Behavioral/Systems/Cognitive

Impaired Stress-Coping and Fear Extinction and Abnormal Corticolimbic Morphology in Serotonin Transporter Knock-Out Mice

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A lesser-expressing form of the human 5-HT transporter (5-HTT) gene has been associated with increased fear and anxiety and vulnerability to the effects of stress. These phenotypic abnormalities are linked to functional and anatomical disturbances in a neural pathway connecting the prefrontal cortex (PFC) and amygdala. Likewise, rodent and nonhuman primate studies indicate a major role for PFC and amygdala in the mediation of fear- and stress-related behaviors. We used a 5-HTT knock-out (KO) mouse to examine the effects of genetically driven loss of 5-HTT function for the following: (1) depression-related behavior in response to repeated stress, and pavlovian fear conditioning, extinction, and extinction recall; and (2) dendritic morphology and spine density of Golgi-stained pyramidal neurons in the infralimbic cortex (IL) and the basolateral amygdala (BLA). 5-HTT KO mice exhibited increased depressive-like immobility after repeated exposure to forced swim stress, compared with wild-type (WT) controls. Whereas fear conditioning and fear extinction was normal, 5-HTT KO mice exhibited a significant deficit in extinction recall. The apical dendritic branches of IL pyramidal neurons in 5-HTT KO mice were significantly increased in length relative to WT mice. Pyramidal neurons in BLA had normal dendritic morphology but significantly greater spine density in 5-HT KO mice compared with WT mice. Together, the present findings demonstrate a specific phenotypic profile of fear- and stress-related deficits in 5-HTT KO mice, accompanied by morphological abnormalities in two key neural loci. These data provide insight into the behavioral sequelae of loss of 5-HTT gene function and identify potential neural substrates underlying these phenotypes.

Key words: **serotonin transporter; gene; stress; prefrontal cortex; amygdala; extinction**

Introduction

Corticolimbic pathways mediating emotion are rich in 5-HT terminals and receptors (Barnes and Sharp, 1999), and both 5-HT and corticolimbic dysfunction are implicated in affective disorders (Hariri and Holmes, 2006). 5-HT neurotransmission is regulated by clearance of 5-HT from the extracellular space by the NaCl-dependent 5-HT transporter (5-HTT) (SERT) (Blakely et al., 1991). The 5-HTT is a principal target for antidepressants (Ballenger, 1999), and drug-free mood and anxiety disorder patients have reduced prefrontal cortex (PFC) 5-HTT binding (Arango et al., 2002). Moreover, a low-expressing polymorphism in the *HTT* promoter region (Lesch et al., 1996) associates with heightened fear and anxiety (Lesch et al., 1996; Bengel et al., 1998; Garpenstrand et al., 2001; Munafo et al., 2005) and risk for posttraumatic stress disorder (PTSD) (Lee et al., 2005) and major depression following stress (Caspi and Moffitt, 2006).

Although these data suggest that deficits in 5-HTT function compromise stress coping, the neural basis of this effect has not been fully ascertained. The low-expressing *HTT* polymorphism is associated with increased amygdala activity in response to fearful stimuli and at rest (Hariri et al., 2002; Canli et al., 2006). This hyperactivation correlates with reduced volume of the anterior cingulate (ACC) region of the PFC, as well as weakened functional coupling between the ACC and amygdala (Pezawas et al., 2005) and increased activation of the ventromedial PFC (vmPFC) (Canli et al., 2005; Heinz et al., 2005; Pezawas et al., 2005).

PFC regulation of responses to emotional stimuli in human imaging studies is consistent with findings from rodent models (Cryan and Holmes, 2005). Inactivation of the infralimbic (IL) and prelimbic (PL) regions of the vmPFC induces learned helplessness behavior after exposure to uncontrollable stress because of loss of PFC inhibitory control over 5-HT responses to stress (Amat et al., 2005). The vmPFC-amygdala circuit also plays an

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integral role in the regulation of fear memory (Quirk and Beer, 2006). The vmPFC and amygdala are reciprocally innervated (Canteras et al., 1992; Bacon et al., 1996; McDonald et al., 1996; Smith et al., 2000; Berretta et al., 2005), and the vmPFC likely participates in modulation of learned fear via input to amygdaloid nuclei (Sotres-Bayon et al., 2004), perhaps by activation of inhibitory neurons in the intercalated nuclei (Berretta et al., 2005). Lesions (Quirk et al., 2000), neurochemical inactivation (Santini et al., 2001; Santini et al., 2004), or stress-induced dendritic retraction in this region produces deficits in fear extinction and/or the recall of extinction (Izquierdo et al., 2006; Miracle et al., 2006) resulting from dysregulation of PFC modulation of the amygdala (Canteras et al., 1992; McDonald et al., 1996; Smith et al., 2000; Berretta et al., 2005). These effects are localized to neurons in the IL (Milad and Quirk, 2002).

Here, we used a 5-HTT knock-out (KO) mouse model (Bengel et al., 1998) to further evaluate the consequences of disruption of 5-HTT function for corticolimbic neuronal morphology and stress- and fear-related behaviors mediated by this circuit. This model has previously provided insight into the consequences of loss of 5-HTT gene function for emotion-related behaviors and for cortical development (Esaki et al., 2005; Hariri and Holmes, 2006; Li, 2006). First, we tested for depression-related behavior and pavlovian fear conditioning, extinction, and extinction recall. Next, we assessed dendritic morphology and spine density of pyramidal neurons in IL and basolateral amygdala.

