Autism gene variant causes hyperserotonemia,
serotonin receptor hypersensitivity, social impairment and repetitive behavior


Departments of *Psychiatry, †Pediatrics, and ‡Pharmacology, †Brain Institute, and †Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, TN 37232; Departments of Physiology, *Neurosciences, and †Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229; Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX 78229; Laboratory of Behavioral Neuroscience, National Institute of Mental Health, Bethesda, MD 20892; *Silvio O. Conte Center for Neuroscience Research, Vanderbilt University, Nashville, TN 37232; Department of Biological Sciences, Vanderbilt University, Nashville, TN 37232; and †Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232

Edited* by Susan G. Amara, University of Pittsburgh School of Medicine, Pittsburgh, PA, and approved February 14, 2012 (received for review July 28, 2011)

Fifty years ago, increased whole-blood serotonin levels, or hyperserotonemia, first linked disrupted 5-HT homeostasis to Autism Spectrum Disorders (ASDs). The 5-HT transporter (SERT) gene (SLC6A4) has been associated with whole blood 5-HT levels and ASD susceptibility. Previously, we identified multiple gain-of-function SERT coding variants in children with ASD. Here we establish that transgenic mice expressing the most common of these variants, SERT Ala56, exhibit elevated, p38 MAPK-dependent transporter phosphorylation, enhanced 5-HT clearance rates and hyperserotonemia. These effects are accompanied by altered basal firing of raphe 5-HT neurons, as well as SHT1A and SHT2A receptor hypersensitivity. Strikingly, SERT Ala56 mice display alterations in social function, communication, and repetitive behavior. Our efforts provide strong support for the hypothesis that altered 5-HT homeostasis can impact risk for ASD traits and provide a model with construct and face validity that can support further analysis of ASD mechanisms and potentially novel treatments.

Autism spectrum disorder (ASD) is a male-predominant disorder that is characterized by deficits in social interactions and communication, as well as repetitive behavior (1). Hyperserotonemia, or increased whole-blood serotonin [i.e., 5-hydroxytryptamine (5-HT)], is a well replicated biomarker that is present in approximately 30% of subjects with ASD (2, 3). Some data suggest an association of hyperserotonemia with stereotyped or self-injurious behavior, but results have been inconsistent (4, 5). Despite the high heritability of whole-blood 5-HT levels (6), a mechanistic connection between hyperserotonemia and specific components of the pathophysiology of ASD remains enigmatic. In blood, 5-HT is contained almost exclusively in platelets (7) that lack 5-HT biosynthetic capacity but accumulate the monoamine via the antidepressant-sensitive serotonin transporter (SERT; 5-HTT). A genome-wide study of whole-blood 5-HT as a quantitative trait found association with the SERT-encoding gene SLC6A4, as well as with ITGB3, which encodes the SERT-interacting protein integrin β3. In both cases, the strongest evidence for association was found in males (8–10). Linkage studies in ASD also implicate the 17q11.2 region containing SLC6A4, again with stronger evidence in males (11, 12).

As common SLC6A4 variants are only modestly associated with ASD (13), we and our colleagues previously screened SLC6A4 for rare variants in multiplex families that demonstrate strong linkage to 17q11.2. In this effort, we identified five rare SERT coding variants, each of which confers increased 5-HT transport in transfected cells as well as in lymphoblasts derived from SERT variant-expressing probands (11, 14, 15). We found the most common of these variants, Ala56 (allele frequency in subjects of European ancestry of 0.5%–1%), to be overtransmitted to autism probands, and to be associated with both rigid-compulsive behavior and sensory aversion (11, 16). No such trait association is seen in families without linkage to this region (17). In transfected cells, SERT Ala56 also demonstrates increased basal phosphorylation and insensitivity to PKG- and p38 MAPK-linked signaling that normally produce increased transporter trafficking and catalytic activation, respectively (15). These findings suggest that homeostatic control of 5-HT may be impaired in some children with ASD. Importantly, model system studies indicate that 5-HT and SERT are important determinants of normal brain development and that early-life perturbations in 5-HT signaling can have enduring effects on behavior (18–21).

To explore the dependence of juvenile and adult behavior on early-life 5-HT manipulation and further understand the impact of the SERT Ala56 variant in vivo, we generated mice expressing SERT Ala56 from the native mouse SLC6A4 locus (22). Although SERT Ala56 mice exhibit normal growth and fertility (22), they display significantly increased CNS 5-HT clearance, enhanced 5-HT receptor sensitivity, and hyperserotonemia. Even more striking, SERT Ala56 animals display alterations in a number of ASD-relevant behaviors.

Results

SERT Function and Synaptic Homeostasis Is Altered in SERT Ala56 Mice. As predicted from studies of SERT Ala56 transfected cells (11, 14, 15), midbrain SERT protein levels in Ala56 mice were found to be identical to those of WT, Gly56 littermate controls (Fig. S1A), results that are paralleled by the results of antagonist


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†To whom correspondence may be addressed. E-mail: j.vee@vanderbilt.edu or randy.blakely@vanderbilt.edu.

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Department of Molecular Physiology and Biophysics, University of Texas Health Science Center, San Antonio, TX 78229; Laboratory of Behavioral Neuroscience, National Institute of Mental Health, Bethesda, MD 20892; Silvio O. Conte Center for Neuroscience Research, Vanderbilt University, Nashville, TN 37232; Department of Biological Sciences, Vanderbilt University, Nashville, TN 37232; and Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232

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binding (Fig. S1B) and immunohistochemical studies (Fig. S2). SERT proteins exhibit significant posttranslational regulation (23), including Ser/Thr phosphorylation that involves PKG and p38 MAPK-linked pathways (24, 25). Consistent with our findings in transfected cells (14, 15), we found phosphorylation of SERT Ala56 to be significantly elevated in midbrain synaptosomes under basal conditions (P = 0.0002; Fig. L4). Moreover, we found that activation of PKG with 8-Bromo-cGMP (8-Br-cGMP) fails to increase phosphorylation of SERT Ala56, whereas a robust increase in phosphorylation is observed for WT SERT (P = 0.0013; Fig. 1B). Basal SERT phosphorylation is dependent on p38 MAPK activity (24), and PKG activation leads to a p38 MAPK-dependent increase in SERT activity (26). The gain of SERT activity following activation of p38 MAPK is paralleled by an increased affinity for 5-HT that can support an enhanced rate of transport at low 5-HT concentrations (14, 15). When we incubated synaptosomes with the p38-MAPK inhibitor PD169316, we found significant reductions in basal phosphorylation of SERT Gly56 mice (P = 0.018; Fig. 1C). Importantly, the inhibitor also normalized the difference in phosphorylation between the WT and mutant transporters. These findings suggest that constitutive phosphorylation of SERT Ala56 precludes the flexibility exhibited by WT SERT to move between low and high activity states in parallel with changes in 5-HT release.

