5a); however, the factor "genotype" did not achieve statistical significance ($F_{2,12}$=1.91, P=0.17). Two data points were removed from the ACTH data set as statistical outliers: one from the $GABA_{B(1a)}^{-/-}$ mice (922 pg/ml) and one from the $GABA_{B(1b)}^{-/-}$ mice (1,176 pg/ml). The genotype did not significantly influence basal ACTH levels (Fig. 5b; H=1.93, P=0.38).

Discussion

The present study aimed to determine the influence of $GABA_{B(1)}$ subunit isoform deletion on innate, unconditioned anxiety-related behaviors in mice. To this end, $GABA_{B(1a)}^{-/-}$, $GABA_{B(1b)}^{-/-}$, and wild-type control mice were investigated in a comprehensive battery of anxiety tests that employed a number of different types of experimental end points including autonomic, passive and active avoidance and ethological parameters. The test battery approach has been advocated for the detection of genuine differences in anxiety phenotypes in mutant mice, as reliance on fewer tests may give rise to erroneous interpretations depending on the specific idiosyncrasies of individual tests or mutations (Cryan and Holmes 2005). Overall, the present study demonstrated that the constitutive genetic deficiency of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoform did not, for the most part, result in an alteration of innate, unconditioned anxiety (Table 3).

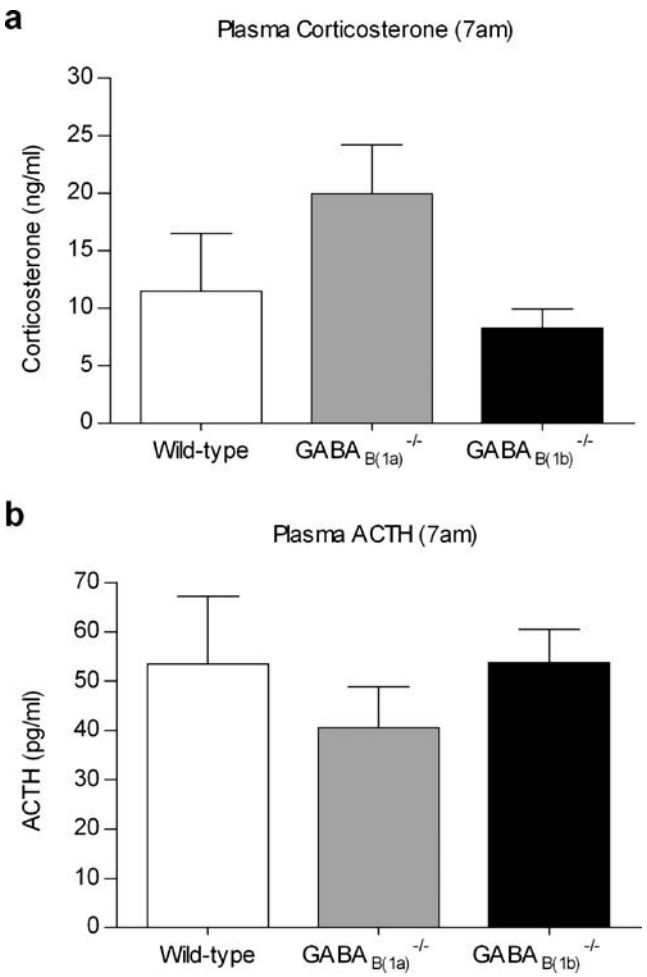

Fig. 5 Hypothalamic–pituitary–adrenal axis characteristics. Deletion of $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms did not significantly influence plasma corticosterone (**a**) or ACTH (**b**) of male mice as measured within 1 h of the start of the circadian light phase (wild-type n=9, $GABA_{B(1a)}^{-/-}$ n=9, $GABA_{B(1b)}^{-/-}$ n=6). *Bars* represent means+SEM

No anxiety-related phenotype was detected for either the $GABA_{B(1a)}^{-/-}$ or $GABA_{B(1b)}^{-/-}$ mice, in experiments with either gender, in the SIH, marble-burying, and elevated plus maze paradigms. Furthermore, in male mice at least, genotype did not influence the levels of HPA axis hormones (Table 3). In the staircase, light–dark box, and elevated zero maze tests, $GABA_{B(1b)}^{-/-}$ mice showed some minor differences in behavior when compared with $GABA_{B(1a)}^{-/-}$ mice. However, given that neither mutant differed from the control wild-type mice in these measures or, for that matter, in the majority of the response parameters measured in these tests, one cannot place too much credence on such subtle effects with relation to anxiety-related behaviors.