Materials and Methods

Animals. 5-HTT KO mice were generated as described previously by replacing a 1.1 kbp fragment of the *htt* gene containing exon 2 with a 1.8 kbp pPNT-neo replacement targeting vector (Bengel et al., 1998). Mice originally from a 129P1 (129P1/ReJ) × C57BL/6J hybrid genetic background were repeatedly backcrossed onto a C57BL/6J for more than eight generations. Male 5-HTT KO mice and nonmutant wild-type (WT) controls were siblings derived from matings between 5-HTT heterozygous parents. Male nonmutant C57BL/6J mice used to assess the effects of fluoxetine in the repeated forced swim test (FST) procedure (see below) were obtained from The Jackson Laboratory (Bar Harbor, ME) at 8–10 weeks of age and tested ~1 week later. All mice were housed (two to five per cage) in same-sex littermate groups in a temperature- and humidity-controlled vivarium under a 12 h light/dark cycle (lights on at 6:00 A.M.). Before testing, mice were given a 1 h acclimation period in the testing room. Experimenters remained blind to experimental variables during testing. Experimental procedures were performed in strict accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and were approved by the local Animal Care and Use Committee.

Repeated forced swim stress. Sixteen 5-HTT KO mice and 15 WT mice were assessed for depression-related behavior using a modified version of the mouse forced swim test in which subjects were repeatedly exposed to the swim stressor (Porsolt et al., 1977). On day 1, mice received a single 15 min FST exposure to a 25-cm-high, 20-cm-diameter cylinder filled with 24 ± 1°C water, as described previously (Boyce-Rustay and Holmes, 2006). On day 2, mice received four consecutive 6 min exposures with a 12–15 min intertrial interval between trials. Passive immobility (cessation of movement except minor involuntary movements of the hindlimbs) was measured using an instantaneous sampling method every 5 s during minutes 2–6 on each day by an observer blind to genotype and converted to a percentage of observations [(freezing observations/total observations) × 100].

A separate experiment was conducted to confirm that both acute and repeated exposure to the test were sensitive to a pharmacological manipulation known to produce alterations in depression-related behavior in the forced swim test in nonmutant mice. This would argue against the possibility that genotype differences during repeated but not acute exposure were an artifact of a relative insensitivity of the former to experimental manipulation. Male C57BL/6J mice were assessed on 6 min FST exposure 30 min after intraperitoneal injection (at a volume of 10 ml/kg body weight) of 0, 5, 10, or 20 mg/kg fluoxetine hydrochloride (Sigma, St. Louis, MO) dissolved in 0.9% saline. Half of the mice were FST-naive, whereas the other half had received a 15 min FST exposure 24 h earlier. Doses were chosen on the basis of previously demonstrated antidepressant-related effects of fluoxetine in C57BL/6J mice (Holmes et al., 2002).

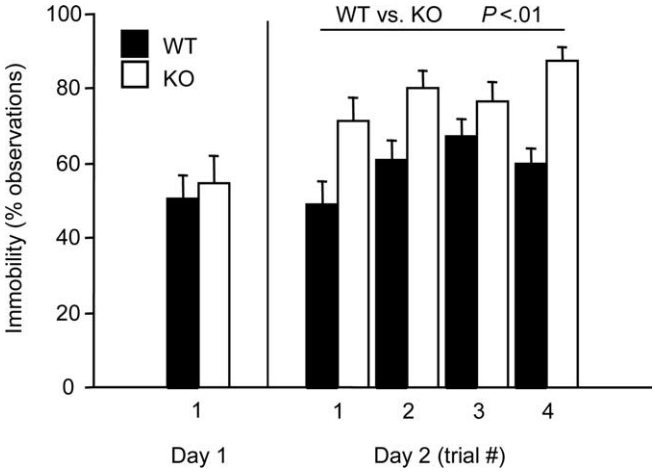

Figure 1. Increased depression-like behavior during repeated stress in 5-HTT KO mice. 5-HTT KO mice showed significantly increased depression-related behavior in response to repeated forced swimming. Data are mean ± SEM percentage observations of immobility (n = 15–16/genotype).

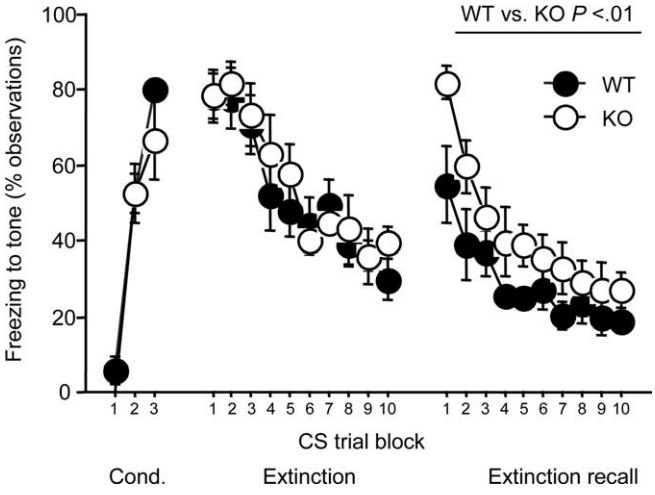

Figure 2. Impaired extinction recall in 5-HTT KO mice. 5-HTT KO mice showed normal conditioning (Cond.), CS-recall, and extinction of a conditioned fear response but exhibited significantly impaired extinction recall compared with WT mice. Data are mean ± SEM percentage observations of freezing during CS trial blocks (n = 6/genotype).