To complement our ex vivo phosphorylation studies and determine whether SERT Ala56 mice exhibit constitutively enhanced SERT activity in vivo, we monitored hippocampal 5-HT clearance by in vivo chronoamperometry (27). In these studies, we observed a significant increase in the rate of 5-HT clearance for Ala56 animals vs. WT littermates (P < 0.0001; Fig. 1D and Fig. S5). Paralleling our findings in synaptosomes, we observed a significant increase in 5-HT clearance following infusion of 8-Br-cGMP in the WT animals but no significant response in SERT Ala56 animals (P = 0.022 and P = 0.009 for time to clear 20% and 80% of maximum 5-HT signal, respectively; Fig. 1E). Despite the significant increase in 5-HT clearance, no change was observed in midbrain or forebrain tissue 5-HT levels (Fig. S4 A and B). In contrast, in whole blood, in which 5-HT is sequestered by platelets that lack the capability to offset 5-HT accumulation with decreased 5-HT synthesis, SERT Ala56 mice exhibited significantly increased 5-HT levels relative to WT littermates (P = 0.02; Fig. 1F).

Genetic or pharmacological reductions in SERT activity produce diminished sensitivity of multiple 5-HT receptors (28). Therefore, we hypothesized that increased CNS 5-HT clearance could lead to decreased synaptic 5-HT availability and a compensatory increase in 5-HT receptor sensitivity. Consistent with this idea, enhanced 5-HT receptor sensitivity occurs in mice overexpressing SERT (29). To explore this hypothesis in our mice, we first examined the
sensitivity of animals to the 5-HT{sub}2A/2C receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), which produces a stereotyped head twitch response mediated by postsynaptic, cortical 5-HT{sub}2A receptors (30). We observed a significantly elevated head twitch response in SERT Ala56 mice compared with WT littermate controls (P < 0.01; Fig. 2A). Next, we treated animals with the 5-HT{sub}1A agonist 8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT), which leads to hypothermia in mice, mediated by 5-HT{sub}1A autoreceptors located on raphe 5-HT neurons (31). As with DOI studies, SERT Ala56 mice displayed a significantly increased sensitivity to 8-OH-DPAT–induced hypothermia compared with WT controls (P < 0.0001; Fig. 2B; baseline temperature shown in Fig. S5A).

To establish a physiological consequence of altered 5-HT receptor signaling in SERT Ala56 mice, we used loose-patch recordings of dorsal raphe 5-HT neurons in midbrain slices to examine basal firing rates and 5HT{sub}1A–mediated suppression of raphe neuron excitability. Location of recordings was first

Fig. 2. Increased receptor sensitivity and altered social, communication, and repetitive behavior in SERT Ala56 knock-in mice. (A) Head twitches recorded by two observers blind to genotype over 15 min following injection of saline solution, the 5-HT{sub}2A agonist DOI, or the specific 5-HT{sub}2A antagonist M-100907 followed by DOI. Two-way repeated-measures ANOVA revealed a significant genotype–drug interaction (F = 6.88, P = 0.0029, n = 10 per genotype), with a significant Bonferroni posttest result only for the difference between WT and SERT Ala56 animals in the DOI condition (P < 0.01). (B) Change in rectal temperature from baseline after administration of the 5-HT{sub}1A receptor agonist 8-OH-DPAT. Piecewise mixed linear model analysis revealed a significant genotype–drug–time interaction over the 30 min from baseline to maximal hypothermia response (F = 292.3, P < 0.0001). (C) Example traces with basal firing rates are shown for cell-attached extracellular recordings of dorsal raphe neurons in midbrain slices (n = 16 per genotype). Unpaired t test with Welch correction for unequal variances (t test to compare variances, F = 4.345, P = 0.0036) revealed a significant decrease in firing rate in the Ala56 animals compared with the WT controls (t = 2.92, P = 0.032). (D) Percent inhibition of firing of dorsal raphe neurons as a function of varying, bath-applied 5-HT concentration. Curve fit analysis against log(5-HT concentration) with variable slope reveals a significant increase in sensitivity to inhibition of firing by 5-HT in the Ala56 animals compared with the WT controls (F = 292.3, P < 0.0001). (E) Pup vocalizations upon separation from the dam for 5 min at postnatal day 7. Mann–Whitney test revealed a significant decrease in ultrasonic vocalizations in the SERT Ala56 animals compared with WT littermate controls (U = 85.5, P = 0.015; WT, n = 15; Ala56, n = 22). (F) Time in each chamber of the three-chamber Crawley sociability test is shown. Animals with four or fewer total entries were excluded from the analysis as a result of inactivity (Fig. S5C). Two-way repeated measures ANOVA revealed a main effect for chamber (F = 23.25, P = 0.0006) and a trend for an interaction between genotype and stimulus (F = 3.92, P = 0.058; WT, n = 11; Ala56, n = 17). Bonferroni posttest revealed a significant preference for the social chamber in the WT (P < 0.01) but not the SERT Ala56 animals (P > 0.05). (G) Wins (frontward exit) for male animals on the tube test. McNemar exact test revealed a significant decrease in wins in the SERT Ala56 animals in contrast with WT littermate controls (P < 0.0001, n = 14 pairings). (H) Time spent performing individual behaviors over 24 h in the home cage. To allow better visualization, time spent sleeping is not shown, but did not differ by genotype. Two-way repeated measures ANOVA of log(bouts/time) revealed a significant genotype effect (F = 5.84, P = 0.027, n = 10 per genotype), with Bonferroni posttest showing a significant genotype difference only for time spent hanging (P < 0.05). (I) Number of bouts of hanging behavior in 24 h in the home cage. t test of log(bouts) revealed a significant increase in bouts of hanging in Ala56 SERT animals in comparison with WT littermate controls (t = 2.567, P = 0.019), with a significant correlation between log(bouts/time) and log(bouts) (Pearson R = 0.749, P < 0.001).

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established by using ePET-1:EFYP transgenic mice (32), centering on neurons of the medial division of the dorsal raphe. Under basal conditions, we observed a decrease in the firing rate of these neurons in SERT Ala56 brain slices (P = 0.0036; Fig. 2C), potentially arising from increased firing suppression by inhibitory 5-HT\(_{1A}\) autoreceptors (33). Consistent with this hypothesis, dose–response studies of raphe neuron inhibition produced by bath-applied 5-HT revealed an enhanced inhibitory potency of bath-applied 5-HT (P < 0.0001; Fig. 2D).

