Identifying Molecular Substrates in a Mouse Model of the Serotonin Transporter × Environment Risk Factor for Anxiety and Depression

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Background: A polymorphism in the serotonin transporter (5-HTT) gene modulates the association between adverse early experiences and risk for major depression in adulthood. Although human imaging studies have begun to elucidate the neural circuits involved in the 5-HTT × environment risk factor, a molecular understanding of this phenomenon is lacking. Such an understanding might help to identify novel targets for the diagnosis and therapy of mood disorders. To address this need, we developed a gene-environment screening paradigm in the mouse.

Methods: We established a mouse model in which a heterozygous null mutation in 5-HTT moderates the effects of poor maternal care on adult anxiety and depression-related behavior. Biochemical analysis of brains from these animals was performed to identify molecular substrates of the gene, environment, and gene × environment effects.

Results: Mice experiencing low maternal care showed deficient γ-aminobutyric acid–A receptor binding in the amygdala and 5-HTT heterozygous null mice showed decreased serotonin turnover in hippocampus and striatum. Strikingly, levels of brain-derived neurotrophic factor (BDNF) messenger RNA in hippocampus were elevated exclusively in 5-HTT heterozygous null mice experiencing poor maternal care, suggesting that developmental programming of hippocampal circuits might underlie the 5-HTT × environment risk factor.

Conclusions: These findings demonstrate that serotonin plays a similar role in modifying the long-term behavioral effects of rearing environment in diverse mammalian species and identifies BDNF as a molecular substrate of this risk factor.

Key Words: 5-HTT, anxiety, depression, gene × environment, maternal behavior

Risk for depression is determined by both genetic and environmental factors (1), particularly adverse childhood experiences (2). The low-functioning allele of a common functional polymorphism (5-HTTLPR short-variant) in the promoter of the serotonin transporter gene (5-HTT, *SLC6A4*) has been associated with increased neuroticism (3) and increased incidence of major depression selectively in persons experiencing childhood maltreatment or other life stress events (4). Functional magnetic resonance imaging (fMRI) studies have shown that the 5-HTTLPR short-variant confers increased baseline neural activity in a wide range of forebrain structures (5), increased neural responses to the presentation of aversive versus neutral stimuli in structures associated with affective processing such as amygdala and hippocampus (6,7), as well as increased functional coupling between amygdala and ventromedial prefrontal cortex during similar tasks (8). Moreover, a recent fMRI study has shown that baseline neural activity in both amygdala and hippocampus correlates positively with the number of self-reported life stress events in short-variant carriers but negatively in long-variant carriers (5). These findings suggest that 5-HTT influences the long-term impact of stressful life experiences on brain activity that, in turn, influences the response to aversive stimuli and contributes to altered behavioral patterns that are risk factors for depression.

Although human imaging studies have begun to elucidate the neural circuits involved in the 5-HTT × environment risk factor, a molecular understanding of this phenomenon is lacking. Such an understanding might help to identify novel targets for the diagnosis and therapy of mood disorders. To address this need, we developed a gene-environment screening paradigm in the mouse (9). In rodents, low maternal care is associated with high anxiety-related behavior and exaggerated stress response in adulthood (10,11). This maternal programming phenomenon in rodents has been proposed to serve as a model for the increased psychopathology and stress hormone responses associated with poor maternal care and adverse childhood environment in humans (12). Thus, we set out to ask whether the 5-HTT × environment risk factor reported for depression in humans could be modeled with maternal programming of anxiety and depression-related behavior in mouse. To study maternal programming in a genetically controlled manner in mice, we established offspring that were exposed to different levels of maternal care but were nevertheless derived from genetically identical parents. We performed reciprocal inter-crosses between two inbred strains, C57BL/6J (B6) and BALB/cByJ (C) that are known to exhibit large differences in maternal care. Females derived from these crosses (B6xC and CxB6) are genetically identical but provide different levels of maternal care, owing to the intergenerational transmission of maternal behavior from mother to daughter (13). Offspring of B6xC mothers are exposed to high maternal care, whereas offspring of CxB6 mothers are exposed to low maternal care (9,10). Low maternal care in our paradigm is associated with increased anxiety-related behavior, consistent

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with previous studies (10,14). By introducing targeted mutations into these mice, we can identify mutations that moderate this maternal programming phenomenon and model early gene × environment risk factors for behavioral traits (15).