Pavlovian fear conditioning, extinction, and extinction recall. An experimentally naive cohort of eight 5-HTT KO and nine WT mice were tested for fear conditioning, extinction, and extinction recall using methods based on those described previously (Cain et al., 2002; Izquierdo et al., 2006). Conditioning was conducted in a 27 × 27 × 11 cm chamber with transparent walls and a metal rod floor, cleaned with a 79.5% water/19.5% ethanol/1% vanilla-extract solution. After a 120 s acclimation period, mice received three pairings (60–120 s variable interpairing interval) of the conditioned stimulus (CS) (30 s, 80 dB, 3 kHz tone) and the unconditioned stimulus (US) (2 s, 0.6 mA scrambled footshock), in which the US was presented during the last 2 s of the CS by the San Diego Instruments (San Diego, CA) Freeze Monitor system. After a 120 s no-

stimulus consolidation period after the final CS–US pairing, mice were returned to the home cage.

Twenty-four hours later, initial CS-recall and subsequent CS-extinction was measured in a novel context (black/white-checkered walls and a solid-Plexiglas, opaque floor, cleaned with a 50% ethanol/50% water solution) housed in a different room from that used for conditioning. After a 120 s acclimation period, the mouse received forty 30 s CS presentations (5 s interstimulus interval). The same procedure was repeated 24 h later to assess extinction recall.

Freezing (no visible movement except respiration) was scored using an instantaneous sampling method every 5 s by an observer blind to genotype and converted to a percentage of observations [(freezing observations/total observations) × 100]. Freezing was averaged over each CS trial during conditioning and averaged into four-trial blocks during freezing extinction and extinction recall.

Histology and morphological analyses. Dendritic and spine analyses were performed on mice at the completion of fear conditioning/extinction/recall. Mice used for forced swim testing were not used for morphological analysis as we have shown previously that this procedure causes dendritic alterations of the PFC of nonmutant mice (Izquierdo et al., 2006). Within 1 h of the completion of extinction recall testing, five 5-HTT KO and six WT mice were overdosed with isoflurane and transcardially perfused with saline. Brains were removed and processed for Golgi histology using Glaser and Van der Loos' modified Golgi stain (Glaser and Van der Loos, 1981). Briefly, tissue was immersed in Golgi–Cox solution for 10 d. Brains were then dehydrated, infiltrated with a graded series of celloidins, and embedded in 8% celloidin. Coronal sections were cut at 200 μm on a sliding microtome (Leica Histoslide 2000; Leica, Nussloch, Germany). Free-floating sections were then alkalinized, developed in Kodak (Rochester, NY) D-19, fixed in Kodak Rapid Fix (solution B omitted), dehydrated, mounted, and coverslipped.

IL was identified by its position on the medial wall of the rostral cortex, its location ventral to PL, and its characteristic cytoarchitecture: IL is markedly thinner than prelimbic cortex and has fewer, less well-defined layers (Paxinos and Franklin, 2001). Basolateral amygdala (BLA) analysis was restricted to pyramidal neurons located between 8.0 and 2.0 mm posterior to bregma. Within this region, BLA is readily identified in Golgi-stained material, as the external capsule branches into two smaller fiber tracts that define the dorsal, medial, and lateral borders of the BLA. Likewise, axon fibers clearly delineate basal amygdala from BLA. Pyramidal neurons within IL and BLA were defined by the presence of a distinct, single apical dendrite (for IL, extending from the apex of the soma toward the pial surface of the cortex), two or more basilar dendritic trees extending from the base of the soma, and dendritic spines (see Figs. 3*a*, 6*a*).

Dendritic analyses. Neurons selected for reconstruction were located in the middle third of the section, did not have truncated branches, and were unobscured by neighboring neurons and glia, with dendrites that were easily discriminable by focusing through the depth of the tissue. Within each region examined, 8–11 neurons were drawn for each mouse; this number yielded an average within-animal error for total dendritic length of ~12.1 ± 0.5% for IL neurons and ~9.2 ± 0.6% for BLA neurons. Neurons were drawn at 400–600×, and morphology of apical and basilar arbors was quantified in three dimensions using a computer-based neuron tracing system (Neurolucida; MBF Bioscience, Williston, VT) (for IL, see Fig. 3*a*; for BLA, see Fig. 6*a*) with the experimenter blind to genotype. To rule out artifactual differences in dendritic morphology resulting from differential sampling across cortical layers, the soma-to-pial-surface distance was measured in each neuron and compared across genotypes using a *t* test (*ns*). For IL neurons, total length and number of basilar and apical dendrites, as well the length and number of terminal branches were compared across genotypes using *t* tests. To assess differences in the amount and location of dendritic material, a three-dimensional version of a Sholl analysis (Larkman, 1991) was performed in which the number of intersections of dendrites with 10 μm concentric spheres centered on the soma was measured; for statistical and graphical purposes, the counts of intersections were summed over pairs of radii.

