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Abnormal anxiety-related behavior in serotonin transporter null mutant mice: the influence of genetic background

A. Holmes[*,†], Q. Li[‡], D. L. Murphy[§], E. Gold[†] and J. N. Crawley[†]

[†]Laboratory of Behavioral Neuroscience, National Institute of Mental Health, NIH, Bethesda, MD, [‡]Department of Psychiatry and Behavior Science, University of Texas Medical Branch, Galveston, TX, [§]Laboratory of Clinical Science, National Institute of Mental Health, NIH, Bethesda, MD, USA

**Corresponding author: A. Holmes, Laboratory of Behavioral Neuroscience, National Institute of Mental Health, Building 10, Room 4D11, Bethesda, MD 20892–1375, USA. E-mail: aholmes@intra.nimh.nih.gov*

Serotonin transporter (5-HTT) null mutant mice provide a model system to study the role genetic variation in the 5-HTT plays in the regulation of emotion. Anxiety-like behaviors were assessed in 5-HTT null mutants with the mutation placed on either a B6 congenic or a 129S6 congenic background. Replicating previous findings, B6 congenic 5-HTT null mutants exhibited increased anxiety-like behavior and reduced exploratory locomotion on the light ↔ dark exploration and elevated plus-maze tests. In contrast, 129S6 congenic 5-HTT null mutant mice showed no phenotypic abnormalities on either test. 5-HTT null mutants on the 129S6 background showed reduced 5-HT_{1A} receptor binding (as measured by quantitative autoradiography) and reduced 5-HT_{1A} receptor function (as measured by 8-OH-DPAT-indcued hypothermia). These data confirm that the 5-HTT null mutation produced alterations in brain 5-HT function in mice on the 129S6 background, thereby discounting the possibility that the absence of an abnormal anxiety-like phenotype in these mice was due to a suppression of the mutation by 129 modifier genes. Anxiety-like behaviors in the light ↔ dark exploration and elevated plus-maze tests were significantly higher in 129S6 congenic +/+ mice as compared to B6 congenic +/+ mice. This suggests that high baseline anxiety-like behavior in the 129S6 strain might have precluded detection of the anxiety-like effects of the 5-HTT null mutation on this background. Present findings provide further evidence linking genetic variation in the 5-HTT to abnormalities in mood and anxiety. Furthermore, these data highlight the utility of conducting behavioral phenotyping of mutant mice on multiple genetic backgrounds.

Keywords: 5-HT_{1A} receptor, anxiety, gene, genetic background, inbred strains, mouse, serotonin, serotonin transporter

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The serotonin transporter (5-HTT) is a cell membrane protein that regulates serotonin signaling via reuptake of extracellular fluid serotonin (Blakely *et al*. 1991; Ramamoorthy *et al*. 1993). There is a wealth of literature implicating the 5-HTT in the etiology and treatment of stress-related psychiatric disorders. Serotonin reuptake inhibitors that block the functional activity of the 5-HTT are clinically effective as antidepressants and anxiolytics (Ballenger 1999; Kugaya *et al*. 2003; Stein & Berk 2000). In depression and anxiety disorders, 5-HTT binding is reduced in postmortem brain tissue, living brain and platelets, suggesting a pathophysiological role for the 5-HTT in these diseases (Arango *et al*. 2001; Briley *et al*. 1980; Malison *et al*. 1998; Nemeroff *et al*. 1994; Willeit *et al*. 2000). In the context of this evidence, genetically encoded variation in 5-HTT function is an attractive candidate for abnormalities in mood and anxiety (Hariri & Weinberger 2003; Lesch & Mossner 1998; Murphy *et al*. 2001).

A 44-base pair insertion/deletion polymorphism in the regulatory region of the human serotonin transporter gene (serotonin transporter linked polymorphic region; *HTTLPR; SLC6A4*) has been identified and related to variance in 5-HTT mRNA, 5-HTT binding sites and platelet serotonin reuptake (Greenberg *et al*. 1999; Lesch *et al*. 1996). Demonstration of a strong association between the *HTTLPR* and 5-HTT availability in the brain remains controversial (Heinz *et al*. 1998; Jacobsen *et al*. 2000; Little *et al*. 1998; Mann *et al*. 2000; Naylor *et al*. 1998; Shioe *et al*. 2003). Nonetheless, a number of studies have found that the low-expressing (short) form of the *HTTLPR* is associated with heightened trait anxiety/dysphoria, exaggerated neural responses to fear and increased risk of depression following adverse life events (Caspi *et al*. 2003; Greenberg *et al*. 2000; Hariri *et al*. 2002; Lesch *et al*. 1996; Mazzanti *et al*. 1998).

To further study the role of the 5-HTT in mediation of anxiety and other behaviors, 5-HTT null mutant mice were

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generated (Bengel *et al*. 1998). 5-HTT null mutant mice demonstrate a range of behavioral and neurophysiological abnormalities that resemble symptoms of mood and anxiety disorders (Holmes *et al*. 2003c). 5-HTT null mutants show reduced aggression (Holmes *et al*. 2002a), reduced locomotor activity (Holmes *et al*. 2002a), altered responses to antidepressants (Holmes *et al*. 2002d) and psychostimulants (Bengel *et al*. 1998; Sora *et al*. 2001), reduced REM sleep (Wisor *et al*. 2003), altered analgesic responses (Vogel *et al*. 2003), and exaggerated neuroendocrine and adrenomedullary responses to stress (Li *et al*. 2000; Tjurmina *et al*. 2002). Extending these findings, we recently reported that 5-HTT null mutant mice exhibit increased anxiety-like behavior and reduced exploratory locomotion on a battery of tests (Holmes *et al*. 2003b). The abnormal anxiety-like phenotype was observed in 5-HTT null mutant mice backcrossed onto a C57BL/6J (B6) genetic background. Interestingly, preliminary analysis of 5-HTT null mutant mice backcrossed onto a 129SvEvTac (129S6) failed to reveal a consistent abnormal anxiety-related phenotype and thereby suggested an influence of genetic background on the mutation (Holmes *et al*. 2001).

The potential influence of genetic background is an important issue in behavioral phenotyping of mutant mice. Inbred strains used in the generation and maintenance of mutant mice may differ from one another in genes that regulate behaviors relevant to the expression of phenotypic abnormalities caused by the mutation (Banbury Conference on Genetic Background in Mice 1997; Caldarone *et al*. 2000; Crawley *et al*. 1997; Crawley 2000; Crusio 1996; Gerlai 1996; Le Roy *et al*. 2000; Phillips *et al*. 1999; Wolfer & Lipp 2000; Wolfer *et al*. 2002). Previous studies that have examined mutant behavioral phenotypes on multiple genetic backgrounds have found major effects of background strain (Bowers *et al*. 1999; Kelly *et al*. 1998; Le Roy *et al*. 2000; Thiele *et al*. 2000). Interactions between a mutation and genetic background may be particularly salient to the study of a candidate gene, such as the 5-HTT, in a complex behavioral trait such as anxiety. This is because, while it is estimated that genetic factors contribute approximately 30–70% of the risk for anxiety disorders (Kendler 2001), no single gene accounts for more than a small proportion of the overall risk (Lesch 2001). Rather, traits such as anxiety are likely to result from epistatic gene–gene interactions (Crabbe 2001; Murphy *et al*. 2003; Plomin *et al*. 1994) and gene–environment interactions (Barr *et al*. 2003; Caspi *et al*. 2003; Crabbe *et al*. 1999). Thus, functional interactions between mouse mutations affecting anxiety-like behavior and genetic background may be of relevance to understanding the polygenic nature of anxiety states.

In the present study, 5-HTT null mutant mice were repeatedly backcrossed onto either a B6 or 129S6 genetic background for at least 12 generations to produce two congenic 5-HTT null mutant lines. To test whether genetic background influenced abnormal anxiety-like and exploratory phenotypes in 5-HTT null mutant mice, we conducted a cross-background comparison of 5-HTT null mutants. Male and female 5-HTT null mutants on the B6 congenic and 129S6 congenic backgrounds, and their respective +/+ littermate controls, were compared using two tests for anxiety-like behavior, the elevated plus-maze and light ↔ dark exploration tests. Results showed that behavioral abnormalities were evident in 5-HTT null mutant mice on the B6, but not the 129S6, congenic background. Therefore, in a second part of the study, we sought to confirm that the 5-HTT null mutation was penetrant on the 129S6 genetic background. To this end, 5-HTT null mutants on the 129 background were assessed for 5-HT_{1A} receptor expression/function as measured via quantitative autoradiography for 5-HT_{1A} receptor binding and assessment of 5-HT_{1A} receptor agonist-induced hypothermia. Decreased 5-HT_{1A} receptor expression/function has been reliably demonstrated in 5-HTT null mutant mice on various genetic backgrounds (Fabre *et al*. 2000; Gobbi *et al*. 2001; Li *et al*. 1999; Li *et al*. 2000; Mannoury la Cour *et al*. 2001) and provides a robust marker for serotonergic abnormalities resulting from the 5-HTT null mutation. Decreased 5-HT_{1A} receptor expression/function in 129S6-background null mutants would demonstrate that, despite the absence of behavioral abnormalities, the null mutation was indeed penetrant in mutants on this background.