SERT Ala56 Mice Show Abnormal Social, Communication, and Repetitive Behavior. Impaired social communication is often the first sign of ASD (34). To obtain a measure of early social communication, we measured ultrasonic vocalizations in pups that were separated from their dam at postnatal day 7. We observed a twofold decrease in vocalizations in SERT Ala56 pups in contrast to Gly56 littermate controls (P = 0.015; Fig. 2G). As body temperature could affect vocalization, we measured body temperatures in 7-day-old pups and found no differences between SERT Ala56 pups and Gly56 littermate controls (Fig. S5B).

As adults, SERT Ala56 and WT littermates exhibit a low baseline level of amulatory activity in novel environments (Fig. S6 A–C), typical of 129S substrains (35, 36). Thus, in analyses of adult animals that are dependent upon exploratory behavior, we included only data from mice with sufficient activity levels to allow a valid comparison between time spent in different arms or chambers (Fig. S6 B and C). In these studies, we observed no differences in anxiety-like behavior on the elevated plus-maze or between SERT Ala56 pups and Gly56 littermate controls (Fig. S5B). As adults, SERT Ala56 and WT littermates exhibit a low baseline level of amulatory activity in novel environments (Fig. S6 A–C), typical of 129S substrains (35, 36). Thus, in analyses of adult animals that are dependent upon exploratory behavior, we included only data from mice with sufficient activity levels to allow a valid comparison between time spent in different arms or chambers (Fig. S6 B and C). In these studies, we observed no differences in anxiety-like behavior on the elevated plus-maze or between SERT Ala56 pups and Gly56 littermate controls (Fig. S5B).

To evaluate adult social interaction in a task that does not require high levels of amulatory activity, we implemented the tube test of social dominance (38). Mouse models of other disorders that display ASD traits, including Rett and Fragile X syndromes (38, 39), show altered behavior on this task. After being trained to progress forward through an empty tube to be returned to their home cage, mice encounter an unfamiliar mouse that has entered from the opposite end of the tube. In these experiments, we found that SERT Ala56 animals more often withdrew from the tube upon encountering an age- and sex-matched WT littermate control (P < 0.0001; Fig. 2F).

In our studies that identified multiple, gain-of-function SERT variants in ASD subjects, we found the SERT Ala56 variant to be associated with rigid-compulsive behavior and sensory aversion in ASD (11). Several tests of sensorimotor function display deficits in subjects with ASD, including prepulse inhibition (40), a sensorimotor gating test that can be applied in mice. In a comparison with WT littermate controls, SERT Ala56 mice displayed a genotype by prepulse amplitude interaction effect on acoustic startle amplitude and prepulse inhibition of startle, reflecting an increased startle response at baseline that attenuated with increasing prepulse amplitudes (Fig. S8 A and B). To assess spontaneous repetitive behavior, we performed noninvasive, automated monitoring of animals in the home cage. Although many behaviors were found to be normal in these studies, we observed that SERT Ala56 mice spent a significantly greater time hanging from the wire cage lid relative to WT littermates (P < 0.05; Fig. 2H). Time hanging was significantly correlated with the number of bouts of hanging (Pearson \( R = 0.749, P < 0.0001 \)). Indeed, examination of recordings revealed that SERT Ala56 animals climbed up to hang briefly on the wire lid and then returned back to the floor of the cage, repeating this behavior an average of approximately 1,000 times over a 24-h recording period (P = 0.019; Fig. 2I). Other potential repetitive behaviors, including grooming, were not found in the home cage (Fig. 2H). We also did not see abnormalities in marble burying (Fig. S9), a test of repetitive behavior proposed to be relevant to ASD (41).

Discussion

These studies describe biochemical, physiological and behavioral traits that derive from the conversion of a single amino acid, Gly56, in SERT. Although conversion of Gly to Ala is a relatively minor change of structure, the SERT N terminus supports multiple SERT protein associations that may be impacted (23, 42–44). In this regard, SERT associates with proteins that influence the transporter’s phosphorylation state, including the catalytic subunit of the Ser/Thr phosphatase PP2A (45) and PKG1 (46); although sites supporting these associations have not been defined. As the Ala56 variant does not create or alter a canonical phosphorylation site, we suspect that the alteration modifies the secondary structure of the N terminus to permit enhanced access of one or more kinases (or restricted access of a protein phosphatase) to either the N terminus itself or a nearby cytoplasmic phosphorylation site. The SERT N terminus is directly connected to the transmembrane domain 1, a domain that participates in 5-HT binding during the translocation process (47, 48). We hypothesize therefore that SERT Ala56-induced changes in N-terminal structure, protein associations, or phosphorylation, lock the transporter in a high-affinity conformation for 5-HT. Such an effect could lead to diminished availability of 5-HT for signaling and effectively eliminate the flexibility needed for SERT activity to track changes in 5-HT release. As proper control of 5-HT availability is vital to normal brain development (18–21), constitutively diminished 5-HT availability could lead to alterations in brain wiring and enduring changes in behavior.

The pattern of alterations in whole blood 5-HT levels, midbrain 5-HT neuron firing, and receptor sensitivities in the SERT Ala56 mouse reflects homeostatic changes in response to the primary change in SERT. Hyperserotonemia in the Ala56 mouse is consistent with the role of SERT in platelet 5-HT uptake and with prior studies showing that mice lacking SERT show essentially no whole blood 5-HT (9, 49). Although no changes were found in brain tissue 5-HT levels in the SERT Ala56 mice, we suspect that tryptophan hydroxylase activity can be readily modified to reduce 5-HT biosynthetic capacity. Platelets lack this mechanism of homeostatic control, as they do not synthesize 5-HT but rather accumulate 5-HT released by duodenal enterochromaffin cells as they circulate through the gut (49). Moreover, tissue levels are a poor correlate of the synaptic availability of 5-HT, which likely depends more on smaller, readily releasable pools of neurotransmitter and the inherent excitability of 5-HT neurons. Our findings of altered basal firing of raphe neurons in vitro and increased sensitivity of SERT Ala56 animals to challenge with 5HT\(_{1A}\) and 5HT\(_{2A}\) receptor agonists provides critical evidence that the changes seen in 5-HT clearance translate into behaviorally relevant changes in 5-HT signaling.