Deletion of either $GABA_{B(1)}$ subunit isoforms significantly influenced a number of parameters in the elevated zero maze in female mice. Locomotor activity can influence aspects of performance in exploratory-based tests of anxiety (Cryan and Holmes 2005); for example, a hyperactive phenotype may falsely suggest anxiolysis, and likewise, hypoactivity anxiogenesis. $GABA_{B(1b)}^{-/-}$ mice (both females and males) were previously demonstrated to be hyperactive in a novel environment, while the distance traveled by $GABA_{B(1a)}^{-/-}$ mice was not different to that of the wild-type controls (Jacobson et al. 2006a). However, these observations are at odds with the reductions in exploratory activity by both mutant strains relative to the wild-types in the elevated zero maze in the present study, as indicated by an increased latency to enter the open-sided area in both female mutant genotypes and by the reduced line crossings made by the $GABA_{B(1b)}^{-/-}$ females. Furthermore, the ethological measures of head-dipping and rearing were also reduced in both $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ female mice in this test. Reductions in the parameters of latency to enter an innately aversive area, head-dipping over the edges of elevated apparatuses, and rearing have been interpreted as heightened anxious responses in various apparatuses, including the elevated zero maze (Belzung 1999; Homanics et al. 1999; Rodgers 1997; Rodgers and Johnson 1995; Shepherd et al. 1994). The elevated zero maze has been considered as a more sensitive test than the elevated plus

Table 3 Summary of results for the behavioral assessment of innate anxiety in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice

Parameter	Endpoint category	Males		Females	
		$GABA_{B(1a)}^{-/-}$	$GABA_{B(1b)}^{-/-}$	$GABA_{B(1a)}^{-/-}$	$GABA_{B(1b)}^{-/-}$
Stress-induced hyperthermia					
SIH	Autonomic	x	x	x	x
Staircase					
Steps	Passive avoidance	x	x	x	x
Rears	Ethological	x	x	x	x
Ratio	Combined	x	x	x	x
Boli and urine	Autonomic	x	x	x	x
Light–dark box					
Latency	Active avoidance	x	x	x	x
Time in light	Passive avoidance	x	x	x	x
Transitions	Passive avoidance	x	x	x	x
Marble burying					
Marbles buried	Active avoidance	x	x	x	x
Elevated plus maze					
Open arm	Passive avoidance	x	x	x	x
Closed arm	Passive avoidance	x	x	x	x
Ratio	Passive avoidance	x	x	x	x
Total arm	Passive avoidance	x	x	x	x
Time open	Passive avoidance	x	x	x	x
Elevated zero maze					
Latency	Passive avoidance	x	x	↑	↑
Time open	Passive avoidance	x	x	x	↑
Line X	Passive avoidance	x	x	x	↑
Head dips	Ethological	x	x	↑	↑
Rears	Ethological	x	x	↑	↑
SAP	Ethological	x	x	x	x
Basal corticosterone	Autonomic	x	x	–	–
Basal ACTH	Autonomic	x	x	–	–

x = No alteration in phenotype, ↑ = anxiogenic-like phenotype, relative to wild-type controls

maze, mainly due to the elimination of the central square of the elevated plus maze, which can produce difficulties in data interpretation (Lee and Rodgers 1990; Shepherd et al. 1994), and to the facilitation of locomotor exploration given by the circular track of the zero maze, thus eliminating corners in which the mice may barricade themselves (Rodgers 1997; Shepherd et al. 1994).Therefore, the present findings suggest a subtle, sex-specific role for the $GABA_{B(1)}$ isoforms in innate anxiety. It should be noted, however, that in the light–dark box test, $GABA_{B(1b)}^{-/-}$ female mice spent an increased amount of time in the light side relative to the $GABA_{B(1a)}^{-/-}$ female mice, which is an anxiolytic-like response. Together with the data from other anxiety paradigms where no robust anxiety phenotype was observed, these results indicate that the influence of $GABA_{B(1)}$ isoforms on anxiety in female mice is most likely highly dependent on the environment.

Failure to enter the dark compartment of the light–dark box was shown by a small number of both male and female mice, and occurred to a reasonably similar level in wild-type and mutant mice. The proportion of mice of each genotype making 0 or 1 total arm entries in the elevated plus maze was also similar to that of the light–dark box, although only in female mice. These behaviors may reflect "freezing", neophobic avoidance of newly discovered compartments, or failure to explore extensively enough to find additional compartments (Rodgers 1997) and, thus, may indicate anxiety in these animals. As genotype did not significantly influence these behaviors, they may be a consequence of the background strain. The BALB/c substrains have previously been reported as anxious in comparison to other mouse strains (Belzung 1999; Belzung and Griebel 2001; Cryan and Holmes 2005; Griebel et al. 2000). It could therefore also be posited that innate anxiety may have been near-maximal in the background strain for the $GABA_{B(1)}$ isoform mutant mice; thus, further increases in anxiety induced by the genetic manipulations may be difficult to detect (Crawley et al. 1997). However, this was certainly not the case with either of the $GABA_{B(1)}^{-/-}$ or $GABA_{B(2)}^{-/-}$ mice, both of which were also maintained on a BALB/c background (Gassmann et al. 2004; Schuler et al. 2001) and both of which show profoundly increased anxiety relative to wild-type controls in exploratory anxiety tests (Mombereau et al. 2004a,b, 2005). $GABA_{B(1)}^{-/-}$ mice were substantially more anxious than wild-types in the light–dark box (Mombereau et al. 2004a,b) and staircase

tests (Mombereau et al. 2004a), while in the elevated zero maze, all $GABA_{B(1)}^{-/-}$ mice jumped from the maze, a response indicating heightened flight or panic behavior (Mombereau et al. 2004a). This clearly indicates that the tests used in the present study are suitable to detect increases in anxious behaviors in the BALB/c strain.