With this paradigm, we sought to determine whether a heterozygous null mutation in mouse 5-HTT (16–18) could modulate susceptibility to the long-term behavioral effects of altered maternal care. Our findings demonstrate that maternal programming of anxiety and depression-related behavior is enhanced in mice carrying a heterozygous null mutation in 5-HTT in a way that mimics the 5-HTT × environment risk factor for depression described in humans. Moreover, biochemical analysis of these mice allowed us to identify molecular substrates of the 5-HTT, environment, and 5-HTT × environment effects that point to alterations in hippocampal function as an underlying risk factor for depression and anxiety.

Methods and Materials

Animals

Breeding to obtain B6x(B6xC) and B6x(CxB6) offspring for behavioral and biochemical phenotyping was performed as previously described (9) except that F1 females were mated with 5-HTT +/− male mice (>10 generation backcross to C57BL/6J) at 12 weeks of age (litters: B6xC, $n = 13$; CxB6, $n = 14$; litter size: B6xC, 10.2 ± .34; CxB6, 9.85 ± .59; t test, $p = .59$). Mice were housed on a 12:12 light/dark cycle with lights on at 7:00 AM until 2 weeks before behavioral testing, when the mice were moved to a room close to the testing room and switched to a reverse 12:12 dark/light cycle with lights off at 10:30 AM for the remainder of the testing period as previously described (9). Genotyping for the 5-HTT null (19) and Tph2-P447R alleles were performed by polymerase chain reaction of tail biopsies as previously described (20,21). All work with animals was performed in accordance with European Molecular Biology Laboratory (EMBL) and Italian guidelines for the ethical treatment of animals under approval of the Italian Ministry of Health (decree number #25/2004-B).

Behavioral Testing

Maternal observations were performed on F1 hybrid mothers of the two pedigrees (B6xC and CxB6) for 3 hours during the light period and 1 hour during the dark period each day from postnatal day 1 to 7 and every other day thereafter until postnatal day 19 as previously described (9). Testing in the open field and elevated-plus maze was performed as previously described (9). All mice were male and 8–10 weeks of age at the beginning of testing, and behavioral tests were separated by 2-week intervals to reduce inter-test interactions and performed in the following order: open field, elevated-plus maze, tail suspension, ambiguous cue fear conditioning. The tail suspension test was carried out according to the method of Steru *et al.* (22). The mice were individually hung by the tail with an adhesive tape placed approximately 1 cm from the tip of the tail attached to a wooden stick and hanging 40 cm above the floor. Periods of immobility were scored during a 6-min test session with the aid of Observer software (Noldus, Wageningen, Netherlands). Fear conditioning was carried out according to Tsetsenis *et al.* (23). The training session lasted 18 min, with three .5-mA, 1-sec shocks delivered at 219, 579, and 939 sec. A small exposed light bulb (28 V DC, 100 mA) was used as the light cue and was presented three times for 20 sec, co-terminating with foot shock. The tone (85 dB, 3 kHz) was presented five times for 20 sec, three times co-terminating with light onset and two times alone at 360 and 720 sec. Freezing was scored during 3-min habituation to the testing chamber, during 3-min tone presentation, and during 3-min light presentation.

Neurochemical Analysis

Neurochemical analysis was carried out, as previously described (24), on tissue punches from frozen brains of mice selected by discriminant analysis to show group-representative behavior. Punches were obtained from coronal brain slices of the left hemisphere no thicker than 300 μm according to Puglisi-Allegra *et al.* (24). Whole brains were used for the analysis of Tph2 genotype effects. For autoradiography frozen 16-μm coronal sections from the right hemisphere of brains used for high-pressure liquid chromatography (HPLC) analysis earlier were thaw-mounted onto gel-coated slides and stored at −80°C. Non-selective benzodiazepine receptor binding (for α1, α2, α3, α5 subtypes) was performed on sections containing frontal cortex, dorsal hippocampus, and central nucleus of the amygdala. Slides were thawed and pre-incubated in buffer (.17 mol/L Tris–hydrochloric acid, pH 7.7) at 0°−4°C for 40 min and then incubated at room temperature for 60 min in buffer containing 2 nmol/L [^{3}H]-flunitrazepam, a nonselective benzodiazepine receptor agonist (85.2 Ci/mmol, PerkinElmer, Boston, Massachusetts). The sections were then washed three times in ice-cold buffer followed by ice-cold water, left to dry overnight, and exposed to Kodak MS film (Kodak, Rochester, New York) for 14 days. Quantitative measurements were made with Image J software (National Institutes of Health, Bethesda, Maryland) and [^{3}H] microscales. Quantitative autoradiographic study of 5-HTT protein expression levels in hippocampus and amygdala was preformed with the selective radioligand ^{125}I-IDAM (25) as previously described (26). For in situ hybridization frozen 16 μm sections from the same right hemisphere as described earlier were used for brain-derived neurotrophic factor (BDNF) in situ hybridization following the protocol of Nibuya *et al.* (27). The rat BDNF complementary DNA clone was a gift of R. Duman, Yale University School of Medicine, New Haven, Connecticut.