Materials and methods

Subjects

Serotonin transporter (5-HTT) null mutant mice were originally generated using 129P1/ReJ (129P1) embryonic stem cells microinjected into C57BL/6J (B6) blastocysts (Bengel *et al*. 1998). One cohort of mice was backcrossed onto a B6 genetic background for 12–15 generations (B6 congenic). The 129SvEvTac 129S6 strain was used to backcross another cohort of mutant mice for 13 generations (129S6 congenic). To minimize potential genotype-related variation in parental behavior and early life experience (e.g., Bale *et al*. 2002), all subjects were generated from heterozygous 5-HTT +/− × 5-HTT +/− matings. Post-weaning, littermates were reared together and housed together in same-sex groups, in a temperature and humidity controlled vivarium, under a 12-h light/dark cycle (lights on at 06.00). Mice from the B6 congenic and 129S6 congenic cohorts were housed side-by-side in the same holding room. B6 congenic mice used in the present experiments were 13 female and 10 male 5-HTT −/− mice, 11 female and 15 male 5-HTT +/− mice and 11 female and 11 male +/+ controls. 129S6 congenic mice were 11 female and 12 male 5-HTT −/− mice, 14 female and 12 male 5-HTT +/− mice and 14 female and 13 male +/+ controls.

There is clear evidence that behavioral profiles on tests including the light ↔ dark exploration test and elevated plus-maze are altered in mice with repeated exposure to the same test (e.g., Dawson *et al*. 1994; Holmes & Rodgers 1998; Holmes *et al*. 2001). Relatively little is known about

the potential carry-over effect of experience of one test for anxiety-like behavior on behavior in another (McIlwain *et al*. 2001). In the present study, 20–24 week-old B6 and 129S6 congenic cohorts were simultaneously assessed on the light ↔ dark exploration test and, one week later, the elevated plus-maze test. Both tests were conducted during the light phase of the light/dark cycle (06.00–18.00). To ensure that the experimenter remained blind to genotype during testing, subjects were only identified by coded ear tag numbers. An additional cohort of mice was backcrossed onto a 129S6 genetic background for six generations. These mice were assessed for 5-HT_{1A} receptor agonist-induced hypothermia and 5-HT_{1A} receptor autoradiography. There were 16 female and 16 male 5-HTT −/− mice, 27 female and 24 male 5-HTT +/− mice, and 15 female and 13 male +/+ controls. All experimental procedures were approved by the National Institute of Mental Health Animal Care and Use Committee, and followed the NIH guidelines outlined in *Using Animals in Intramural Research*.

Light ↔ dark exploration test

The light ↔ dark exploration test was conducted as previously described (Crawley 1981; Holmes *et al*. 2002b, 2003a, 2003b; Mathis *et al*. 1995). The apparatus consisted of a polypropylene cage (44 × 21 × 21 cm) separated into two compartments by a partition, which had a small opening (12 × 5 cm) at floor level. The larger compartment (28 cm long) was open-topped, transparent and brightly illuminated by a desk lamp (∼1000 lux). The smaller compartment (14 cm long) was close-topped and painted black. In our previous study with 5-HTT null mutants on a B6 background, we found that placing 5-HTT −/− mice in the light compartment at the beginning of testing produced a suppression of behavior in a high proportion of 5-HTT −/− mice (but not +/+ littermate controls). This response had the effect of producing a false-positive, anxiolytic-like, 'preference' for the light compartment (Holmes *et al*. 2003b). Therefore, for the purposes of the present study, all mice were initially placed within the dark compartment, and then allowed to freely explore the apparatus for 10 min. Mice were tested in an order pseudorandomly counterbalanced for genotype, gender and genetic background. The number of light ↔ dark transitions between the two compartments, and the total time spent in the dark compartment, was automatically recorded via photocells located at the intercompartmental partition, connected to a custom-built data analyzer (Bruce Smith, George Dold and colleagues, Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

Elevated plus-maze

The elevated plus-maze was conducted as previously described (Bale *et al*. 2002; File 2001; Holmes *et al*. 2002b, 2003a, 2003b). The apparatus (San Diego Instruments, San Diego, CA) comprised two open arms (30 × 5 cm) and two closed arms (30 × 5 × 15 cm) that extended from a common central platform (5 × 5 cm). A small raised lip (0.5 cm) around the edges of the open arms helped to prevent mice from slipping off. The apparatus was constructed from polypropylene and Plexiglas (white floor, clear walls) and elevated to a height of 38 cm above floor level. Mice were placed on the center square, facing an open arm, and allowed to freely explore the apparatus under even overhead fluorescent lighting (∼200 lux) for 5 min. Mice were tested in an order pseudorandomly counterbalanced for genotype, gender and genetic background. Standard behaviors measured were open and closed arm entries (entry = all 4 paws in an arm) and time spent in the open arms. Ethological behaviors (Holmes & Rodgers 1998; Holmes *et al*. 2000; Rodgers *et al*. 2002a, 2003) measured were rears in the closed arms, head dips over the sides of the open arms, stretched attend postures within the center square or closed arms (forward stretching and retraction of the body without forward ambulation) and the number of fecal boli emitted. Behavior was scored by an experienced observer (intrarater reliability >0.90, as previously determined; Holmes & Rodgers 1998), using behavioral scoring software (Hindsight, Scientific Programming Services, Wokingham, UK).

5-HT_{1A} receptor agonist-induced hypothermia (129S6-background)

The effects of the 5-HT_{1A} receptor agonist 8-OH-DPAT on core body temperature were assessed as an assay for 5-HT_{1A} receptor function (Li *et al*. 1999). To obtain a baseline reading, core body temperature was measured once every 10 min for a total of 3 separate measurements. 10 min after the third measurement, mice were subcutaneously injected with either saline or WAY 100635 (1 mg/kg). Core body temperature was assessed 10 min and then 20 min later. After a further 10 min had elapsed, all mice were injected subcutaneously with 8-OH-DPAT (0.1 mg/kg). Core body temperature was assessed once every 10 min for a total of six further measurements.

5-HT_{1A} receptor quantitative autoradiography (129S6-background)

Two weeks after testing for 8-OH-DPAT-induced hypothermia, mice were killed by rapid decapitation. Brains were removed and frozen immediately in dry, ice-cooled isopentyl alcohol for 10 seconds, transferred to dry ice for 10 min until completely frozen and then stored at ∼80 °C. Brains were cut into 16 μm-thick coronal sections in a cryostat. The sections were thaw-mounted onto chromalum/gelatin-coated glass slides and stored at ∼80 °C. Each slide contained brain sections from one mouse from each genotype. Four levels of sections were collected: striatum (bregma 0.98–0.50 mm), medial hypothalamus (bregma 0.7 to ∼1.06 mm), caudal hypothalamus (bregma ∼1.34 to ∼1.94 mm) and midbrain (bregma ∼4.36 to ∼4.84 mm) according to a mouse brain atlas (Franklin & Paxinos 1997).

^{125}I-MPPI binding sites in the brain sections were determined by autoradiographic assay, as described previously

(Li *et al*. 2001). Briefly, the slides were thawed and dried in a desiccator at room temperature before assay. The brain sections were preincubated for 30 min in an assay buffer (50 mM Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$) and then incubated with ^{125}I-MPPI (0.14 nM in assay buffer) for 2 h at room temperature. Nonspecific binding was defined in the presence of 10^{-5} M 5-HT. Slides were then washed twice with the assay buffer at 4 °C for 15 min and rinsed with cold ddH_2O. After being air blow-dried, the slides were exposed to Kodak Biomax MR film (Eastman Kodak Co., New York, NY). Films containing hippocampus and dorsal raphe slices were exposed for 1 day. Films containing all other regions were exposed for 3 days. A set of ^{125}I microscales (Amersham Biosciences, Piscataway, NJ) was exposed with the slides to calibrate the optic density into fmol/mg of tissue equivalent. Following exposure, brain images were digitized and analyzed using AIS image software (Imaging research Inc., Ontario, Canada). The gray scale density readings were calibrated to fmol/mg of tissue equivalent using the ^{125}I microscale. Specific ^{125}I-MPPI binding in each brain region was determined by subtracting the non-specific binding sites from the total binding sites in each region. Data for each individual subject and brain region are the mean average of four adjacent sections.