The anxious phenotype of the aforementioned $GABA_{B(1)}^{-/-}$ mice differs considerably from that of the specific $GABA_{B(1)}$ isoform-deficient mice in the present study. Both $GABA_{B(1)}^{-/-}$ and $GABA_{B(2)}^{-/-}$ mice show spontaneous seizures, hyperalgesia, hyperlocomotion, memory impairments, and significantly elevated exploratory anxiety (Gassmann et al. 2004; Mombereau et al. 2004a,b, 2005; Schuler et al. 2001). In these mutant mice, classical $GABA_B$ receptor agonist responses are abolished (Gassmann et al. 2004; Kaupmann et al. 2003; Schuler et al. 2001). In contrast, there is only partial $GABA_B$ receptor loss in the $GABA_{B(1a)}$ and $GABA_{B(1b)}^{-/-}$ mice (Vigot et al. 2006), and some residual agonist-induced function remains, albeit blunted, in both the $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice (Jacobson et al. 2006a; Perez-Garci et al. 2006; Shaban et al. 2006; Vigot et al. 2006). Together with the lack of overt anxious phenotype of the $GABA_{B(1)}$ isoform mutant mice in the present study, these findings demonstrate that at least some heterodimeric $GABA_B$ receptor function is essential for the prevention of an increase in anxiety behavior and, furthermore, that the presence of either the $GABA_{B(1a)}$ or $GABA_{B(1b)}$ isoforms can fulfill this task.

The indistinct innate anxiety phenotype of $GABA_{B(1b)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice demonstrated in the present study contrasts with their reported phenotypes in aversive taste memory (Jacobson et al. 2006b) and conditioned freezing paradigms (Shaban et al. 2006). $GABA_{B(1a)}^{-/-}$ mice did not acquire a CTA to a saccharin solution paired with LiCl-induced malaise, while $GABA_{B(1b)}^{-/-}$ mice acquire CTA well, but were profoundly impaired in the extinction of this aversion (Jacobson et al. 2006b). In conditioned freezing, $GABA_{B(1a)}^{-/-}$ mice show subsequent generalized freezing to sound cues irrespective of whether they were paired with high-intensity (0.9 mA) foot shocks during conditioning (Shaban et al. 2006). In theory, it should not necessarily be expected that conditioned and unconditioned tests of fear and anxiety should demonstrate similar phenotypes or, indeed, even to produce similar phenotypes within each of these categories (Rodgers 1997). Certainly, dissociations between aversive conditioning and exploratory anxiety has also been reported for other mutant mice, such as forebrain-selective glycine transporter 1-deficient mice (Yee et al. 2006) and 5-HT_{1A} receptor knockout mice (Klemenhagen et al. 2006), although it should be noted that in each of these studies only one unconditioned anxiety paradigm was utilized. However, the differentiation between conditioned and unconditioned responses appears particularly clearly delineated with $GABA_{B(1)}$ isoform-deficient mice. CTA is well-known as an aversive, associative learning and memory paradigm (Akirav 2006; Akirav et al. 2006; Berman and Dudai 2001; Bermudez-Rattoni 2004; Lamprecht et al. 1997; Welzl et al. 2001) and has recently been applied to the study of anxiety disorders associated with altered emotional learning (Cryan and Holmes 2005; Guitton and Dudai 2004; Yasoshima and Yamamoto 2005). Conditioned freezing in rodents is thought to model emotive cognition aspects of human anxiety disorders such as posttraumatic stress disorder and panic disorder (Barad 2005; Cryan and Holmes 2005; Delgado et al. 2006; Ledgerwood et al. 2005; Ressler et al. 2004). It should also be noted that the $GABA_{B(1a)}$ isoform has been demonstrated as necessary for both hippocampal (Vigot et al. 2006) and amygdala (Shaban et al. 2006) long-term potentiation, and the ability to discriminate successfully between novel and familiar objects in a mouse object recognition paradigm—a task which requires the presence of an intact hippocampus (Broadbent et al. 2004; Clark et al. 2000). Together, these findings suggest that the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms of the $GABA_{B(1)}$ subunit have specific relevance for anxiety with a cognitive component, rather than for innate anxiety per se. Indeed, it remains possible that the specific and differential deficiencies in emotive learning and memory in these mutant mice could be a product predominantly of cognitive impairments, rather than one of emotive processing in itself.

In conclusion, previous studies have demonstrated that genetic ablation of all functional $GABA_B$ receptors results in increases in unconditioned anxiety behavior (Cryan and Kaupmann 2005). Results of the present study suggest that this not due to the specific loss of either one of the predominant $GABA_{B(1)}$ subunit isoforms and that the role for the isoforms in innate anxiety is relatively indistinct. In contrast, these isoforms appear to be more explicitly involved in anxious behaviors that are associated with a cognitive component.

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