Statistical Analysis

Analysis of the main effects of rearing environment, 5-HTT genotype, Tph2 background, and interactions between these variables on behavioral and biochemical measures was performed with multivariate analysis of variance (MANOVA) followed by analysis of variance (ANOVA) and, in cases of significance ($p < .05$), either Duncan or planned comparison post hoc testing. Fear conditioning data were analyzed with mixed MANOVA, where the different testing conditions were considered a repeated factor and 5-HTT genotype, Tph2 background, and rearing environment were considered independent factors. Repeated-measure ANOVA was performed to analyze the effect of the rearing environment and its interaction with time on licking and grooming behavior of F1 mothers. All statistical analyses were carried out with the help of Statistica (StatSoft, Tulsa, Oklahoma).

Results

Modeling the 5-HTT × Environment Risk Factor in Mouse

To test whether a mutation in 5-HTT could moderate maternal programming, B6xC and CxB6 females were backcrossed to males carrying a heterozygous null mutation in 5-HTT (Figure 1A; 19). In this way, offspring from the two pedigrees were either wild-type or heterozygous at the 5-HTT locus and were exposed

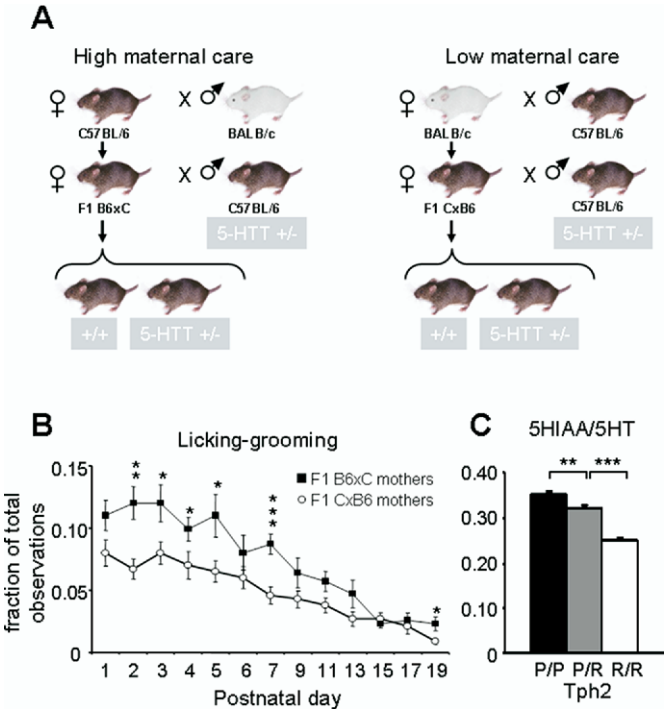

Figure 1. Breeding scheme used for testing interactions between heterozygous null mutation in serotonin transporter (5-HTT) and rearing environment. **(A)** Reciprocal inter-crosses between C57BL/6J and BALB/cByJ mice produced genetically identical F1 hybrid female offspring (B6xC and CxB6) with epigenetically inherited differences in maternal behavior. Backcrossing F1 hybrid females from the two pedigrees to C57BL/6J males carrying a heterozygous null mutation in the 5-HTT +/− produced offspring either wild-type or heterozygous for 5-HTT and exposed to high or low maternal care. **(B)** Despite being genetically identical, B6xC females perform significantly more licking and grooming of their offspring than CxB6 females (B6xC, $n = 13$; CxB6, $n = 14$). **(C)** Serotonin (5-HT) turnover as assessed by the ratio of whole brain tissue levels of the 5-HT metabolite, 5-hydroxyindole acetic acid, to serotonin (5-HIAA/5-HT) is dose-dependently increased in Tph2-447P versus Tph2-447R mice (P/P, $n = 7$; P/R, $n = 16$; R/R, $n = 8$). *$p < .05$, **$p < .01$, ***$p < .001$.