Drugs

8-hydroxy-2-(di-*n*-propylamin)tetralin (8-OH-DPAT) (RBI, Natick, MA) and *N*-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride (WAY 100635) (Wyeth Research, Princeton, NJ) were injected subcutaneously in a saline vehicle at a volume of 5 ml/kg body weight. Doses of 0.1 mg/kg 8-OH-DPAT and 1.0 mg/kg WAY 100635 were chosen on the basis of previous findings (Li *et al*. 1999) and pilot studies, which showed that a relatively high dose of WAY 100635 was required to block the hypothermia-inducing effects of 8-OH-DPAT in 129S6 mice. ^{125}I-MPPI is 4-[2′-[N-(2′-pyridinyl)-iodobenzamido]ethyl]piperazine.

Statistics

Behavioral data from the light ↔ dark exploration and elevated plus-maze tests were analyzed using three-way (genotype × genetic background × gender) analysis of variance (ANOVA) and Newman-Keuls posthoc comparisons where appropriate, using StatView (SAS Institute Inc., Cary, NC). Core body temperature data were analyzed using three-way (genotype × drug treatment × time point) ANOVA, with repeated measures for time point. Statistical significance was set at $P = 0.05$.

Results

Light ↔ dark exploration test

Light ↔ dark exploration test results are shown in Fig. 1. There were significant main effects of genotype ($F_{2,135} = 15.77$, $P < 0.001$), genetic background ($F_{1,135} = 50.83$, $P < 0.001$), gender ($F_{1,135} = 7.51$, $P < 0.01$) and a significant genotype × genetic background interaction ($F_{2,135} = 3.17$, $P < 0.05$), but no other interactions ($P > 0.25$), for light ↔ dark transitions. There were significant main effects of genotype ($F_{2,135} = 9.93$, $P < 0.001$), genetic background ($F_{1,135} = 38.42$, $P < 0.001$), gender ($F_{1,135} = 5.25$, $P < 0.05$) and a significant genotype × genetic background interaction ($F_{2,135} = 8.06$, $P < 0.001$), but no other interactions ($P > 0.76$), for percent time in the light compartment.

Newman-Keuls *post hoc* analyses indicated that male and female B6 congenic 5-HTT −/− mice spent significantly less time in the light compartment and made significantly fewer light ↔ dark transitions, as compared to B6 congenic +/+ controls (all $P < 0.01$). Female B6 congenic 5-HTT +/− mice also spent significantly less time in the light compartment and made significantly fewer light ↔ dark transitions than female +/+ controls (both $P < 0.05$). There were no significant differences between 129S6 congenic 5-HTT +/− or 5-HTT −/− mice and 129S6 congenic +/+ controls (regardless of gender), for light ↔ dark transitions or percent time in the light compartment. Newman-Keuls *post hoc* comparisons across genetic background showed that male and female 129S6 congenic +/+ mice spent significantly less time in the light compartment and made significantly fewer light ↔ dark transitions than same-gender B6 congenic +/+ mice (all $P < 0.01$). Similarly, male and female 129S6 congenic 5-HTT +/− mice showed significantly fewer light ↔ dark transitions than B6 congenic 5-HTT +/− mice (all $P < 0.01$). Male 129S6 congenic 5-HTT +/− and 5-HTT −/− mice spent significantly less percent time in the light compartment than male B6 congenic 5-HTT +/− and 5-HTT −/− mice (both $P < 0.05$).

Elevated plus-maze

Standard measures of elevated plus-maze behavior are shown in Fig. 2. There was a significant main effect of genotype for percent open time ($F_{2,120} = 7.58$, $P < 0.001$), entries into the open arms ($F_{2,120} = 9.81$, $P < 0.001$), closed entries ($F_{2,120} = 9.89$, $P < 0.001$) and total entries ($F_{2,120} = 13.73$, $P < 0.001$). There was a significant main effect of genetic background for percent open time ($F_{1,120} = 6.82$, $P = 0.01$), entries into the open arms ($F_{2,120} = 15.10$, $P < 0.001$), closed entries ($F_{1,120} = 9.23$, $P < 0.01$) and total entries ($F_{1,120} = 17.23$, $P < 0.001$). There was a significant main effect of gender for entries into the open arms ($F_{2,120} = 4.62$, $P < 0.05$), but not other measures ($P > 0.09$). There was a significant genotype × genetic background interaction for percent open time ($F_{1,120} = 3.85$, $P < 0.05$) and entries into the open arms ($F_{2,120} = 3.44$, $p < 0.05$), but not closed entries ($F_{2,120} = 0.23$, $P = 0.79$) or total entries ($F_{2,120} = 1.27$, $P = 0.28$). There were no other significant interactions between variables for any standard measure ($P > 0.09$).

Newman-Keuls *post hoc* analyses indicated that male and female B6 congenic 5-HTT −/− mice showed significantly less

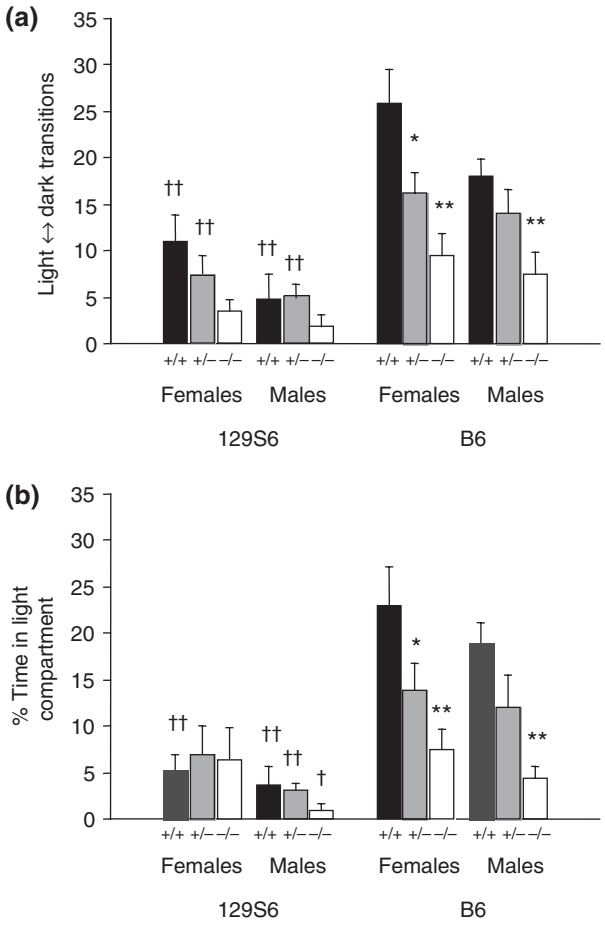

Figure 1: Genetic background determined the detection of abnormal anxiety-related and exploratory phenotypes in 5-HTT null mutant mice in the light ↔ dark exploration test. B6 congenic 5-HTT −/− mice (a) made fewer light ↔ dark transitions and (b) spent less time in the light compartment, as compared to B6 congenic +/+ controls. Both male and female 5-HTT null mutants showed a gene-dose effect, with greater reductions in these measures evident in 5-HTT −/− than 5-HTT +/− mice. 129S6 congenic 5-HTT null mutant mice were no different from 129S6 congenic +/+ controls on either measure (a,b). 129S6 congenic 5-HTT +/− and +/+ mice (a) made fewer light ↔ dark transitions and (b) spent less time in the light compartment, as compared to B6 congenic 5-HTT +/− and +/+ counterparts. Male 129S6 congenic 5-HTT −/− mice spent significantly less (b) time in the light compartment, as compared to B6 congenic 5-HTT −/− mice. $n =$ 10–15 per genotype, per gender, per genetic background. $^{**}P < 0.01$, $^{*}P < 0.05$ B6 congenic 5-HTT −/− or 5-HTT +/− vs. B6 congenic +/+ controls. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ 129S6 congenic vs. B6 congenic mice of the same genotype.

percent open time, and total entries, than B6 congenic +/+ controls (all $P < 0.05$). Male B6 congenic 5-HTT −/− and 5-HTT +/− mice also showed significantly less open entries than B6 congenic +/+ controls (both $P < 0.01$). Female B6 congenic 5-HTT −/− mice made significantly fewer closed entries than B6 congenic +/+ controls ($P < 0.05$). Male 129S6 congenic 5-HTT −/− mice made significantly fewer closed entries and total entries than 129S6 congenic +/+ controls (both $P < 0.05$). Newman-Keuls *post hoc* comparisons across genetic background indicated that male and female 129S6 congenic +/+ mice showed significantly fewer open entries and total entries, as compared to same-gender B6 congenic +/+ mice (both $P < 0.05$). In addition, female 129S6 congenic +/+ mice made significantly fewer closed entries than female B6 congenic +/+ counterparts ($P < 0.05$). There were no significant differences between 129S6 congenic 5-HTT +/− or 5-HTT −/− mice and B6 congenic 5-HTT +/− or 5-HTT −/− mice (all $P > 0.05$).