Substantial ethological differences exist between mice and humans, and scientists have, to date, generated only a few mouse models derived from gene variants identified in ASD (50–54). It is thus not possible to assert that a particular set of behavioral abnormalities directly models ASD in a mouse. We believe that, at this time, it is more reasonable to identify how genetic variation impacts mouse behavior, with the goal of identifying underlying changes in brain function that may be conserved in man and which can promote an understanding of the deficits arising in ASD. Given that SERT Ala56 represents a susceptibility variant, rather than a highly penetrant, monogenic cause of ASD (11, 17), we do not expect animals expressing the variant to model all aspects of ASD.
The impact of susceptibility variants is expected to vary depending on the presence of other genetic or environmental factors. Thus, the biochemical, physiological, and behavioral changes seen in an animal model of a susceptibility variant could offer many, or few, parallels to the human disorder. Further, individuals with ASD show considerable heterogeneity in clinical symptoms and genetic susceptibility, and an animal model of a susceptibility variant could therefore show some features that arise in only a subset of individuals (55). Further research is needed to understand how other genetic or environmental factors modulate the phenotypes that we observed in the SERT Ala56 mice, as well as the interaction between this variant and other risk factors in individuals with ASD (11).

We find potential parallels of ASD-associated deficits in the SERT Ala56 mice. ASD is a disorder of pediatric onset. The decrease in ultrasonic vocalizations we observe in SERT Ala56 pups suggests an early emergence of the impact of 5-HT on the capacity or drive for communication. Social interaction deficits in ASD persist into adulthood. The tube test represents an ethologically valid mouse social interaction with a binary outcome that may be particularly sensitive to changes in social proficiency (39). Interestingly, Duvall and colleagues (56) identified a male-pre-dominant, quantitative trait locus for social responsiveness in multiplex ASD families on chromosome 17q, including the SLC6A4 gene region. Consistent with this, we observed a lack of preference by adult SERT Ala56 mice for a social stimulus in the three-chamber test. Finally, SERT Ala56 has been associated with sensory aversion in ASD subjects (11). Although we observed only a modest enhancement in the startle response during prepulse inhibition tests, more sensitive studies are needed that examine the physiological properties of the sensory fields of mice and the ability of animals to integrate sensory information as these functions are known to be under the influence of 5-HT during development and in the adult (19–21).

Rigid-compulsive behavior (57) is significantly elevated in SERT Ala56 carriers and in a combined group comprising SERT Ala56 carriers and other carriers of rare, hyperfunctional SERT coding variants (11). It is difficult to predict how a genetic determinant of rigid-compulsive behavior in humans will manifest in an animal model. The repetitive bouts of hanging from the wire cage lid we observe in these mice may represent a novel parallel of the repetitive, stereotyped, nonfunctional routines that are common in ASD (1), although other interpretations are possible. Importantly, the repetitive hanging phenotype was identified in the context of many normal behaviors and in the animals’ home cage, suggesting that we detected a spontaneous, rather than experimentally induced, repetitive behavior.

The biochemical, physiological, and behavioral results in the SERT Ala56 animals also have some important limitations. First, it is not clear how the specific change from Gly56 to Ala56 leads to increased p38-MAPK–sensitive basal phosphorylation, whether by way of altered SERT tertiary structure or disrupted protein–protein interactions in the N-terminal domain where Gly56 is expressed. Further work is needed to understand these mechanisms, including identifying the residues at which phosphorylation occurs and the kinases or phosphatases that act at these residues. Second, we studied only homozygous animals to maximize our ability to detect phenotypes. Future studies will be needed to understand whether similar biochemical, physiological, and behavioral phenotypes are found in animals with only one copy of the SERT Ala56 variant. Third, the low activity level appears to be a result of the inbred strain background (35, 36) and does not differ by genotype. When coupled with the ultrasonic vocalization and tube test results, however, the overall data are consistent with a change in social function. Further experiments conducted on other inbred strain backgrounds may clarify the lack of preference for social stimuli in the SERT Ala56 mice. Finally, in contrast to increased grooming behavior observed in some other mouse models of ASD-associated genetic variation (52, 54, 58), the increased climbing/hanging behavior we observed in the SERT Ala56 mice is a repetitive behavior that has not previously been described to our knowledge. The difference in the pattern of observed repetitive behavior in the SERT Ala56 mice, in contrast to other models of ASD susceptibility, could reflect the fact that expression of this susceptibility variant is limited to a single, neurochemically defined pathway. Further experiments will be necessary to connect this behavior to changes in underlying circuits.

As autism is a neurodevelopmental disorder, it will be important to now investigate the temporal profile and developmental requirements for constitutively elevated 5-HT transport in the changes we observe in the SERT Ala56 mice. Excess 5-HT clearance during early stages of development could influence neuronal migration, axonal projections, and synapse development in these mice, as indicated by other developmental manipulations that target 5-HT signaling (19, 20, 59). Our constitutively expressed variant also does not speak to the important sites of expression of SERT Ala56 in dictating phenotypes. SERT is not only expressed in the developing and mature brain but also in gut, platelet, adrenal gland, immune cells, pancreas, and lung. Moreover, Boman and coworkers have shown that the placentas, which expresses high levels of SERT (60, 61), is a source of forebrain 5-HT during gestation and is important for normal axonal trajectories (62). Modulating 5-HT levels or transporter function to assess the reversibility of these phenotypes could yield insight into the developmental impact of increased and dysregulated SERT function. Ultimately, studies that allow for temporal and spatial control of the SERT Ala56 variant are needed to answer these questions. Finally, ASD is a disorder with few therapies and none that consistently reverse major deficits. We believe that the SERT Ala56 model offers an opportunity to pursue genetic and pharmacological studies that can both probe ASD mechanisms and possibly identify novel therapeutic targets.

Materials and Methods
All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University, Medical University of South Carolina, or University of Texas Health Science Center Institutional Animal Care and Use Committee. SERT Ala56 knock-in mice were constructed as previously described (22). Details of methods related to synaptosome preparation, Western blotting, citoplasmic binding, SERT phosphorylation, HPLC, immunohistochemistry, in vivo electrochemical recordings, slice preparation, electrophysiological recordings, and behavioral experiments are provided in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Mice. Serotonin [5-hydroxytryptamine (5-HT)] transporter (SERT) Ala56 knock-in mice were constructed as previously described on a 129S6/SvEvTac genetic background (1). Briefly, a targeting construct was created containing exons 2 to 5 of the mouse 129S6/SvEvTac Slc6a4 gene with a floxed neomycin-resistance cassette inserted between exons 4 and 5 (SERT Ala56-Neo). This construct was electroporated into mouse 129S6/SvEvTac embryonic stem cells, and correct targeting was identified by PCR screening and confirmed by Southern blot (1). Following blastocyst injection, resulting chimeric animals were bred with 129S6/SvEvTac female mice to generate germline transmission. To remove the floxed neomycin-resistance cassette, the knock-in mice were crossed with 129S4/Jae protamine-Cre mice, yielding the SERT Ala56 line used for experiments. All mice used in experiments were progeny of heterozygous pairings that were housed by sex with three to five mixed-genotype littermate animals per cage. Mice used within each experiment were greater than 8 wk of age and were matched by birth date to generate cohorts of animals varying by no more than 4 wk.