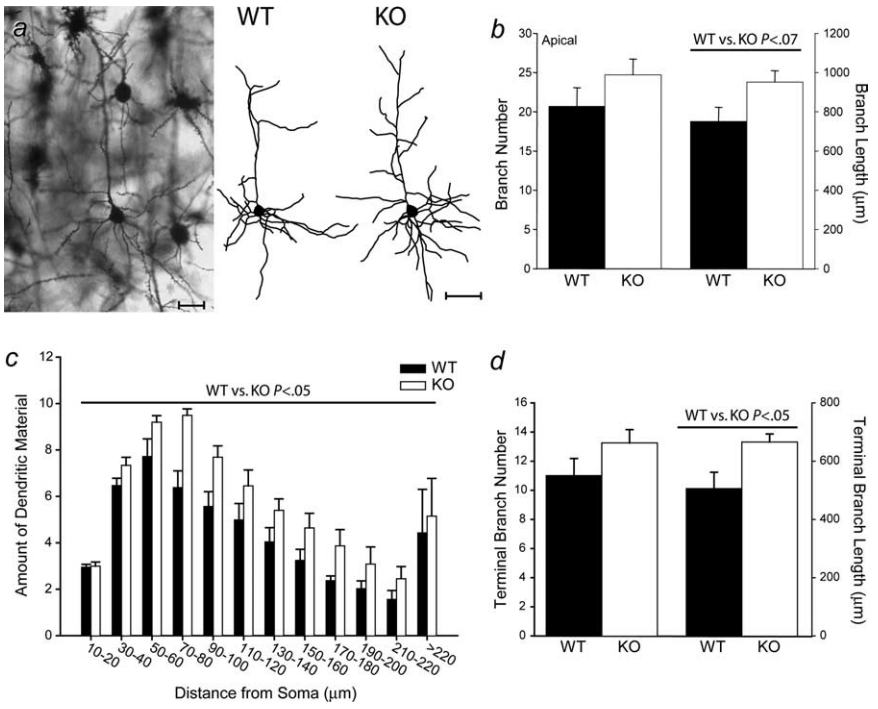

Figure 3. Abnormal apical dendritic morphology of IL pyramidal neurons in 5-HTT KO mice. ***a***, Golgi-stained pyramidal neuron in IL and computer-assisted reconstructions of representative neurons in WT and 5-HTT KO mice. Scale bars, 50 μm. ***b***, Overall length, but not overall number, of apical dendritic branches in IL pyramidal neurons was marginally greater in 5-HTT KO than WT mice. ***c***, Apical dendritic material was significantly greater in 5-HTT KO relative to WT mice, and this effect was uniform across the apical arbor. ***d***, Length, but not number, of apical terminal branches was significantly greater in 5-HTT KO than WT mice. Data in Figures 3–6 are mean ± SEM and $n = 5–6$/genotype.

For each neuron, one dendritic tree containing at least one third-order branch (for IL basilar and BLA dendrites) or at least one horizontal segment (for apical dendrites) was chosen. One to two branches at each order were drawn, and spines were counted at 1000× using a computer-based neuron tracing system (Neurolucida, MBF Biosciences). In IL, branch lengths sampled averaged 47.03 ± 3.21, 40.76 ± 2.67, and 32.98 ± 4.40 μm in length for trunk, oblique, and horizontal segments of IL apical dendrites, respectively, and 7.63 ± 0.46, 19.61 ± 0.71, 25.57 ± 1.40, and 29.69 ± 2.45 μm for first- through fourth-order basilar dendrites. In BLA, branches sampled averaged 11.40 ± 0.97, 25.62 ± 1.61, 31.27 ± 2.23, and 32.46 ± 1.63 μm for first- through fourth-order den-

Spine density analyses. Spines were counted on dendritic branches from six to eight neurons per region in each animal; this number yielded a within-animal error for spine density of ~17% in IL and 18% in BLA and thus was considered to provide a representative sample of spine densities on IL and BLA pyramidal neurons. In IL, separate analyses were performed for apical and basilar dendrites. For apical dendrites, spines were counted on apical trunk, oblique, and horizontal segments separately, because spine density has been shown to vary across these dendritic compartments (Peters and Kaiserman-Abramof, 1970). At least 95% of the basilar branches of IL pyramidal cells of WT mice were fourth-order or lower. Therefore, spines were counted on first-, second-, third-, and fourth-order branches. In BLA, spines were also counted on first- through fourth-order branches, because these make up at least 87% of the dendritic arbor of BLA pyramidal neurons in WT mice.