Ethological measures of elevated plus-maze behavior are shown in Fig. 3. There was a significant main effect of genotype for head dips ($F_{2,120} = 13.47$, $P < 0.001$), but not rears ($F_{1,120} = 2.04$, $P = 0.13$), stretched attend postures ($F_{2,120} = 1.84$, $P = 0.16$) or fecal boli ($F_{1,119} = 0.33$, $P = 0.72$). There was a significant main effect of genetic background for rears ($F_{2,120} = 177.82$, $P < 0.001$), head dips ($F_{1,120} = 52.32$, $P < 0.001$), stretched attend postures ($F_{1,120} = 23.93$, $P < 0.001$) and fecal boli ($F_{2,119} = 95.70$, $P < 0.001$). There was a significant main effect of gender for head dips ($F_{1,120} = 4.39$, $P < 0.05$), stretched attend postures ($F_{1,120} = 4.70$, $P = 0.03$), but not for rears ($F_{1,120} = 0.74$, $P = 0.39$) or fecal boli ($F_{1,119} = 1.89$, $P = 0.17$). There was a significant genotype×genetic background interaction for head dips ($F_{2,120} = 12.62$, $P < 0.001$) and fecal boli ($F_{2,119} = 4.43$, $P = 0.01$), but not rears ($F_{2,120} = 0.32$, $P = 0.72$) or stretched attend postures ($F_{2,120} = 1.62$, $P < 0.001$). There were no other significant interactions between variables for any ethological measure ($P > 0.19$).

Newman-Keuls *post hoc* analyses indicated that male and female B6 congenic 5-HTT −/− mice and male 5-HTT +/− mice made significantly fewer head dips than B6 congenic +/+ controls (all $P < 0.05$). There were no significant differences between 129S6 congenic 5-HTT null mutant mice and 129S6 congenic +/+ controls for any of the ethological measures. Newman-Keuls *post hoc* comparisons across genetic background revealed that, regardless of genotype or gender, 129S6 congenic mice showed significantly fewer rears and (except male 5-HTT +/− mice) emitted significantly more fecal boli than their B6 congenic counterparts (all $P < 0.05$). Regardless of genotype, male 129S6 congenic mice showed significantly more stretched attend postures than male B6 congenic counterparts (all $P < 0.05$). Male and female 129S6 congenic 5-HTT +/− and +/+ mice made significantly fewer head dips than their B6 congenic counterparts (all $P < 0.01$).

5-HT$_{1A}$ receptor agonist-induced hypothermia (129S6-background)

The hypothermia-inducing effects of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, in mice on the 129S6 background are shown in Fig. 4. Male and female mice were tested separately and therefore their data were analyzed separately.

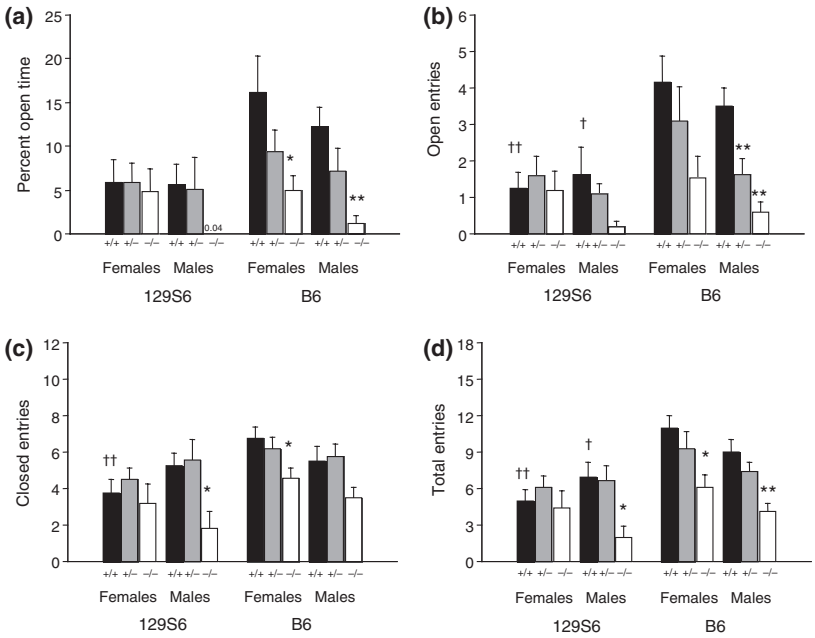

Figure 2: Genetic background determined the detection of abnormal anxiety-related and exploratory phenotypes in 5-HTT null mutant mice on standard behaviors in the elevated plus-maze. B6 congenic 5-HTT –/– mice showed (a) less percent open time, and made fewer (b) open entries (males, not females), (c) closed entries (females, not males) and (d) total entries, as compared to B6 congenic +/+ controls. Both male and female 5-HTT null mutants showed a gene-dose effect, with greater and more consistent reductions in these measures evident in 5-HTT –/– than 5-HTT +/– mice. Male 129S6 congenic 5-HTT null mutant mice (c) made fewer closed entries as compared to 129S6 congenic +/+ controls, but were no different on any other measure (a,b,d). 129S6 congenic +/+ mice made fewer (b) open entries (c) closed entries (females, not males) and (d) total entries, as compared to B6 congenic +/+ counterparts. There were no differences between 129S6 congenic 5-HTT +/– or 5-HTT –/– mice and their B6 congenic 5-HTT +/– or 5-HTT –/– counterparts on any measure (a-d). $n = 10$–13 per genotype, per gender, per genetic background. $^{**}P < 0.01$, $^{*}P < 0.05$ B6 congenic 5-HTT –/– or 5-HTT +/– vs. congenic +/+ controls of the same genetic background. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ 129S6 congenic vs. B6 congenic mice of the same genotype.

Females

There was a significant effect of genotype on baseline core body temperature prior to any drug treatment (i.e., time point 1, Fig. 4) ($F_{2,53} = 8.11$, $P < 0.001$). Newman-Keuls *post hoc* analysis showed that core body temperature was significantly higher in female 5-HTT –/– mice than +/+ controls at this time point ($P < 0.01$). There was a significant main effect of genotype ($F_{2,106} = 86.96$, $P < 0.001$), and a significant drug × genotype interaction ($F_{2,106} = 8.14$, $P < 0.001$), but no main effect of drug treatment (saline vs. WAY 100635) ($F_{1,106} = 0.48$, $P = 0.49$) for core body temperature prior to treatment with 8-OH-DPAT (i.e., time points 3 and 4, Fig. 4). Newman-Keuls *post hoc* analysis showed that core body temperature was significantly higher in female 5-HTT –/– mice than +/+ controls at both of these time points (both $P < 0.01$). In addition, core body temperature in 5-HTT –/– mice treated with WAY 100635 was significantly higher than in 5-HTT –/– mice treated with saline at these time points (both $P < 0.05$).

There were significant main effects of genotype ($F_{2,50} = 51.93$, $P < 0.001$), drug treatment ($F_{1,50} = 36.25$, $P < 0.001$) and time point ($F_{5,250} = 14.77$, $P < 0.001$) for core body temperature following treatment with 8-OH-DPAT (i.e., time points 6–11, Fig. 4). There were also significant genotype × time point ($F_{10,250} = 2.68$, $P < 0.01$), drug treatment × time point ($F_{5,250} = 7.46$, $P < 0.001$), and genotype × drug treatment × time point ($F_{10,250} = 2.27$, $P = 0.02$) interactions for core body temperature. Separate ANOVAS for each genotype showed that 8-OH-DPAT significantly reduced core body temperature in female 5-HTT +/– mice ($F_{5,55} = 9.53$, $P < 0.001$) and +/+ controls ($F_{5,35} = 13.37$, $P < 0.001$), but not 5-HTT –/– mice ($F_{5,42} = 0.43$, $P = 0.83$). Newman-Keuls *post hoc* analysis showed that core body temperature was significantly higher in saline-pretreated female 5-HTT –/– mice at every time point (all $P < 0.01$) and in 5-HTT +/– mice at every time point (all $P < 0.05$) except 10, as compared to saline-pretreated +/+ controls. In mice pretreated with WAY 100635, separate ANOVAS for each genotype showed that 8-OH-DPAT had no effect on core body temperature in female 5-HTT –/– mice ($F_{5,30} = 0.64$, $P = 0.67$), 5-HTT +/– mice ($F_{5,60} = 2.23$, $P = 0.06$) or +/+ controls ($F_{5,30} = 0.51$, $P = 0.77$). However, core body temperature in 5-HTT –/– mice pretreated with WAY 100635