Synaptosome Preparation. WT and SERT Ala56 mice were rapidly decapitated, and the midbrain was immediately homogenized in 10 volumes (wt/vol) of cold 0.32 M sucrose using a Teflon-glass homogenizer under chilled water and centrifuged at 1,000 × g for 15 min at 4 °C. The resulting supernatant was centrifuged at 15,000 × g for 20 min and the pellet was washed by resuspending in 0.32 M sucrose and frozen for immunoblotting and binding assays or used immediately for phosphorylation assays. Protein assays used either the BCA method (Pierce) or DC protein assay (Bio-Rad).

Western Blotting. Mouse midbrain synaptosomes were solubilized with 250 μL RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1:100; Sigma). Supernatants of detergent extracts (50 mg) were separated by 10% SDS/PAGE, electroblotted to PVDF membranes, and immunoblotted with affinity purified SERT antibody 48 as described previously (2) (1:1,000). Immunoblotting for β-actin followed the same procedure with primary antibody anti–β-actin (1:50,000; Sigma). Optical densities of the bands were quantified with ImageJ software 1.4 (National Institutes of Health) (3) after obtaining multiple gel exposures to ensure linearity of data capture.

[3H]Citalopram Binding. Mouse midbrain synaptosomes were centrifuged at 15,000 × g for 20 min, and the pellet was lysed in binding buffer of 50 mM Tris(hydroxymethyl)aminomethane and 100 mM NaCl, pH 7.4. Each tube was prepared with 200 ng synaptosome protein in 500 μL binding buffer. Five nanomolar [3H]citalopram was then added to each tube and incubated on ice for 20 min. Samples were harvested onto GF/B Whatman filters using a Brandel harvester. Filters were washed three times with ice-cold binding buffer and then immersed in scintillation liquid for 8 h before scintillation spectrometry (Beckman Coulter). Counts were corrected for nonspecific uptake by using parallel samples preincubated at 4 °C for 10 min with fluoxetine 500 μM.

SERT Phosphorylation. Synaptosomes were suspended in modified Krebsbicarbonate buffer (25 mM NaHCO3, 124 mM NaCl, 5 mM KCl, 5 mM MgSO4, 10 mM glucose, pH 7.3) that was saturated with 95% O2/5% CO2 for 60 min at 22 °C. SERT basal phosphorylation was conducted by using 250 to 300 μg P2-synaptosomal protein with 5.0 mCi 32P carrier-free orthophosphate per milligram protein with continuous shaking at 37 °C for 1 h. To study the effect of 8-Bromo-cGMP (8-Br-cGMP) or PD169316, synaptosomes were preincubated with 5.0 mCi 32P carrier-free orthophosphate per milligram protein for 30 min at 37 °C, followed by the addition of 100 μM 8-Br-cGMP, 20 μM PD169316, or vehicle with incubation for another 30 min at 37 °C. Samples were then centrifuged at 15,000 × g for 20 min, and the pellet was resuspended in RIPA buffer supplemented with protease inhibitors (protease inhibitor mixture; Sigma) and phosphatase inhibitors (10 mM sodium fluoride, 50 μM sodium pyrophosphate, 5 mM sodium orthovanadate, and 1 μM okadaic acid) by passing through a 25-gauge needle 10 times. The clear supernatant obtained after centrifuging the solubilized synaptosomes at 25,000 × g for 40 min at 4 °C was subjected to immunoprecipitation with SR-12 antibody as described previously (4, 5). The immunoadsorbents captured by protein A sepharose beads were washed with RIPA buffer before the addition of 60 μL Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 20% (vol/vol) glycerol, 2% (wt/vol) SDS, and 5% (vol/vol) 2-mercaptoethanol] and incubated for 30 min at 22 °C. The eluates were subjected to SDS/PAGE (10% wt/vol) and the 32P-radio-labeled SERT proteins were detected on autoradiograms. Quantification from digitized autoradiograms was evaluated on multiple exposures (4 and 7 d) of the film by using ImageJ software (National Institutes of Health) (3).

HPLC. Levels of 5-HT were quantified by previously described HPLC electrochemical detection methods (6). Trunk blood and brain samples were harvested by rapid decapitation. Blood samples were collected into microcentrifuge tubes containing 25 μL of ACD anticoagulant solution A (BD). Trunk blood and brain samples were frozen on dry ice and maintained at −20 °C until analysis. Biogenic amines were determined by a specific HPLC assay using an Anacon (oxidation, 0.7) electrochemical detector (Antec). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10% aqueous methanol, and 0.5% acetic acid at a flow rate of 0.5 mL/min. The column oven was maintained at 60 °C. Amine elution times were 2.0 min for 5-HIAA, 3.0 min for DOPAC, and 3.3 min for HVA. Peaks were detected electrochemically with a PM1 carbon fiber microelectrode. Amine concentrations were quantified using 5-HT as an internal standard.

Immunohistochemistry. Mouse were deeply anesthetized with sodium pentobarbital and intracardially perfused with 4% paraformaldehyde. Brains were postfixed overnight, transferred to 30% sucrose until sunk, and sectioned on a sliding freezing microtome to a thickness of 40 μm. After blocking in 3% normal donkey serum and 0.1% Triton X-100 in PBS solution, pH 7.4, sections were incubated in anti-SERT (1:1,000; Abcam) and anti-tryptophan hydroxylase (1:1,000; Millipore) antibodies overnight at 4 °C. After washing with PBS solution, sections were incubated in DyLight 488-conjugated goat anti-rabbit (1:200; Jackson ImmunoResearch) and DyLight 549-conjugated donkey anti-sheep (1:200; Jackson ImmunoResearch) secondary antibodies for 2 h at room temperature.