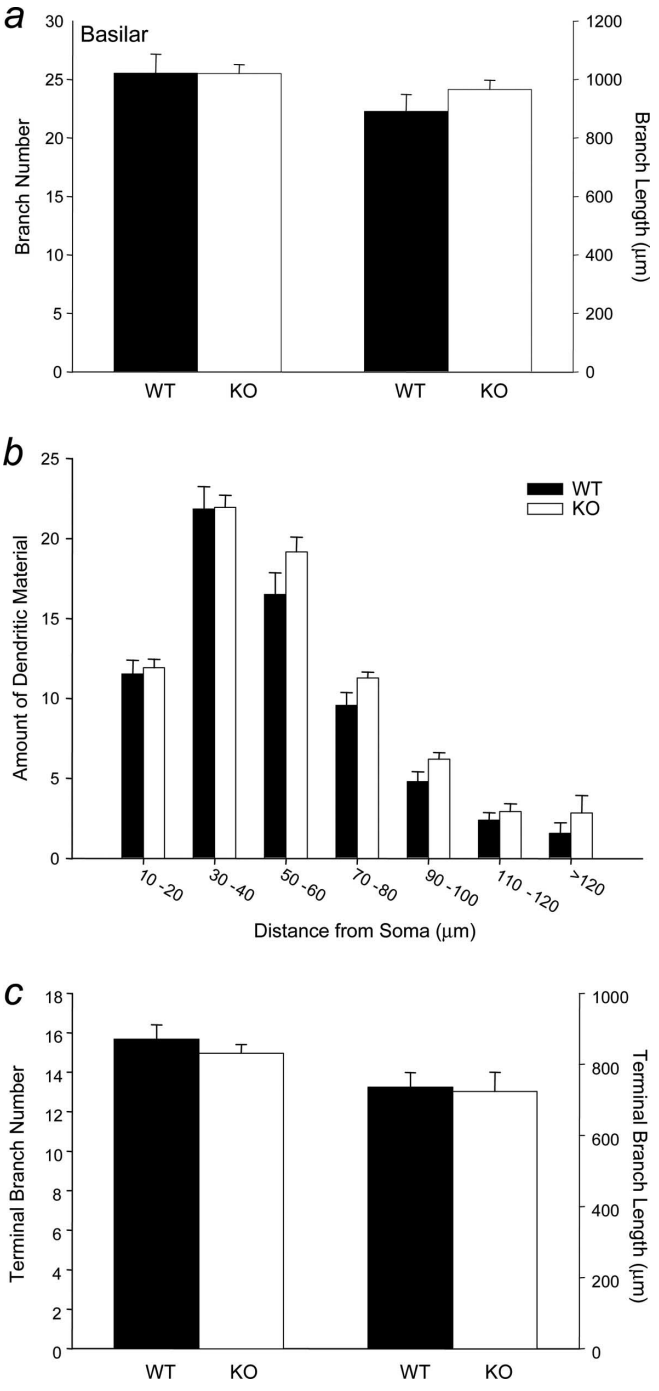

Figure 4. Normal basilar dendritic morphology of IL pyramidal neurons in 5-HTT KO mice. ***a***, Neither overall length nor number of IL basilar dendritic branches differed between 5-HTT KO and WT mice. ***b***, Genotypes did not differ in amount or distribution of dendritic material. ***c***, Neither length nor number of basilar terminal branches was different between genotypes.

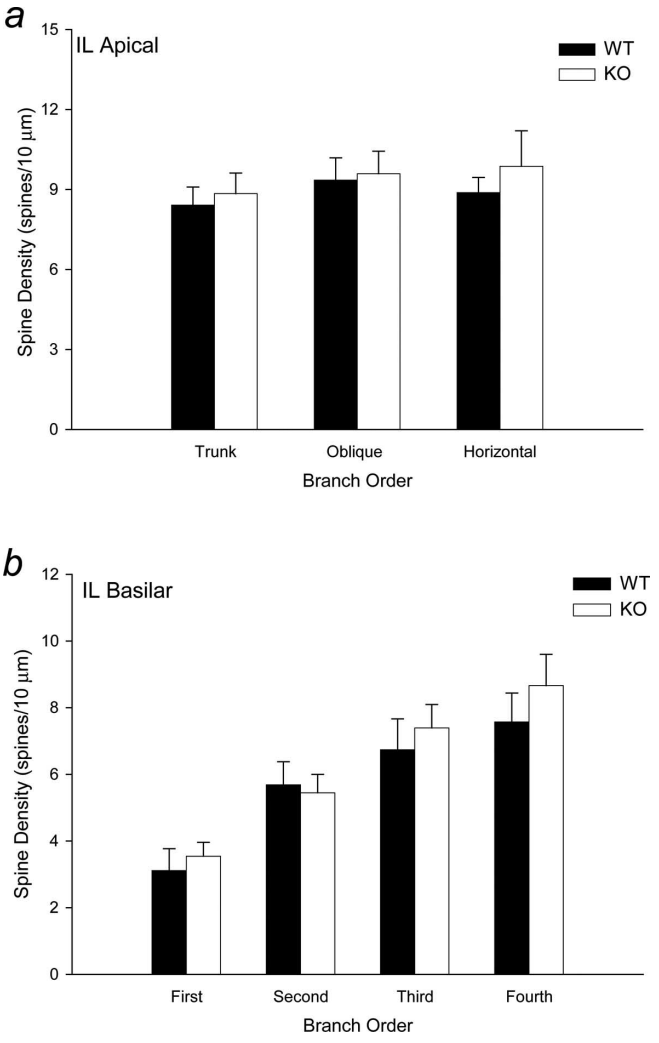

Figure 5. Normal spine density on IL pyramidal neurons in 5-HTT KO mice. Spine density on apical (***a***) and basilar (***b***) dendrites of IL pyramidal neurons did not differ between genotypes.

drites. Spines were identified based on the morphological criteria for "mushroom" and "thin" spines described by Peters and Kaiserman-Abramof (1970): only protrusions perpendicular to the dendritic shaft and possessing a clear neck and bulbous head were counted (see Fig. 7*a*). Because spine density varies with thickness of the dendrite (and therefore branch order), the lengths of dendritic segments were recorded, and spine densities (spines per 10 μm) for each branch order were calculated separately.

Statistical analysis. The effect of genotype on percentage immobility during day 1 forced swim exposure, on percentage freezing during fear conditioning, and on IL and BLA dendrite length and number was analyzed using *t* tests. The effects of genotype × day 2 forced swim trial number on percentage immobility; the effects of genotype × CS extinction-trial on percentage freezing; the effects of genotype × distance from soma on amount and location of dendritic material; and the effects of genotype × branch order on dendritic spine density were analyzed using two-factor ANOVA with repeated measures. Significant *F* tests were followed up with Bonferroni comparisons.

Results

Increased depression-like behavior during repeated stress in 5-HTT KO mice

5-HTT KO mice showed increased behavioral despair after repeated exposure to the forced swim test. Immobility was not different between 5-HTT KO and WT mice during initial exposure (*ns*). In contrast, on re-exposure to the test 24 h later, 5-HTT KO mice showed significantly greater immobility than WT mice ($F_{(1,87)} = 11.42$; $p < 0.01$) (Fig. 1). Generally, there was an increase in immobility with repeated exposure during day 2 ($F_{(29,87)} = 5.34$; $p < 0.01$).