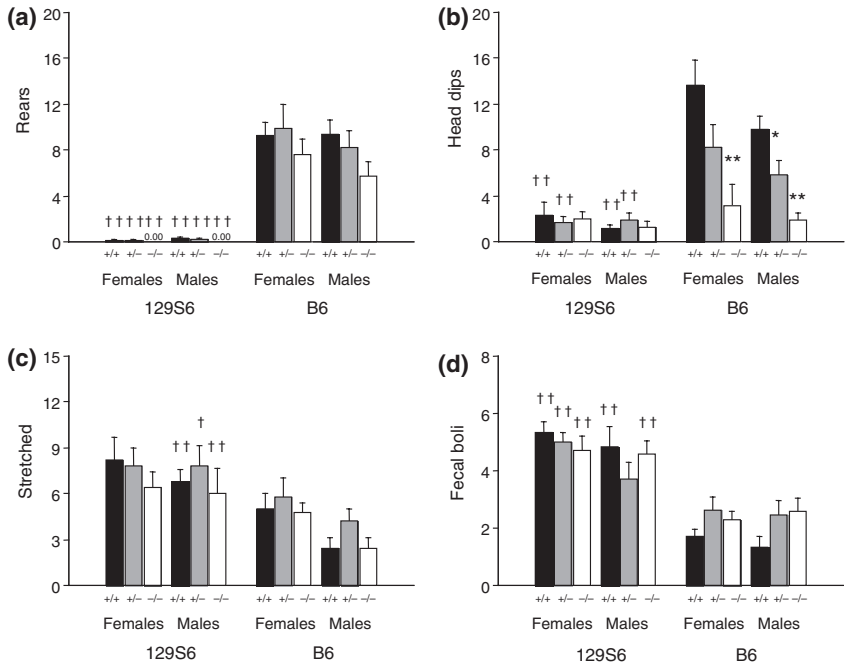

Figure 3: Genetic background determined the detection of abnormal anxiety-related and exploratory phenotypes in 5-HTT null mutant mice on ethological behaviors in the elevated plus-maze. B6 congenic 5-HTT −/− mice (b) made fewer head dips as compared to B6 congenic +/+ controls, but were normal on (a) rears (c) stretched attend postures, and (d) fecal boli. 129S6 congenic 5-HTT null mutant mice were no different from 129S6 congenic +/+ controls on any measure (a-d). 129S6 congenic 5-HTT +/− and +/+ mice made fewer (a) rears (b) head dips (c) stretched attend postures (males, not females), and (except 5-HTT +/− males) (d) emitted more fecal boli, as compared to their B6 congenic 5-HTT +/− and +/+ counterparts. 129S6 congenic 5-HTT −/− mice (a) made more rears and (d) emitted more fecal boli, as compared to B6 congenic 5-HTT −/− mice. $n = 10$–13 per genotype, per gender, per genetic background. $^{**}P < 0.01$, $^{*}P < 0.05$ B6 congenic 5-HTT −/− or 5-HTT +/− vs. congenic +/+ controls of the same genetic background. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ 129S6 congenic vs. B6 congenic mice of the same genotype.

was significantly higher than in 5-HTT −/− mice treated with saline at every time point (all $P < 0.05$).

Males

There was no significant effect of genotype on baseline core body temperature prior to any drug treatment (i.e., time point 1, Fig. 4) ($F_{2,50} = 2.68$, $P = 0.08$). There was a significant main effect of genotype ($F_{2,100} = 24.19$, $P < 0.001$) and a significant drug × genotype interaction ($F_{2,100} = 4.29$, $P = 0.02$), but no main effect of drug treatment (saline vs. WAY 100635) ($F_{1,100} = 0.11$, $P = 0.17$) for core body temperature prior to treatment with 8-OH-DPAT (i.e., time points 3 and 4, Fig. 4). Newman-Keuls *post hoc* analysis showed that core body temperature was significantly higher in male 5-HTT −/− mice than +/+ controls at both time points (both $P < 0.05$). In addition, core body temperature in 5-HTT −/− mice pretreated with WAY 100635 was significantly higher than in 5-HTT −/− mice pretreated with saline at time point 3 ($P < 0.05$).

There were significant main effects of genotype ($F_{2,47} = 37.66$, $P < 0.001$), drug treatment ($F_{1,47} = 69.63$, $P < 0.001$) and time point ($F_{5,235} = 16.84$, $P < 0.001$) for core body temperature following treatment with 8-OH-DPAT (i.e., time points 6–11, Fig. 4). There were also significant genotype × time point ($F_{10,235} = 4.70$, $P < 0.01$), drug treatment × time point ($F_{5,235} = 16.32$, $P < 0.001$) and genotype × drug treatment × time point ($F_{10,250} = 4.89$, $P < 0.001$) interactions for core body temperature. Separate ANOVAs for each genotype showed that 8-OH-DPAT significantly reduced core body temperature in male 5-HTT +/− mice ($F_{5,50} = 14.86$, $P < 0.001$) and +/+ controls ($F_{5,25} = 11.80$, $P < 0.001$), but not 5-HTT −/− mice ($F_{5,35} = 0.93$, $P = 0.47$). Newman-Keuls *post hoc* analysis showed that core body temperature was significantly higher in saline-pretreated male 5-HTT −/− mice than in saline-pretreated +/+ controls at every time point (all $P < 0.01$). In mice pretreated with WAY 100635, separate ANOVAs for each genotype showed that 8-OH-DPAT had no effect on core body temperature in male 5-HTT −/− mice ($F_{5,35} = 0.29$, $P = 0.91$), 5-HTT +/− mice ($F_{5,60} = 1.85$, $P = 0.12$) or +/+ controls ($F_{5,30} = 0.73$, $P = 0.61$). Core body temperature in male 5-HTT −/− mice pretreated with WAY 100635 was significantly higher than in male 5-HTT −/− mice pretreated with saline at all time points (all $P < 0.05$) except 10.

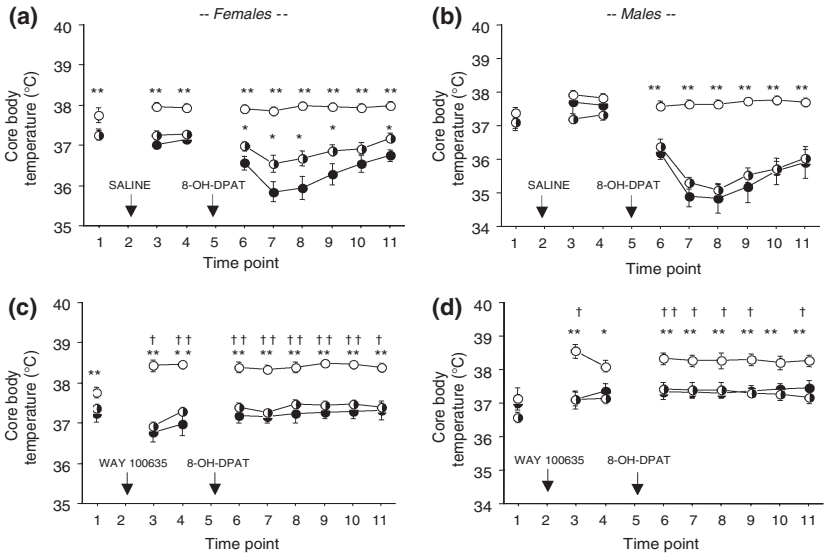

Figure 4: 5-HTT null mutant mice on a 129S6 genetic background showed a loss of the hypothermia response to the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT. 8-OH-DPAT (0.1 mg/kg) produced a significant decrease in core body temperature in (a) female and (b) male 5-HTT +/− mice and +/+ controls at all time points (except 10 in female 5-HTT +/− mice) post-treatment. Pre-treatment with the 5-HT$_{1A}$ receptor antagonist, WAY 100635 (1.0 mg/kg), blocked the hypothermia-inducing effects of 8-OH-DPAT in (c) female and (d) male 5-HTT +/− mice and +/+ controls. Pre-treatment with WAY 100635 produced a significant increase in core body temperature in (c) female and (d) male 5-HTT −/− mice, as compared to 5-HTT −/− mice pretreated with saline, at all time points (except 10 in females) post 8-OH-DPAT-treatment. $n = 6$–13 per genotype, per gender, per treatment. $^{**}P < 0.01$, $^{*}P < 0.05$ 5-HTT +/− and 5-HTT −/− mice vs. +/+ controls receiving the same treatment. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ 5-HTT −/− mice pretreated with WAY 100635 vs. 5-HTT −/− mice pretreated with saline. 5-HTT −/− (empty circles), 5-HTT +/− (semifilled circles), +/+ (filled circles).