In Vivo Electrochemical Recordings. Clearance of 5-HT was measured by in vivo chronoamperometry as described previously (7, 8). SERT Ala56 and littermate SERT Gly56 mice (20–30 g) were anesthetized by i.p. injection (2 mL/kg body weight) of chloralose (35 mg/kg) and urethane (350 mg/kg) and placed into a stereotaxic frame. High-speed chronoamperometric recordings were

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made using the FAST-12 system (Quanteon). Oxidation potentials consisted of 100-ms pulses of +0.55 V separated by a 900-ms interval at 0.0 V. An Ag/AgCl reference electrode was positioned in the extracellular fluid of the ipsilateral superficial cortex. Oxidation and reduction currents were digitally integrated during the last 80 ms of each 100-ms voltage pulse. Nafion-coated carbon fiber electrodes were pretested for selectivity ratio for 5-HT over 5-hydroxyindoleacetic acid greater than 500:1 and a linear response ($r^2 > 0.9$) to 5-HT. The detection limit for the measurement of 5-HT in these experiments averaged 35 ± 10 nM ($n = 75$).

In vivo electrochemical recording assemblies consisted of a Nafion-coated, single carbon fiber electrode attached to a glass micropipette such that their tips were separated by approximately 200 μm. The barrel was filled with 5-HT (200 μM) in artificial cerebrospinal fluid (aCSF), pH 7.4. The electrode–micropipette recording assembly was lowered into the CA3 region of the dorsal hippocampus (anterior–posterior, −1.94; medial–lateral, +2.0; dorsal–ventral, −2.0) (9). Exogenous 5-HT was applied by pressure-ejection (5–25 psi for 0.25–3 s), in volumes ranging from 4 to 170 nL to produce signal amplitudes ranging from 0.1 to 4.2 μM in randomized order between mice. An electrolytic lesion was made for histological verification of electrode localization at the end of each experiment.

To evaluate the effect of 8-Br-cGMP on 5-HT clearance in WT and SERT Ala56 mice, 5-HT was first pressure-ejected in the CA3 region of hippocampus to achieve signals with amplitudes less than 1.0 μM (range, 0.44–0.90 μM; micropipette barrel concentration, 200 μM). Signal amplitudes did not differ between genotypes (WT, 0.61 ± 0.06 μM, $n = 7$; G56A, 0.69 ± 0.06 μM, $n = 6$). When three reproducible 5-HT signals had been attained, 8-Br-cGMP was applied (micropipette barrel concentration, 12.5 μM; 25 nL ejected to deliver 0.5 pmol). Five minutes later, 5-HT was locally applied, and then again at 10 min and at 10-min intervals thereafter for 1 h. Two time course parameters were analyzed: $T_{50}$ and $T_{80}$, defined as the time for 5-HT to be cleared by 20% and 80%, respectively, of the peak signal amplitude. $T_{50}$ reflects 5-HT at a concentration at which SERT-mediated 5-HT clearance is near $V_{\text{max}}$ whereas $T_{80}$ provides an index of 5-HT clearance at a concentration approximating the $K_m$ for SERT-mediated 5-HT uptake. For both parameters, baseline 5-HT clearance time was fastest in SERT Ala56 mice; however, this reached significance only for the $T_{80}$ parameter ($T_{80}$ WT, 123 ± 13 s; G56A, 81 ± 5 s; $t$ test for independent samples, $t_{12} = 2.22$, $P = 0.0166$; $T_{80}$, WT, 25 ± 4 s; WT, 16 ± 2 s; $t_{11} = 1.806$, $P = 0.0984$). Because of the difference in clearance time between genotypes, 8-Br-cGMP treatment data were analyzed as a percent of baseline (i.e., pre–8-Br-cGMP time course parameters).

**Data Analyses.** The oxidation current was converted to μM units of 5-HT concentration by using the calibration factor determined in vitro. $V_{\text{max}}$ and apparent transporter affinity ($K_T$) values for 5-HT clearance were determined by fitting a one-site hyperbolic function to a plot of clearance rate (TC) vs. signal amplitude (Prism 5; GraphPad), where TC is defined as the slope of the decay curve from 20% to 60% of maximal signal amplitude, i.e., the most linear portion of the decay. Each point on the curves used for kinetic analyses of 5-HT clearance was derived from three to six mice. $K_T$ values were corrected for a volume fraction ($\alpha$) of 0.20 (10).

**Slice Preparation and Electrophysiological Recordings.** Following rapid decapitation, brains were removed and blocked in cold sucrose-substituted aCSF. Solutions and procedures were the same as those detailed elsewhere (11). Coronal, midbrain slices (200-μm thickness) containing the dorsal raphe nucleus were cut on a brain slicer (Campden Instruments) at ice-cold temperature and maintained in aCSF at room temperature for 1 h before recording. Neurons were visualized with an Axioskop microscope (Carl Zeiss) equipped for near-IR differential interference contrast imaging. Recording pipettes with resistance of 3 to 5 MΩ were filled with filtered, normal Heps-buffered saline solution (12). Cell-attached extracellular recordings were performed at 0 mV voltage-clamp condition at 35 °C. Raphe neurons were identified as likely serotoninergic based on suppression of spontaneous spike frequency by 5-HT (4–μM). Spontaneous, basal firing rate was measured as the average of a 100-s record. The inhibition of the firing at various 5-HT concentrations was normalized by the basal firing frequency. Electrophysiological signals were processed and controlled by an Axopatch 1D amplifier and pClamp 10.0 software (Molecular Devices) in gap-free mode. The signals were low-pass filtered at 1 kHz, high-pass filtered at 1 Hz, and digitized at 5 kHz. All solutions contained 3 μM phenylephrine hydrochloride and 40 μM L-tryptophan to maintain the spontaneous firing rate in the absence of noradrenergic tone and tryptophan availability (13).

**Behavioral Experiments.** Four cohorts of adult male mice born consecutively to heterozygous SERT Ala56/Gly56 pairs were used for behavioral experiments. The first cohort of male SERT Ala56 ($n = 22$) and SERT Gly56 (WT littermate controls, $n = 15$) animals underwent the testing reported here in the following sequence: elevated plus-maze, open field activity, Crawley three-chamber sociability, acoustic startle and prepulse inhibition of acoustic startle, Morris water maze, tube test for social dominance, forced swim test, and 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT)-induced hypothermia. The second cohort of male SERT Ala56 ($n = 12$) and SERT Gly56 (WT) animals underwent the testing reported here in the following sequence: Crawley sociability, acoustic startle and prepulse inhibition, tube test, and 1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane (DOI)-induced head twitch response. A third cohort of male SERT Ala56 ($n = 13$) and SERT Gly56 animals ($n = 15$) underwent the testing reported here in the following sequence: radar, a general examination of health and sensorimotor characteristics (14), and home cage monitoring ($n = 10$ of each genotype). A fourth cohort of male SERT Ala56 ($n = 10$) and SERT Gly56 ($n = 10$) underwent the marble burying test. A cohort of mixed-sex postnatal day 7 pups born consecutively to heterozygous SERT Ala56/Gly56 pairs was used for the ultrasonic vocalization experiment. A separate cohort of mixed-sex postnatal day 7 pups born consecutively to heterozygous SERT Ala56/Gly56 pairs was used for the pup temperature measurements.