Analysis of the effects of treatment of nonmutant C57BL/6J mice with the reference antidepressant fluoxetine before either day 1 or day 2 forced swim exposure indicated that differences between 5-HTT KO and WT mice on day 2 but not day 1 of testing were not attributable to poor validity of the modified

procedure. Fluoxetine produced a significant "antidepressant-like" reduction in immobility in nonmutant C57BL/6J regardless of whether mice had been exposed previously to the FST (main effect of dose, $F_{(3,63)} = 12.05$, $p < 0.01$; dose × previous exposure interaction, *ns*), whereas immobility was generally lower in mice receiving the first compared with the second exposure ($F_{(1,63)} = 13.36$; $p < 0.01$) (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Impaired extinction recall in 5-HTT KO mice

5-HTT KO mice exhibited a selective deficit in extinction recall. During conditioning, freezing was not different between 5-HTT KO and WT mice either before, during CS presentation, or immediately after the final CS–US pairing (all *ns*). Freezing during initial CS recall (WT, 78.3%; KO, 78.4% during first trial-block) and extinction was also not different between genotypes (*ns*), and both genotypes showed significantly reduced freezing with repeated presentation of the CS ($F_{(10,90)} = 19.20$; $p < 0.01$). Although there was no significant difference in freezing during extinction, visual inspection of the data indicated higher freezing in 5-HTT KO mice at the end of the session (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Therefore, to exclude the possibility that any genotype differences in freezing seen during extinction recall were caused by differences in success of extinction learning, rather than recall, genotypes were matched for freezing levels on the final extinction trial-block by excluding three WT and two 5-HTT KO mice from the analysis. Results showed that matching for extinction learning, 5-HTT KO mice exhibited significantly more freezing than WT mice during extinction recall (WT, 54.8%; KO, 81.9%) ($F_{(1,10)} = 5.40$; $p < 0.05$) (Fig. 2). With repeated CS presentations, both WT and 5-HTT KO mice showed significantly reduced freezing ($F_{(10,90)} = 15.81$; $p < 0.01$).

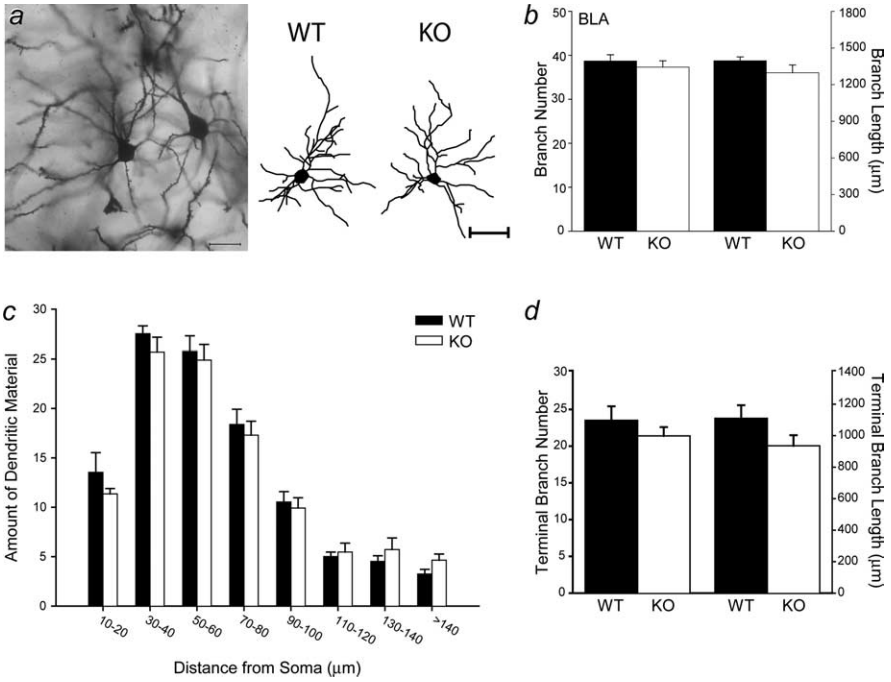

Figure 6. Normal dendritic morphology of BLA pyramidal neurons in 5-HTT KO mice. ***a***, Golgi-stained pyramidal neurons in BLA and computer-assisted reconstructions of representative neurons in WT and 5-HTT KO mice. Scale bars, 50 μm. ***b***, Neither overall length nor number of BLA dendritic branches differed between genotypes. ***c***, Genotypes did not differ in amount and distribution of dendritic material. ***d***, Neither length nor number of terminal branches was different between genotypes.

Increased dendritic length in IL, increased spine density in BLA of 5-HTT KO mice

In IL, overall apical dendritic length was increased by 22% in 5-HTT KO mice relative to WT controls, and this difference approached significance (Fig. 3*b*) ($t_9 = 2.10$; $p < 0.07$). Analysis of the amount and distribution of apical dendritic material confirmed a significant increase in 5-HTT KO mice relative to WT mice ($F_{(1,99)} = 5.03$; $p < 0.05$), which was a result of increased length of terminal branches (Fig. 3) ($t_9 = 2.38$; $p < 0.05$). In contrast to these morphological changes in apical dendrites, basilar dendrites of IL pyramidal neurons did not differ between genotypes (Fig. 4). In contrast, spine density did not significantly differ between genotypes at any branch order for either apical or basilar dendrites (Fig. 5*a*,*b*) (all *ns*).

Dendritic morphology of BLA neurons did not differ significantly between genotypes in overall branch number or length (Fig. 6*a*,*b*), distribution of dendritic material (Fig. 6*c*), or terminal branch number or length (Fig. 6*d*) (all *ns*). However, spine density was significantly increased by 24% in 5-HTT KO mice relative to WT controls (Fig. 7) ($F_{(1,9)} = 7.08$; $p < 0.05$). Changes in spine density varied across branch orders ($F_{(3,27)} = 7.01$; $p < 0.05$), with 31 and 61% increases in spine density on first- and fourth-order branches ($t_9 = 9.50$ and 8.86, respectively; $p < 0.05$).