5-HT$_{1A}$ receptor quantitative autoradiography (129S6-background)

5-HT$_{1A}$ receptor quantitative autoradiography results are shown in Table 1 and Fig. 5. Brain sections from male and female mice were analyzed separately and therefore their data were analyzed separately.

Females

There was a significant effect of genotype on 5-HT$_{1A}$ receptor binding density in all brain regions analyzed (all $P < 0.05$), except the hippocampus. Newman-Keuls *post hoc* analyses showed that binding density was significantly lower in female 5-HTT −/−, as compared to female +/+ controls, in midbrain raphe (dorsal and median), amygdala (AC, BMA, MeA, Aco, but not BLA), hypothalamic (AH, LHN, VMH, DMH, but not PVN) and septal nuclei (MS, Ld, but not LSI) (all $P < 0.05$).

Males

There was a significant effect of genotype on 5-HT$_{1A}$ receptor binding density in all brain regions analyzed (all $P < 0.05$), except the hippocampus. Newman-Keuls *post hoc* analyses showed that binding density was significantly lower in male 5-HTT −/−, as compared to male +/+ controls, in midbrain raphe (dorsal and median), amygdala (AC, BMA, MeA, but not Aco, BLA), hypothalamic (AH, LHN, DMH, but not VMH, PVN) and septal nuclei (Ld, but not LSI, MS) (all $P < 0.05$).

Discussion

The present findings demonstrate a robust influence of genetic background strain on anxiety-related and exploratory locomotor abnormalities resulting from targeted null mutation of the mouse 5-HTT gene (*htt*). Marked effects of genetic background on abnormal behavioral phenotypes in 5-HTT null mutant mice were observed in mice that underwent repeated backcrosses onto either a B6 or a 129S6 genetic background for at least 12 generations; thereby producing two separate congenic lines. Male and female 5-HTT −/− mice, 5-HTT +/− mice and +/+ littermates on the 129S6 or B6 congenic backgrounds were compared using the light ↔ dark exploration and elevated plus-maze tests for anxiety-like behavior.

Results indicated that in the light ↔ dark exploration test male and female B6 congenic 5-HTT −/− mice showed a significant reduction in time spent in the aversive light compartment and significantly fewer light ↔ dark transitions than B6 congenic +/+ littermate controls, a profile consistent with increased anxiety-like behavior. B6 congenic 5-HTT +/−

Table 1: 5-HTT null mutant mice on a 129S6 genetic background showed lower densities of ^{125}-I-MPPI-labeled 5-HT_{1A} receptor binding in several brain regions. Quantitative autoradiographic analysis demonstrated reduced 5-HT_{1A} receptor binding density in midbrain, amygdala, hypothalamic and septal nuclei in female and male 5-HTT −/− mice, as compared to +/+ controls. Data are means ± SEM expressed in fmol/mg tissue equivalent. $n = 6$–7 per genotype, per gender. See Fig. 5 for key to abbreviations

	Females			Males		
	+/+	+/−	−/−	+/+	+/−	−/−
Midbrain raphe						
DR	351.2 ± 14.9	377.2 ± 28.8	243.3 ± 12.0**	350.0 ± 23.9	365.9 ± 9.7	265.0 ± 4.9**
MR	192.3 ± 9.5	158.2 ± 9.9	104.4 ± 15.5**	174.3 ± 12.8	157.1 ± 9.9	121.1 ± 7.2*
Amygdala						
BLA	43.6 ± 4.9	48.1 ± 3.1	38.9 ± 2.9	42.8 ± 5.0	46.9 ± 2.9	38.9 ± 2.6
AC	82.0 ± 6.4	86.4 ± 5.5	61.1 ± 3.8*	85.5 ± 6.4	86.0 ± 2.6	65.1 ± 2.0**
BMA	108.2 ± 4.9	106.6 ± 4.3	79.1 ± 1.9**	119.7 ± 8.3	118.7 ± 3.3	93.7 ± 4.8*
MeA	92.4 ± 3.5	96.3 ± 3.1	68.5 ± 3.7**	102.5 ± 7.8	99.2 ± 3.1	73.0 ± 3.8**
ACo	130.5 ± 3.2	126.8 ± 5.3	99.8 ± 7.6**	132.6 ± 9.3	124.9 ± 3.0	104.3 ± 8.7
Hippocampus						
CA1	370.8 ± 5.7	364.4 ± 14.9	343.9 ± 10.1	406.9 ± 11.6	393.2 ± 13.6	383.6 ± 9.1
Hypothalamus						
PVN	20.6 ± 4.7	31.6 ± 6.2	21.8 ± 5.7	47.1 ± 6.1	46.5 ± 3.2	30.9 ± 5.3
AH	82.7 ± 3.3	81.4 ± 3.1	53.1 ± 4.4**	94.7 ± 6.0	90.9 ± 2.3	69.6 ± 2.1**
LHN	30.8 ± 1.4	30.4 ± 2.0	24.4 ± 0.7*	39.7 ± 3.8	39.0 ± 2.6	26.4 ± 1.4*
VMH	63.1 ± 4.9	59.6 ± 8.2	40.1 ± 3.2*	54.9 ± 9.1	42.5 ± 2.0	32.9 ± 2.4
DMH	61.8 ± 1.9	57.0 ± 5.5	41.9 ± 1.3**	68.3 ± 5.2	63.1 ± 3.1	48.2 ± 1.9**
Septum						
LSI	387.5 ± 11.7	381.2 ± 16.3	293.3 ± 11.3**	413.9 ± 18.3	409.9 ± 13.8	312.9 ± 11.6**
Ld	201.8 ± 23.8	170.4 ± 15.0	158.1 ± 9.9	227.0 ± 16.6	227.7 ± 8.3	198.5 ± 9.4
MS	460.7 ± 14.7	433.1 ± 17.1	357.9 ± 29.4*	457.4 ± 32.0	422.5 ± 32.7	383.1 ± 17.9

*$P < 0.05$; **$P < 0.01$ for 5-HTT −/− mice vs. same-sex +/+ controls.

showed reduced time spent in the light compartment and fewer light ↔ dark transitions than +/+ controls, although these differences were statistically significant in female, but not male, mice. Behavioral abnormalities in B6 congenic 5-HTT null mutant mice were confirmed in the elevated plus-maze. In this test, B6 congenic 5-HTT −/− mice spent significantly less time in the aversive open arms than B6 congenic +/+ littermates. B6 congenic 5-HTT −/− mice also showed a significant (males) or a non-significant trend (females) for fewer open arm entries than +/+ littermates. In addition, B6 congenic 5-HTT −/− mice made significantly fewer head dips than +/+ controls, a measure of 'directed exploration' (Griebel *et al*. 2000; Holmes & Rodgers 1998), that is increased by anxiolytics (e.g., Griebel *et al*. 2000; Rodgers *et al*. 2002b, 2003) and decreased by exposure to stressors (e.g., Tsuji *et al*. 2000). As in the light ↔ dark exploration test, gene-dosage-dependent abnormalities were observed in the elevated plus-maze, i.e., B6 congenic 5-HTT +/− mice showed greater avoidance of the open arms and fewer head-dips than +/+ littermates. While these differences were statistically robust in male 5-HTT +/− mice, the same pattern of behavioral differences was evident in females, suggesting that gender variations in anxiety-related abnormalities in the mutant mice were relatively minor. Taken together, these data replicate our previous finding that 5-HTT null mutant mice backcrossed onto a B6 background for eight generations exhibit anxiety-related behavioral abnormalities in the elevated plus-maze, light ↔ dark exploration, emergence and open field tests (Holmes *et al*. 2003b). These findings in the mouse provide further support for the low-expressing (short) form of the *HTTLPR* as a major candidate gene for mood and anxiety disorders (Caspi *et al*. 2003; Greenberg *et al*. 2000; Hariri *et al*. 2002; Lesch *et al*. 1996; Mazzanti *et al*. 1998).