**Elevated Plus-Maze.** The elevated plus-maze was conducted as described previously (6) under red light conditions in the first 4 h of the dark period to increase locomotion. The plus-maze consists of four arms, 10 × 10 × 30 cm, connected in a plus configuration and elevated approximately 50 cm. Two of the arms have walls 20 cm high, and two arms have no walls. Mice were placed in the center of the maze at the beginning of the 5-min session. The position of the mouse was tracked three times per second, and data were analyzed in real time on a Macintosh computer using Image EP (15) (O’Hara), a modification of the public-domain ImageJ software (National Institutes of Health) (3). Mice were placed in the center of the maze at the beginning of the 5-min session. When the number of entries into the open and closed arms was noted to follow a bimodal distribution (Fig. S5B), animals with four or fewer entries ($n = 6$ Ala56, $n = 5$ Gly56) were excluded from the primary analysis.

**Open Field Activity.** Exploratory locomotor activity was evaluated by using activity monitors measuring 27.9 × 27.9 cm (MED Associates). Each apparatus contains 16 photocells in each horizontal direction, as well as 16 photocells elevated 4.0 cm to measure rearing. Exploratory locomotor activity was evaluated
as described previously (6) for 90 min under red light conditions in the first 4 h of the dark period.

Crawley Sociability Test. Social behavior was evaluated in a three-chamber polycarbonate apparatus with 4-inch sliding gates separating the 7 × 9-inch chambers (14). The subject mouse was initially allowed to explore all three chambers for 10 min to acclimate to the apparatus. A stimulus mouse (social stimulus) was then introduced inside an inverted wire pencil cup (Spectrum Diversified Designs) in one side chamber with a clean empty pencil cup (inanimate stimulus) introduced in the opposite side chamber. The stimulus mouse was an adult male WT mouse, previously habituated to the pencil cup in six 30-min sessions across 3 d. The subject mouse was then allowed to explore all three chambers for 10 min. A research assistant blinded to mouse genotype coded videos for time spent in each chamber. When the number of entries was noted to follow a bimodal distribution (Fig. SSC), analysis of time spent in each stimulus chamber (social and inanimate) by repeated-measures two-way ANOVA with Bonferroni post-tests was performed excluding animals with four or fewer total entries during the sociability portion of the test (n = 14 Ala56, n = 12 Gly56).

Acoustic Startle and Prepulse Inhibition. Acoustic startle response and prepulse inhibition of acoustic startle were measured by using the Acoustic Startle Reflex Test Compartments (MED Associates). Mice were acclimated to background white noise of 65 dB for 5 min in a Plexiglas holding cylinder. Mice were then presented with seven trial types in six discrete, randomized blocks of trials for a total of 42 trials with an intertrial interval of 10 to 20 s. One trial measured baseline movement and one trial measured response to the 120-dB, 50-ms startle stimulus alone. The other five trials used an acoustic prepulse of 74, 78, 82, 86, or 90 dB preceding the acoustic startle stimulus by 100 ms. Startle amplitude was measured every millisecond over a 65-ms period beginning at the onset of the startle stimulus. The dependent variable was the maximum startle over the sampling period. Prepulse inhibition was calculated by dividing the difference between baseline startle and startle following prepulse by baseline startle.

Morris Water Maze. The water maze was a 122-cm circular pool in a room with multiple visual cues. Data were analyzed in real time on a Macintosh computer by using Image WM (O’Hara), a modification of the public-domain ImageJ software (National Institutes of Health) (3), which was used to track the mice for the length of time and swimming distance to a stationary submerged platform from four randomly ordered start locations. A subset of mice from cohort 1 (n = 10 per genotype) was used in the Morris water maze task, as described previously (6). Four trials per day were given until each mouse within the cohort achieved an average time of less than 15 s to find the platform across four consecutive trials. On the final day of testing, a 1-min probe trial with no platform was used to measure the time spent in the quadrant of the pool that previously contained the platform. Reversal learning was tested the following week by switching the platform to the diagonally opposite position in the pool and repeating the aforementioned procedure.

Tube Test. Cohort 1 male mice used for the Morris water maze were paired with each other in the tube test for social dominance. Separately, animals from cohort 2 were paired with one another on the tube test. The apparatus is a 30-cm-long, 3.5-cm-diameter clear acrylic tube with small acrylic funnels added to each end to facilitate entry into the tube. On two separate days before testing, each mouse was exposed to the tube, with progress through the tube resulting in the mouse being returned to the home cage. Mice that did not initially enter the tube were encouraged to run forward with a gentle pull of the tail. Some mice (n = 8 of 22 Ala56/Ala56 and n = 7 of 20 Gly56/Gly56 animals) did not progress through the tube when attempting habituation, either taking more than 1 min to exit the tube, freezing in the tube, or backing out. These mice were not entered into pairings for the tube test. For the tube test bouts, one SERT Ala56 and one SERT Gly56 mouse of the same sex and age cohort but from different home cages were placed at the opposite ends of the tube and released. A subject was declared a “winner” when its opponent backed out of the tube. Each mouse was tested against four to five individuals from other cages. Trials were repeated with each mouse beginning at either end to avoid position bias, for a total of 72 bouts in male cohort 1 and 68 bouts in male cohort 2. Wins and losses for each genotype were analyzed by McNemar exact test.

Forced Swim Test. Mice received an i.p. injection of fluoxetine 10 mg/kg or saline solution. After a 30-min delay for the drug to take effect, mice were gently lowered into a transparent Plexiglas cylinder (20-cm diameter) filled halfway with water (25 ± 1 °C) for a 6-min session, as described previously (16). The presence/absence of immobility (cessation of limb movements except minor involuntary movements of the hind limbs) was scored by stopwatch from 120 to 360 s, which corresponds to the time period that is sensitive to treatment with serotonin reuptake inhibitors (17).