to either high or low maternal care. To confirm high and low maternal care behavior in mothers from the two pedigrees, we performed systematic observations of maternal behavior in the home cage from birth to postnatal day 21 (P21). The B6xC mothers displayed significantly higher levels of licking and grooming behavior than CxB6 mothers [$F(1,25) = 14.58$; $p = .001$], consistent with previous reports (9,10), an effect that was greatest during the first 2 postnatal weeks [repeated measure ANOVA: $F(12,300) = 1.93$; $p = .031$; Figure 1B; 9,10].

Because the B6 and C strains carry different alleles of a functional polymorphism in the serotonin synthetic enzyme, tryptophan hydroxylase 2 (Tph2-Pro447Arg, 20), offspring from the two pedigrees were either $Tph2^{P/P}$ or $Tph2^{P/R}$ (Supplement 1). Mutations in 5-HTT block the re-uptake of serotonin (5-HT) from the extra-cellular space, and this function depends critically on levels of available 5-HT. To determine whether the Tph2-Pro447Arg polymorphism could perturb 5-HT homeostasis in our offspring and thus potentially alter the penetrance and/or expressivity of the 5-HTT mutation, we performed neurochemical analyses of 5-HT and its metabolite, 5-hydroxy-indole acetic acid (5-HIAA), in extracts from whole brains of $Tph2^{P/P}$ and $Tph2^{P/R}$ animals. We found, consistent with previous reports (20), significantly reduced 5-HT turnover (5-HIAA/5-HT) in $Tph2^{P/R}$ mice compared with $Tph2^{P/P}$ mice [$F(1,28) = 32.77$, $p = .000$; Figure 1C]. Thus, in subsequent analyses we treated $Tph2^{P/P}$ and $Tph2^{P/R}$ offspring as distinct genetic backgrounds providing high and low levels of 5-HT synthesis, respectively.

5-HTT Modulates the Effect of Rearing Environment on Adult Behavior

We have shown previously that mice exposed to low maternal care show increased anxiety-related behavior in the open field and elevated-plus maze (9). Thus, we first evaluated whether 5-HTT could moderate the maternal programming of anxiety-related behavior in our model. Mice from the low maternal care group demonstrated significantly increased anxiety-related behavior (consistent with our previous results [9]), showing increased avoidance in the open field [time in center, $F(1,124) = 3.99$, $p = .041$] and increased risk assessment [sniffing, $F(1,125) = 60.55$, $p = .000$] and decreased risk seeking [unprotected head dips, $F(1,125) = 11.17$, $p = .001$] in the elevated-plus maze (Supplement 2). No significant effect of maternal care on total locomotion in either test was detected (data not shown). Analysis of the effects of 5-HTT genotype and Tph2 background on maternal programming revealed a significant three-way interaction among genotype, background, and maternal care for avoidance measures in the open field [time in center, $F(1,127) = 4.56$, $p = .035$; time in corners, $F(1,127) = 4.15$, $p = .043$] and elevated plus maze [time in central platform, $F(1,125) = 13.26$, $p = .000$; time in closed arms, $F(1,125) = 7.75$; $p = .006$]. Notably, significant interactions between 5-HTT genotype and maternal care were found only for mice on the $Tph2^{P/P}$ background, with the highest avoidance behavior seen in 5-HTT +/− mice experiencing low maternal care (Figures 2A–2D and Supplement 3). Our finding that this gene × environment effect