The precise behavioral nature of the abnormalities in B6 congenic 5-HTT null mutant mice remains to be fully determined. 5-HTT null mutants not only showed alterations on measures of anxiety-like behavior, but also exhibited reductions in measures of exploratory locomotion, such as fewer plus-maze total and closed entries relative to +/+ controls. In this context we have previously shown that 5-HTT null mutant mice demonstrated reduced locomotor activity not only in the open field test, but also in the nonaversive environment of the home cage (Holmes *et al*. 2002a; Holmes *et al*. 2003b). Therefore, both increased anxiety-like behavior and reduced exploratory locomotion may contribute to the present behavioral profiles of B6 congenic 5-HTT null mutants in the elevated plus-maze and light ↔ dark exploration test. To more accurately define the fear and anxiety-related behavioral abnormalities in 5-HTT null mutant mice, it will be important to assess these mice on tasks that are not

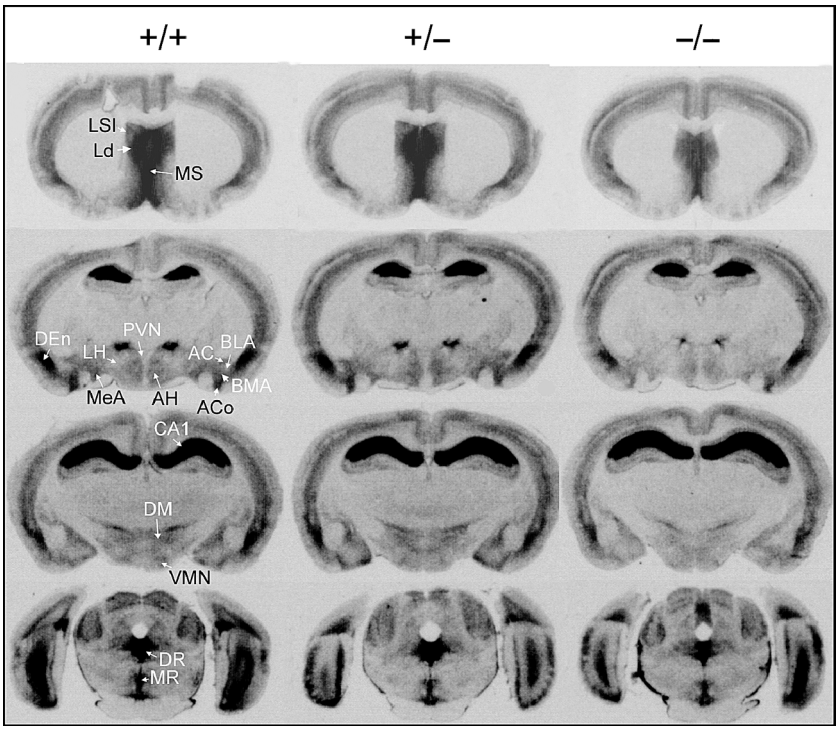

Figure 5: 5-HTT null mutant mice on a 129S6 genetic background showed lower densities of ^{125}I-MPPI-labeled 5-HT$_{1A}$ receptor binding in several brain regions. Quantitative autoradiographic analysis demonstrated reduced 5-HT$_{1A}$ receptor binding density in midbrain, amygdala, hypothalamic and septal nuclei in female and male 5-HTT −/− mice, as compared to +/+ controls. See Table 1 for complete results. Brain sections from top to bottom are striatum (bregma 0.98–0.50 mm), medium hypothalamus (bregma 0.7 to ∼1.06 mm), caudal hypothalamus (bregma ∼1.34 to ∼1.94 mm) and midbrain (bregma ∼4.36 to ∼4.84 mm). AC, central amygdaloid nucleus; ACo, anterior cortical amygdaloid nucleus; AH, anterior hypothalamic nucleus; BLA, basolateral amygdaloid nucleus, anterior; BMA, basomedial amygdaloid; CA1, CA1 subfield of hippocampus; Ce, central amygdaloid nucleus; DMN, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; Ld, lambdoid septal zone; LH, lateral hypothalamic nucleus; LSI, lateral septal nucleus, intermediate; MeA, medial amygdaloid nucleus; MR, medial raphe nucleus; MS, medial septal nucleus; PVN, paraventricular hypothalamic nucleus; VMN, ventromedial hypothalamic nucleus.

contingent upon normal exploratory locomotion (e.g., Vogel conflict test, fear-potentiated startle, fear conditioned freezing).

The major novel finding of the present study was that, while B6 congenic 5-HTT null mutant mice exhibited clear phenotypic abnormalities on the elevated plus-maze and light ↔ dark exploration tests, few behavioral abnormalities were detected in 5-HTT null mutant mice backcrossed onto a 129S6 congenic background. In the light ↔ dark exploration test, there were no significant differences in either time spent in the light compartment or light ↔ dark transitions between 129S6 congenic 5-HTT null mutants and their 129S6 congenic +/+ littermate controls. Similarly, in the elevated plus-maze, 129S6 congenic 5-HTT −/− mice were no different from 129S6 congenic +/+ controls on standard or ethological measures of anxiety-like behavior. These findings confirm evidence from preliminary studies demonstrating the absence of a robust increase in anxiety-like behavior in 5-HTT null mutant mice backcrossed onto the 129S6 background for six generations (Holmes *et al*. 2001).

There are three possible explanations for the observation of an abnormal anxiety-like phenotype in B6 congenic, but not 129S6 congenic, 5-HTT null mutant mice, as illustrated in Fig. 6. The first and most parsimonious explanation is that a 'ceiling effect' of high baseline anxiety-like behavior in the 129S6 strain prevented the detection of further increases in anxiety-like behavior caused by the 5-HTT null mutation (Banbury Conference on Genetic Background in Mice 1997; Caldarone *et al*. 2000; Contet *et al*. 2001; Crawley *et al*. 1997; Graves *et al*. 2002; Holmes *et al*. 2002b; Wolfer *et al*. 1997). While there are substantial genetic and behavioral differences between different 129 substrains (Simpson *et al*. 1997), certain 129 substrains, including 129S6 and 129P1, exhibit increased anxiety-like behavior and low exploration relative to other strains such as B6 (Bolivar *et al*. 2000; Contet *et al*. 2001; Cook *et al*. 2002; Holmes *et al*. 2002b; Homanics *et al*. 1999; Logue *et al*. 1997; Montkowski *et al*. 1997; Rogers *et al*. 1999; Rodgers *et al*. 2002a,2002b; Voikar *et al*. 2001).

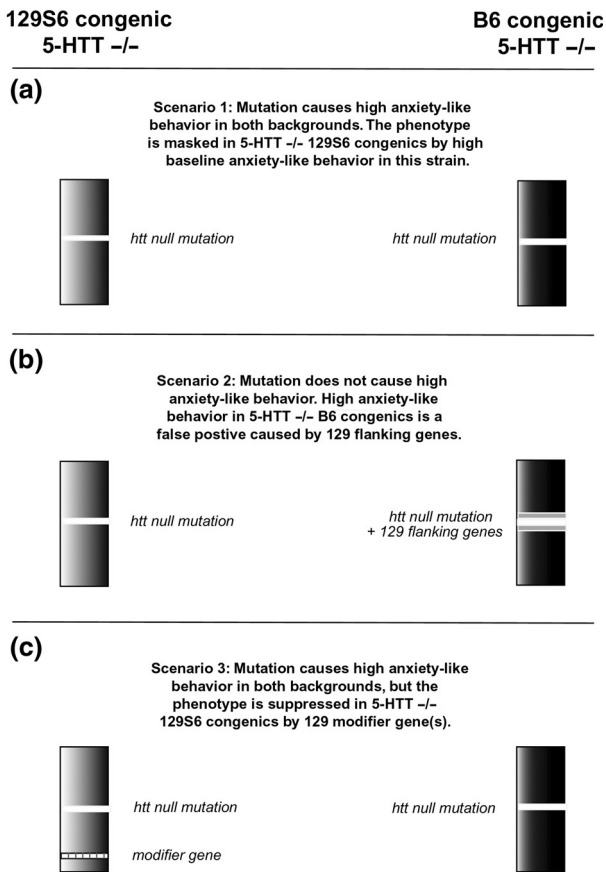

Figure 6: An abnormal anxiety-related phenotype in B6 congenic 5-HTT null mutant mice, but not 129S6 congenic 5-HTT null mutant mice could be caused by one of three possible scenarios. (a) The 5-HTT null mutation causes an increase in anxiety-like behavior in both B6 congenic and 129S6 congenic mice, but the phenotype is masked by abnormally high baseline anxiety-like behavior in 129S6 congenics. (b) The 5-HTT null mutation does not cause an abnormal anxiety-related phenotype in either B6 congenic or 129S6 congenic mice. The abnormal anxiety-related phenotype in B6 congenic mice is a false-positive caused by 129 embryonic stem cell donor genes in the flanking region surrounding the target locus. (c) The 5-HTT null mutation causes an abnormal anxiety-related phenotype in B6 congenic mice. An abnormal anxiety-related phenotype in 129S6 congenic mice is suppressed by an unknown modifier gene(s) present in the 129 genetic background.