Discussion

The present study demonstrates two novel findings. First, genetically driven loss of 5-HTT function produced significant deficits on two measures of responsivity to environmental trauma and stress. Second, these behavioral abnormalities were associated with morphological abnormalities in two key loci of a corticolimbic circuit mediating the processing of emotional stimuli.

5-HTT KO mice exhibited levels of freezing before, during, and immediately after fear conditioning that were similar to WT; demonstrating normal acquisition of associative fear memory in the KO mice. The fear memory was then extinguished by repeatedly presenting the fear-associated stimulus in the absence of aversive outcome (Pavlov, 1927). During the first session of extinction learning, 5-HTT KO and WT mice exhibited similar progressive reductions in conditioned freezing. In contrast, whereas both genotypes showed an expected spontaneous recovery of the fear response when tested for recall of the extinction memory 24 h later, the response was markedly higher in 5-HTT KO mice than in WT mice. 5-HTT KO mice were subsequently able to (re)extinguish to WT levels with additional repeated exposure to the conditioned stimulus. Thus, these data demonstrate a significant and selective impairment in extinction recall in 5-HT KO mice, and extend previous findings that 5-HTT KO mice display increased anxiety-like behaviors, whereas transgenic overexpression of the 5-HTT decreases these behaviors (Ansorge et al., 2004; Hariri and Holmes, 2006; Jennings et al., 2006). A deficit in the ability to acquire or retain extinction memory is a cardinal feature of anxiety disorders such as PTSD (American

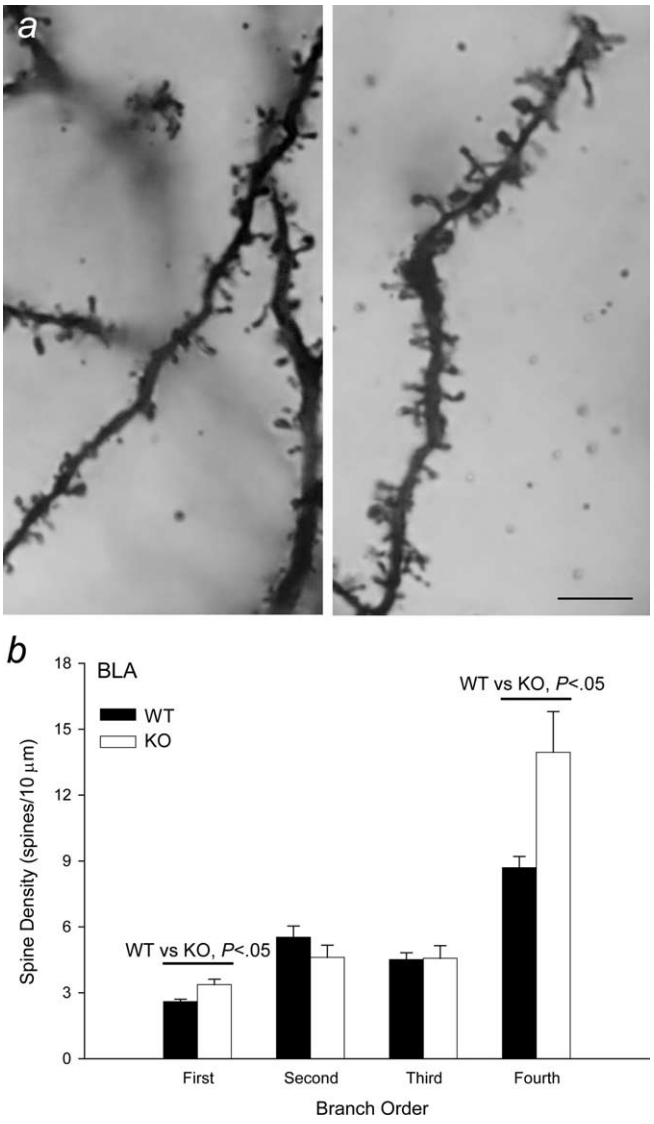

Figure 7. Abnormal spine density on BLA pyramidal neurons in 5-HTT KO mice. ***a***, Dendritic spines on a fourth-order branch of a Golgi-stained pyramidal neuron in a WT mouse (left) and a 5-HTT KO mouse (right). Several digital light micrographs were taken at different Z-levels and merged to increase the number of spines in focus. Scale bar, 5 μm. ***b***, Spine density was significantly increased on first- and fourth-order dendrites in 5-HTT KO mice.

Psychiatric Association, 1994). Interestingly, in this context, a recent study found that human beings carrying a low-expressing polymorphic variant of the promoter region of the *HTT* gene show elevated rates of PTSD (Lee et al., 2005), suggesting that genetically driven loss of 5-HTT function may be a risk factor for the disease.