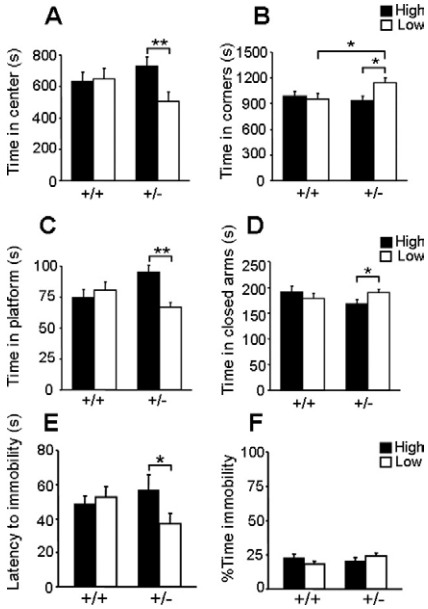

Figure 2. Heterozygous null mutation in serotonin transporter moderates the effect of rearing environment on anxiety and depression-related behavior. Anxiety-related behavioral measures were obtained from the **(A,B)** open field and **(C,D)** elevated-plus maze tests. Depression-related measures were obtained from the **(E,F)** tail suspension test. For the open field and elevated-plus maze data only $Tph2^{P/P}$ mice are shown (+/+, High, $n = 15$; +/+, Low, $n = 13$; +/−, High, $n = 15$; +/−, Low, $n = 18$). For the tail suspension test data from both backgrounds were included (+/+, High, $n = 21$; +/+, Low, $n = 22$; +/−, High, $n = 21$; +/−, Low, $n = 23$). *$p < .05$, **$p < .01$.

is greater in the Tph2$^{P/P}$ than in the Tph2$^{P/R}$ background is consistent with a role for increased 5-HT synthesis in amplifying the penetrance and/or expressivity of the 5-HTT mutation.

Next we examined whether the 5-HTT mutation could moderate the impact of poor maternal care on depression-related behavior. We found that mice carrying the heterozygous 5-HTT null allele and exposed to poor maternal care showed significantly shorter latencies to immobility in the tail-suspension test (28), a model of behavioral despair that has been extensively used to assess depression-related behavior in mice [genotype × maternal care: $F(1,83) = 4.10$; $p = .049$; Figure 2E; (29)]. No differences in percent time immobile were observed (Figure 2F). Although latency to immobility is not a canonical measure of behavioral despair in the tail suspension test, we found that acute treatment with the antidepressant fluoxetine specifically increased latency to immobility without an effect on percent time immobile in our mice (Supplement 4). Interestingly, in this test, Tph2 background did not further modify the interaction between genotype and maternal care (data not shown). These data support the conclusion that the 5-HTT × environment effect modulates susceptibility to a form of behavioral inhibition characterized by increased anxiety and depression-related behavior.

Human fMRI studies have implicated altered neural activity in amygdala and hippocampus in the 5-HTT × environment risk factor for depression (5–8). To more selectively examine amygdala- and hippocampus-dependent fear-related behavior in our mouse model, we tested mice in a partial cue fear conditioning paradigm that assesses an animal's bias in responding to ambiguous threatening cues (23,30). A bias in responding to threatening cues is a hallmark of human anxiety and has been correlated with rumination, a risk factor for major depression (5,31). Mice were simultaneously conditioned to a perfect and partial cue (3 × tone–light–shock and 2 × tone alone), and freezing behavior during cue presentation was quantified 24 hours later in an unfamiliar context. As expected, mice showed lower amounts of freezing to the partially conditioned cue (tone) when compared with the perfectly conditioned cue (light). However, freezing to the partially conditioned cue but not the perfect cue was significantly higher in low maternal care mice [maternal care effect: $F(1,32) = 11.46$, $p = .002$], suggesting a bias in responding to ambiguous cues and a failure to discriminate between ambiguous and non-ambiguous contingencies in these mice (Figures 3A and 3B). Freezing to the partially conditioned cue is hippocampal-dependent, whereas freezing to both partial and perfect cues is amygdala-dependent (23). The increased responding of low maternal care mice was further enhanced by the 5-HTT

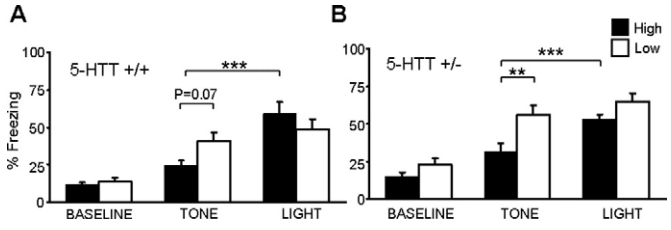

Figure 3. Heterozygous null mutation in serotonin transporter (5-HTT) moderates the effect of rearing environment on ambiguous cue fear conditioning. Percentage of time freezing to the partial (tone) but not perfect (light) conditioned cue was increased in low compared with high maternal care mice. The effect of low maternal care on freezing to the tone was significantly greater in **(B)** 5-HTT +/− mice when compared with **(A)** 5-HTT +/+ littermates (+/+, High, $n = 9$; +/+, Low, $n = 8$; +/−, High, $n = 10$; +/−, Low, $n = 9$). $^{**}p < .01$, $^{***}p < .001$.