Consistent with these earlier findings, comparisons between 129S6 congenic +/+ and B6 congenic +/+ mice indicated evidence of higher anxiety-like behavior and lower exploration in the former. Male and female 129S6 congenic +/+ mice made fewer open entries, head dips and rears in the elevated plus-maze and, in the light ↔ dark exploration test, spent less time in the dark compartment and made fewer light ↔ dark transitions, as compared to B6 +/+ mice congenic mice. In addition, 129S6 mice showed lower plus-maze total entries and (in females) closed entries, consistent with previous observations of a profound hypolocomotion in some 129 substrains (Holmes *et al*. 2002b; Rodgers *et al*. 2002a, 2002b). However, indicating that 129S6 congenic mice were not simply less active than B6 mice in these tests, male and female 129S6 mice exhibited significantly more risk assessment (stretched attend postures) in the elevated plus-maze, as reported previously (Rodgers *et al*. 2002a, 2002b). Furthermore, although defecation can be an ambiguous index of emotionality due to potential strain differences in gastro-intestinal motility (Holmes 2001), 129S6 mice emitted more fecal boli than B6 mice in the elevated plus-maze, further supporting a greater anxiety-like response in this strain. Despite these extreme baseline scores in 129S6 congenic +/+ mice, 129S6 congenic 5-HTT null mutant mice did show some behavioral changes, including significantly fewer plus-maze closed entries, and statistically non-significant trends indicative of increased anxiety-like behavior in both the light ↔ dark exploration (reduced light ↔ dark transitions) and elevated plus-maze tests (reduced percent open time and open entries). Taken together, these data support the possibility that high anxiety-like behavior and low exploration in the 129S6 congenic background masked abnormalities in these behaviors caused by the 5-HTT null mutation. More definitive support for this conclusion requires further study, perhaps by assessing 129S6 congenic 5-HTT null mutants on behavioral tasks that are less likely to be confounded by high levels of anxiety-like behavior and low exploration in 129S6 mice.

Indeed, at present we are unable to discount alternative explanations for the present findings. It is estimated that a considerable number (~300) of ES cell donor genes will flank a target mutation locus even after 12 generations of backcrossing, as undertaken for the present studies (Bolivar *et al*. 2001; Crusio 1996; Gerlai 1996; Wolfer & Lipp 2000; Wolfer *et al*. 2002). Where polymorphisms in the flanking genes exist between the ES cell donor strain and congenic strain, these may cause phenotypic differences between null mutants and +/+ controls which are falsely attributed to the mutation (Bolivar *et al*. 2001; Gerlai 1996; Wolfer *et al*. 2002). Demonstrating the potential affect of flanking genes, previous studies have suggested that 129 flanking genes can account for motor coordination deficits in dopamine D1 receptor null mutants (Kelly *et al*. 1998), hyper-aggression in nitric oxide synthase null mutants (Le Roy *et al*. 2000) and elevated alcohol-consumption in 5-HT_{1B} receptor null mutants (Phillips *et al*. 1999). In this context, the absence of increased anxiety-like behavior in 129 congenic 5-HTT null mutant mice might actually be a true-negative, while the abnormal phenotype observed in B6 congenic 5-HTT null mutants could be a false-positive caused by 129P1 ES cell donor polymorphism(s) in genes flanking the *htt* targeted gene locus (Fig. 6b). However, arguing against this possibility, quantitative trait loci (QTL) for mouse anxiety-like behavior have not mapped to the vicinity of the mouse *htt* gene

(chromosome 11 at 42 cM) (Flint 2003; Mathis *et al*. 1995; Turri *et al*. 2001). Therefore, putative 129 genes in the *htt* flanking region that could account for the abnormal anxiety-like phenotype in B6 congenic 5-HTT null mutants remain to be identified.

A third hypothetical explanation for the present background differences in the abnormal 5-HTT null mutant anxiety-like phenotype concerns modifier genes. There are examples of increased anxiety-like phenotypes in null mutation mice that are consistently observed across different genetic backgrounds, e.g. 5-HT_{1A} receptor null mutants (Heisler *et al*. 1998; Parks *et al*. 1998; Ramboz *et al*. 1998; but see Olivier *et al*. 2001), and $GABA_A$ γ2 subunit mutants (Crestani *et al*. 1999). However, it is likely that modifier genes in the background will strongly influence the effect of a single gene polymorphism or mutation on complex traits such as anxiety (Crabbe 2001; Crusio 1996; Hariri & Weinberger 2003; Hood *et al*. 2001; Kendler 2001; Lesch 2001; Murphy *et al*. 2003; Nadeau 2001; Plomin *et al*. 1994). Indeed, while there has been little investigation of modifier genes in mouse models relevant to psychiatric disease (Marsh *et al*. 1999; McNamara *et al*. 2003; Thiele *et al*. 2000), epistatic interactions have been frequently observed in studies of other CNS disorders such as neurodegeneration (for review, see Nadeau 2001), and are commonly studied in lower organisms (Bergman & Siegal 2003; Rubinsztein 2002; Stoltenberg & Hirsch 1997). Thus, an alternative explanation for the absence of an increased anxiety-like phenotype in 129S6 congenic 5-HTT null mutants is that modifier genes present on this (and not the B6) background suppressed the penetrance or expressivity of the functional effects of the 5-HTT null mutation (Fig. 6c).

Possible epistatic interactions between the 5-HTT null mutation and 129S6 modifier genes in the mediation of an abnormal anxiety-related phenotype have not yet been identified. As a simple approach to determining whether 129S6 modifier genes suppressed the penetrance of the 5-HTT null mutation, we examined brain 5-HT function in 129S6-background 5-HTT null mutants. As noted in the introduction, reductions in both expression and function of the 5-HT_{1A} receptor have been consistently demonstrated in 5-HTT null mutant mice on various genetic backgrounds (Fabre *et al*. 2000; Gobbi *et al*. 2001; Li *et al*. 1999, 2001; Mannoury la Cour *et al*. 2001), and should be evident in mutants on the 129S6-background if the mutation is functionally penetrant.

Quantitative autoradiographic analysis showed that 5-HT_{1A} receptor binding density was lower in the midbrain raphe nuclei and various amygdala, hypothalamic and septal nuclei in 129S6-background 5-HTT $-/-$ mice than $+/+$ littermates. In addition, these mice showed a complete loss of the hypothermia-inducing effects of the 5-HT_{1A} receptor agonist, 8-OH-DPAT. Although core body temperature was significantly higher prior to treatment with 8-OH-DPAT (possibly an anxiety-like, stress-induced hyperthermia response to injection and rectal probe insertion; Olivier *et al*. 2003), it seems unlikely that genotype differences in basal body temperature could explain such a profound loss of the drug-induced hypothermia. Rather, this effect indicates a significant desensitization of somatodendritic 5-HT_{1A} autoreceptors in these mice. In support of this interpretation, the hypothermia-inducing effects of 8-OH-DPAT observed in $+/+$ controls were blocked by pretreatment with the highly selective 5-HT_{1A} receptor antagonist, WAY 100635. Taken together, these findings demonstrate that 5-HT_{1A} receptor expression and function was reduced in 5-HTT null mutant mice on the 129S6 background and, thereby, confirm that the 5-HTT null mutation is penetrant on the 129S6 background at the level of brain 5-HT. In turn, this suggests that the absence of increased anxiety-like behavior in these 129S6 congenic 5-HTT null mutants is unlikely to be caused by a suppression of functional effects of the 5-HTT null mutation by 129 modifier genes.

In summary, present findings show that 5-HTT null mutant mice exhibit increased anxiety-like behavior and reduced exploratory locomotion in the light $\leftrightarrow$ dark exploration and elevated plus-maze tests. These phenotypic abnormalities were observed in B6 congenic 5-HTT null mutant mice, but were not detected in 129S6 congenic null mutants. Comparison of B6 $+/+$ congenic and 129S6 $+/+$ control groups indicated a higher level of anxiety-like behavior in the 129S6 strain, suggesting that this baseline may have precluded the detection of increased anxiety-like behavior caused by the 5-HTT null mutation. Excluding the possibility that the absence of an abnormal anxiety-related phenotype in 129S6-background 5-HTT null mutant mice was due to a suppression of the functional effects of the mutation, these mice showed expected reductions in the expression and function of the 5-HT_{1A} receptor. Our results highlight the importance of choosing a genetic background strain that provides an appropriate baseline to study a predicted effect of mutant and thereby reduce false-negative findings. Behavioral phenotyping mutant mice on different genetic backgrounds can also provide a research tool for identifying epistatic interactions between candidate genes, such as the *HTT*, and modifiers, with implications for understanding the polygenic basis of mood and anxiety disorders.

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