The human low-expressing variant of the promoter region of the *HTT* gene has also been associated with heightened risk for major depression in response to exposure to stressful life events and neurochemical challenges such as 5-HT depletion (Neumeister et al., 2002; Caspi and Moffitt, 2006). Similarly, nonhuman primates with an ortholog of the same polymorphism exhibit exaggerated neuroendocrine responses to stress and increased alcohol consumption after being reared under stressful conditions compared with normally reared, but genetically similar, counterparts (Barr et al., 2003). In an interesting parallel with these data, the present study found increased depression-like behavior in 5-HTT KO mice after repeated but not acute exposure to forced swim stress. 5-HTT KO mice also exhibited exaggerated behavioral, neuroendocrine, and catecholamine responses to certain mild stressors such as saline injection or predator exposure that are in themselves insufficiently potent to affect WT mice (Tjurmina et al., 2002; Li et al., 2004; Adamec et al., 2006; Li, 2006). Although parallels between phenotypic data in KO mice and higher species must always be made with due caution (Cryan and Holmes, 2005), together these findings are consistent with a growing literature indicating that loss of 5-HTT gene function compromises the capacity to adaptively cope with environmental stress.

A corpus of data implicates dysfunction of a neural circuit connecting the PFC and amygdala in the pathophysiology of depression and anxiety disorders (Siegle et al., 2002; Phillips et al., 2003; Phelps et al., 2004). Furthermore, recent data from neuroimaging studies indicate that the low-expressing *HTT* gene variant is associated with functional abnormalities in this circuit, specifically, amygdala hyperactivity coupled with hypofunction in some regions of the PFC (anterior cingulate) and hyperfunction in others (vmPFC) (Hariri et al., 2002; Canli et al., 2005, 2006; Heinz et al., 2005; Pezawas et al., 2005).

The present data indicate morphological abnormalities in both the BLA and vmPFC (IL cortex) of 5-HTT KO mice. First, although gross morphology of pyramidal neurons in the BLA was normal, 5-HTT KO mice had significantly greater spine density on the fourth-order (and therefore likely terminal) BLA dendritic branches of these neurons, relative to WT. Second, whereas spine density was normal in pyramidal neurons in IL of 5-HTT KO mice, the dendrites of these cells were significantly elongated compared with WT mice.

Although these morphological changes do not provide direct evidence of functional alterations in BLA and vmPFC of 5-HTT KO mice, they provide plausible correlates of neural substrates underlying the behavioral deficits presently observed in these mice. One model is that increased spine density in BLA dendrites may be accompanied by increased excitatory drive of these neurons resulting in amygdala hyperactivity. In turn, via its strong inhibitory input to the amygdala (Canteras et al., 1992; Bacon et al., 1996; McDonald et al., 1996; Quirk et al., 2003; Berretta et al., 2005; Laviolette et al., 2005; Likhtik et al., 2005), increased dendritic extent of IL neurons may be an adaptive response to dampen this hyperactivity.

Alternatively, the primary site of the neural insult could be at the level of the IL rather than the BLA. The specific nature of the behavioral deficits in 5-HTT KO mice would support this hypothesis: the phenotypic profile of increased behavioral despair and poor extinction recall in the KO mice closely resembles that caused by vmPFC, but not BLA inactivation in rats, and fits with the correlation of IL, but not BLA neuron activity, with successful extinction in rats and humans (Phelps et al., 2004; Amat et al., 2005; Kalisch et al., 2006; Quirk et al., 2006). Moreover, loss of 5-HT clearance in these mice (Daws et al., 2006) produces marked increases in extracellular fluid levels of 5-HT in brain regions, including frontal cortex (Mathews et al., 2004), and recent electrophysiological data indicate that activation of serotonergic input to the medial PFC (mPFC) inhibits the majority of pyramidal neurons therein via 5-HT_{1A} receptors (Hajos et al., 2003; Puig et al., 2005). Thus, the principle consequence of increased extracellular 5-HT in the mPFC of 5-HTT KO mice would likely be to overinhibit vmPFC neuronal activity. Taken from this perspective, the increased dendritic extent in IL of these mice would be consistent with a compensatory effort to restore functional integrity of a hypoactive IL, an effort that, according to

the present results, is insufficient to normalize fear- and stress-related deficits. Evidence that differences in the pattern and distribution of cortical neuronal dendrites influences their functional properties (Rall et al., 1992; Mainen and Sejnowksi, 1996; Koch and Segev, 2000), together with the finding that IL morphological alterations in 5-HTT KO were restricted to the terminal branches of apical dendrites that are known to be especially plastic (Cook and Wellman, 2004; Radley et al., 2005; Izquierdo et al., 2006), would fit with a compensatory adaptation to overinhibition.

Although this scheme could potentially account for at least some of the deficits in fear- and stress-related behaviors observed in 5-HTT KO mice, additional studies will be needed to test the model and explore other factors involved. For example, we and others have shown that sensory cortical regions are cytoarchitecturally and functionally abnormal in 5-HTT KO because of excessive 5-HT availability during a critical window of neonatal development (Gaspar et al., 2003; Esaki et al., 2005) and, furthermore, that neonatal disruption of 5-HT or the 5-HTT lead to lifelong abnormalities in mouse anxiety- and stress-related behaviors (Ansorge et al., 2004; Gross and Hen, 2004). This raises the possibility that ontogenic disruption of cortical regions, including vmPFC, may contribute to the fear- and stress-related deficits manifest in 5-HTT KO mice. Another important question for future studies will be whether 5-HTT KO mice have morphological abnormalities in other PFC subregions (e.g., anterior cingulate, prelimbic, and medial orbital cortices) and whether the profile of these mice extends to phenotypic abnormalities associated with dysfunction of these regions in rodents, such as cognitive flexibility and impulse control (Robbins, 2005).

In summary, the present study found that genetically driven loss of 5-HTT function led to deficits in stress-induced depression-related behavior and fear extinction recall that were accompanied by morphological abnormalities in the BLA and vmPFC. These findings support a converging cross-species literature suggesting that relative loss of 5-HTT gene function increases risk for affective disorders by compromising the functional integrity of a key corticolimbic circuit.

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