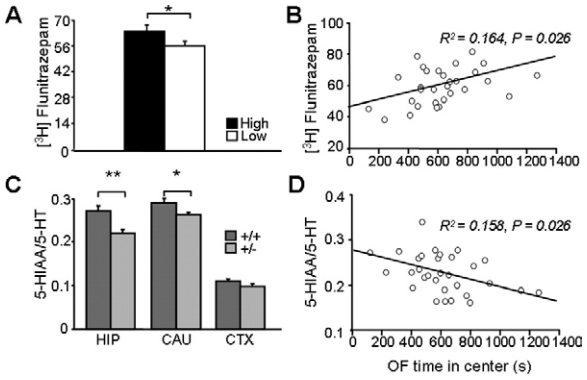

Figure 4. Molecular substrates of serotonin transporter (5-HTT) and rearing environment. **(A)** Binding to the benzodiazepine receptor agonist [^{3}H]-flunitrazepam is decreased in central nucleus of amygdala in mice experiencing low maternal care (relative optical density units; High, $n = 14$; Low, $n = 16$). **(B)** Regression analysis revealed a significant correlation between [^{3}H]-flunitrazepam binding and time in the center of the open field. **(C)** Serotonin turnover (5-HIAA/5-HT) is decreased in hippocampus (HIP) and caudate putamen (CAU), but not frontal cortex (CTX) of 5-HTT +/− mice (+/+, $n = 12$; +/−, $n = 19$). **(D)** Regression analysis revealed a significant correlation between serotonin turnover and time in the center of the open field.

+/− mutation (5-HTT +/+, $p = .07$ vs. 5-HTT +/− $p = .006$). However, increased freezing in 5-HTT +/− mice experiencing low maternal care was not restricted to the ambiguous cue and thus might reflect a more general effect of genotype on brain circuits controlling the processing of aversive conditioned cues.

Molecular Substrates of the Gene × Environment Effect

We hypothesized that molecular substrates within the amygdala and/or hippocampus might underlie the 5-HTT × environment effect. To begin to identify such molecules with our mouse model, we screened a series of candidate downstream substrates that had been shown to be influenced by either 5-HTT genotype or maternal care or both. Previous work has shown that low maternal care in rodents is associated with decreases in γ-aminobutyric acid (GABA)-A receptor binding in the amygdala, and this change has been proposed to underlie the increased anxiety-related behavior seen in these animals (14). To determine whether GABA-A receptors could be a common molecular substrate of 5-HTT and maternal care, we quantified GABA-A receptor binding in brain sections from our mice with the benzodiazepine ligand, ^{3}H-flunitrazepam. Low maternal care was associated with decreased ^{3}H-flunitrazepam binding in the amygdala [$F(1,26) = 4.59$; $p = .041$; Figure 4A], consistent with previous studies, but no effect of 5-HTT genotype or interaction between 5-HTT genotype and maternal care was observed. No difference in ^{3}H-flunitrazepam binding was seen in hippocampus or frontal cortex (Supplement 5). Moreover, regression analysis revealed a significant positive correlation between ^{3}H-flunitrazepam binding in the amygdala and anxiety-related behavior in open field (time in the center: $R^2 = .158$; $R^2_a = .129$; $p = .026$; Figure 4B), suggesting that a deficit in inhibitory neurotransmission in this structure accounts for a significant fraction (approximately13%) of the variation in anxiety-related behavior in our assay. No correlation was seen for non–anxiety-related measures, such as total locomotion in the open field ($R^2 = .033$; $p = .333$).

To examine whether the interaction between 5-HTT and maternal care could occur at the level of 5-HT homeostasis, we analyzed levels of 5-HT and its metabolite, 5-HIAA, in hippocam-

pus, striatum, and frontal cortex of our mice. As expected, levels of 5-HT turnover were significantly reduced in hippocampus [$F(1,27) = 8.64$; $p = .0008$] and striatum [$F(1,29) = 6.06$; $p = .020$] but not frontal cortex of 5-HTT +/− mice compared with wild-type littermates (Figure 4C). However, no significant effect of maternal care or interaction between 5-HTT genotype and maternal care were observed. Quantitative autoradiographic analysis of 5-HTT expression with the selective ligand ^{125}I-IDAM (25) revealed lower levels of 5-HTT protein binding in heterozygous mice compared with wild-type littermates in hippocampus [genotype effect: $F(2, 27) = 26,21$; $p = .0000$; Supplement 6] and amygdala [genotype effect: $F(2,20) = 15,18$; $p = .0001$; Supplement 6] and no significant effect of maternal care or interaction between genotype and maternal care. Nevertheless, we detected a significant negative correlation between 5-HT turnover in hippocampus (but not striatum or frontal cortex) and anxiety-related behavioral measures in the open field (time in center: $R^2 = .139$, $R^2_a = .109$, $p = .042$; Figure 4D) suggesting that changes in 5-HT homeostasis in this structure contribute a small but significant portion (approximately 11%) of the variance in anxiety-related behavior we observed. These findings point to a role for decreased hippocampal 5-HT turnover as a risk factor for anxiety-related behavior and argue for an interaction between 5-HTT and maternal care at a level downstream of 5-HT homeostasis.

Brain-derived neurotrophic factor is a secreted factor crucial for proper neuronal survival, growth, and synaptic plasticity (32) whose expression has been shown to be altered in hippocampus of both homozygous 5-HTT knockout mice (33) and mice exposed to low maternal care (34). These findings suggest that changes in the expression of BDNF could be a common target of 5-HTT and maternal care in our paradigm. In situ hybridization analysis of BDNF messenger RNA (mRNA) in amygdala and hippocampus of our mice revealed a significant interaction of 5-HTT genotype and maternal care on BDNF levels specifically in the CA1 region of the hippocampus [$F(1,14) = 5.55$; $p = .033$]. Strikingly, BDNF mRNA was elevated solely in 5-HTT +/− mice experiencing low maternal care (Figures 5A and 5B and Supplement 7 and Supplement 8). Moreover, levels of BDNF mRNA in hippocampus were positively correlated with anxiety-related behavior in the open field (time in center: $R^2 = .209$, $R^2_a = .159$, $p = .050$; Figure 5C). These findings demonstrate that enhanced hippocampal BDNF activity is a common molecular substrate of 5-HTT genotype and maternal care that explains approximately 16% of the variance in anxiety-related behavior in our paradigm.

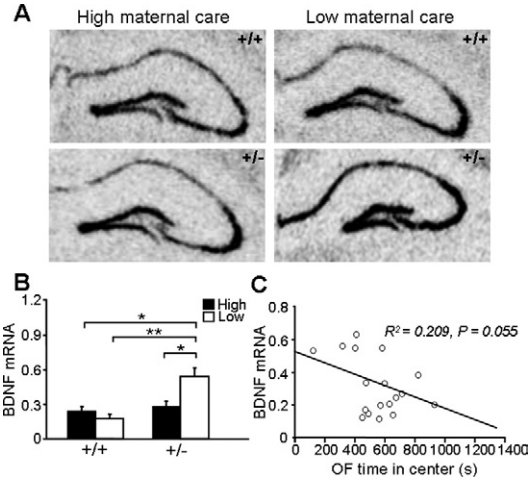

Figure 5. Brain-derived neurotrophic factor (BDNF) is a molecular substrate of both serotonin transporter (5-HTT) and rearing environment. **(A)** Representative in situ hybridization data and **(B)** quantification of BDNF messenger RNA (mRNA) (relative optical density units) in CA1 hippocampus showing increased BDNF expression in 5-HTT +/− mice experiencing low maternal care (+/+, High, $n = 4$; +/+, Low, $n = 3$; +/−, High, $n = 5$; +/−, Low, $n = 6$). **(C)** Regression analysis revealed a correlation between BDNF mRNA and time in the center of the open field. *$p < .05$, **$p < .01$.

Discussion

Our findings of increased anxiety and depression-related behavior in heterozygous 5-HTT knockout mice experiencing low maternal care model the increased risk for major depression seen in individuals carrying the 5-HTTLPR short-variant and exposed to childhood maltreatment (4). These data demonstrate that decreased 5-HT re-uptake capacity is able to exacerbate the long-term behavioral effects of adverse rearing environment across mammalian species and argues for a common molecular mechanism underlying the role of 5-HT in the developmental programming of anxiety- and depression-related behavior.

Unexpectedly, we found that the Tph2-P447R polymorphism moderated some but not all behaviors affected by 5-HTT and maternal care. In the open field, for example, anxiety-related behaviors were found to be increased only in 5-HTT +/− mice experiencing low maternal care on the Tph2$^{P/P}$ background, whereas both the tail suspension and ambiguous cue fear conditioning phenotypes were found to be independent of Tph2 background (Figures 2E, 2F, 3A, and 3B). These findings point to a role for 5-HT synthesis capacity in setting the penetrance and/or expressivity of the 5-HTT heterozygous null mutation under selected environmental circumstances. Presumably, either the threshold for modulation by 5-HT differs for each behavior or, alternatively, distinct serotonergic circuits are involved. One recent association study has described similar interactions between 5-HTTLPR and a single nucleotide polymorphism in Tph2 on human anxiety-related traits (35). Further studies will be required to determine whether this interaction is phenotype-specific. At the same time, it will be important to identify additional polymorphisms in our mouse study that moderate the 5-HTT × environment risk factor. In principle, genome-wide genotyping could be used to identify interacting quantitative trait loci.

Biochemical analysis identified changes in several molecular substrates in response to 5-HTT genotype and/or rearing environment. In the amygdala, low maternal care was associated with reduced GABA-A receptor binding to the benzodiazepine ligand, flunitrazepam (Figure 4A; 14), a finding that is consistent with reduced inhibitory neurotransmission in this structure. Injection of benzodiazepines into the amygdala is anxiolytic in rodents (36), and regression analysis revealed a significant negative correlation between GABA-A receptor binding and anxiety-related behavioral measures in the open field in our mice (Figure 4B). A reduction in inhibitory neurotransmission in the amygdala in low maternal care mice is also consistent with the positive correlation between life stress events and baseline neural activity seen in fMRI studies (5). However, the absence of a 5-HTT genotype effect on this biochemical measure in our mice suggests that the molecular interaction between 5-HTT and rearing environment occurs either downstream of GABA-A receptor function or in another structure.

In the hippocampus, 5-HTT +/− mice showed decreased 5-HT turnover, and regression analysis revealed a significant negative correlation between 5-HT turnover and anxiety-related

measures (Figures 4C and 4D). The functional consequences of altered hippocampal 5-HT turnover are difficult to predict, given the large number of 5-HT receptors expressed in this structure (37). However, the major effect of 5-HT in adult hippocampus is inhibitory (38), and thus reduced turnover might contribute to a disinhibition of neural activity. Changes in amygdala GABA-A receptor binding and hippocampal 5-HT turnover accounted for, respectively, 13% and 11% of the variance in anxiety-related behavior in the open field, suggesting that these molecular substrates explain a significant portion of the behavioral differences we observed.

Finally, our discovery that levels of BDNF mRNA are selectively elevated in the hippocampus of 5-HTT +/− mice exposed to low maternal care (Figures 5A and 5B) raises the possibility that excess BDNF activity contributes to the increased neural activity seen in this brain structure in humans carrying the 5-HTTLPR short-variant and reporting increased life stress events (5). This hypothesis is consistent with human imaging studies showing that increased BDNF activity is associated with increased neural activity and metabolism in the hippocampus during memory tasks (39). Moreover, over-expression of BDNF selectively in the postnatal mouse forebrain causes increased anxiety-related behavior (40), and levels of BDNF protein in the dorsal hippocampus but not amygdala of mice is positively correlated with anxiety-related behavior (41). These results, together with our findings, argue that increased hippocampal BDNF expression is associated with increased hippocampal-dependent anxiety-related behavior.

In summary, we have produced a mouse model of the 5-HTT × environment risk factor for human depression and have used this model to identify molecular substrates underlying this risk factor. Elevated GABA-A receptor expression in amygdala, decreased 5-HT turnover in hippocampus, and enhanced BDNF expression in hippocampus each correlated significantly with the behavioral phenotype seen in our mice. In particular, increased expression of BDNF in CA1 pyramidal neurons was found in mice with reduced 5-HTT function and exposed to low maternal care. This defect was accompanied by an increased bias in the response to threatening cues as assessed by ambiguous cue fear conditioning. Our data suggest that alterations in hippocampal gene expression and function underlie at least part of the interaction between 5-HTT and rearing environment and point to a role for this structure in the increased anxiety and depression-related behavior that is a risk factor for major depression.

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Supplementary material cited in this article is available online.

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