8-OH-DPAT–Induced Hypothermia. A subset of 12 mice per genotype were used. Once every 10 min for 80 min, each mouse had its core body temperature measured with a rectal probe (no. 50314; Stoelting) connected to a BAT-12 thermometer (Physiostemp Instruments). Immediately before the third temperature measurement, mice were administered a 10 mL/kg s.c. injection of 0.1 mg/kg 8-OH-DPAT (Sigma-Aldrich) in PBS solution (recorded as 0 min). Subsequently, the same procedure was used with an equivalent volume of PBS solution only. Finally, the procedure was repeated with the administration of a 10 mL/kg s.c. injection of 0.1 mg/kg WAY-100635 (Sigma-Aldrich) 30 min before injection of 8-OH-DPAT. Data were analyzed by piece-wise mixed-effects linear model by using SAS software (SAS Institute) for the 0- to 30-min time points to evaluate the hypothermia response. Hypoactivity response was not analyzed as a result of the low level of baseline activity.

DOI-Induced Head Twitch Response. A subset of 10 mice per genotype were used for this experiment. Injections (i.p.) of 1.0 mg/kg DOI (Sigma) in PBS solution were administered in a volume of 10 mL/kg. Each mouse was placed in a large glass beaker containing bedding 34 min after injection, and two research assistants who were blind to genotype independently counted head twitches over a 15-min period. More than 1 wk later, the same mice were tested after pretreatment with 10 mL/kg of 0.25 mg/kg M-100907 (gift from Marion Merrell Dow) 17 min before injection with DOI. Finally, mice were tested for head twitch response to PBS solution alone.

Rotarod. Mice were run on an accelerating rotarod (Ugo Basile) on three consecutive days to assess motor learning. On each day, the mice underwent three trials on the rotarod with a 1-min break between trials. Each trial consisted of a maximum of 10 min, with rotation accelerating from four to 40 revolutions per minute.

Home Cage Monitoring. To evaluate possible repetitive behavior, individual mice of each genotype were video-recorded alone in their home cage for 24 h while maintaining their 12 h/12 h light/dark schedule (18). Automated video analysis was conducted by using HomeCageScan (Clever Sys) to index time spent performing individual behaviors. The resulting data were condensed.
into 10 individual behaviors: awaken/sleep, chew/eat/drink, rear, groom, hang, remain low, sniff, stretch, twitch, and walk. To normalize distributions for analysis by two-way repeated-measures ANOVA, data were log_{10} transformed. Bouts of hanging behavior were defined as distinct periods of hanging separated by nonhanging behaviors. The number of bouts per animal was also log_{10} transformed before a t test was performed.

**Marble Burying.** Per a published procedure (19), first, empty clean cages were filled with 4.5 cm corncob bedding (Sani-Chip; Harlan-Teklad). Mice were first habituated to the new cage for 30 min. After habituation, mice were briefly returned to their home cage, and 20 blue glass marbles (15-mm diameter) were evenly placed throughout the test cage in a 4 × 5 arrangement. Test mice were then allowed to explore the test cage freely for 30 min. The number of marbles buried (i.e., less than half the marble still visible) after 30 min was recorded.

**Ultrasonic Vocalization.** Progeny of heterozygous SERT Ala56/Gly56 pairs at postnatal day 7 were used to measure stress-induced communication. Pups were removed from their cage and placed in a Styrofoam chamber with bedding. Ultrasonic vocalizations were measured for 5 min using a Condenser ultrasound microphone (Avisoft-Bioacoustics, Berlin, Germany) and Avisoft SASLab Pro software (Avisoft-Bioacoustics, Berlin, Germany). Thresholds were set to detect only small frequency-modulated vocalizations within a 250-kHz range lasting at least 5 ms and occurring at least 20 ms apart.

**Pup Temperature Measurement.** Progeny of heterozygous SERT Ala56/Gly56 pairs at postnatal day 7 were used to measure body temperature. Pups were removed from their cage and placed in a Styrofoam chamber with bedding. After 5 min, rectal temperatures were measured by using a monochannel rodent thermometer (model BIO-TK885; eb Instruments) and rectal probe (model BIO-SEB BRET-3; eb Instruments).

**Statistical Analysis.** Two-tailed, unpaired Student t test or two-way, repeated-measures ANOVA with Bonferroni post-tests were used to analyze the primary data, except where noted for linear or curve analyses. Specific statistical analyses for each data set are described in Results or in the figure legends.


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**Fig. S1.** No change in midbrain SERT protein expression or citalopram binding in SERT Ala56 knock-in mice. (A) Immunoblot of midbrain synaptosomes for β-actin and SERT (n = 3 per genotype). (B) [3H]Citalopram binding of midbrain synaptosomes normalized to the mean WT value (n = 6 per genotype, two replicates per sample).
Fig. S2. No change in midbrain SERT distribution in SERT Ala56 knock-in mice. (A–C) Representative midbrain confocal images of a WT littermate (SERT Gly56) animal stained for SERT (A) and tryptophan hydroxylase (B). (C) Superimposed image shows both antibodies. (D–F) Representative midbrain confocal images of an SERT Ala56 animal stained for SERT (D) and tryptophan hydroxylase (E). (F) Superimposed image shows both antibodies.

Fig. S3. Elevated 5-HT clearance in hippocampus of SERT Ala56 knock-in mice. Representative oxidation currents for serotonin pressure ejected into the CA3 region of the hippocampus of Ala56 and littermate WT mice. The oxidation currents are converted into micromolar units as described in Materials and Methods.

Fig. S4. No change in midbrain or forebrain 5-HT levels in SERT Ala56 knock-in mice. (A) HPLC measurement of 5-HT levels in the forebrain (unpaired t test, t = 0.79, P = 0.45; WT, n = 7; Ala56, n = 6). (B) HPLC measurement of 5-HT levels in the midbrain (unpaired t test, t = 0.25, P = 0.80; WT, n = 7; Ala56, n = 6).
Fig. S5. No change in body temperature in adult SERT Ala56 knock-in mice at baseline or in SERT Ala56 knock-in pups following 5 min of isolation. (A) Baseline rectal temperature of adult WT and SERT Ala56 mice (unpaired t test, $t = 1.23$, $P = 0.23$; WT, $n = 12$; Ala56, $n = 12$). (B) Rectal temperature of postnatal day 7 WT and SERT Ala56 pups following 5 min of isolation (unpaired t test, $t = 1.16$, $P = 0.26$; WT, $n = 9$; Ala56, $n = 9$).

Fig. S6. No change in activity, anxiety-like behavior, or spatial learning in SERT Ala56 knock-in mice. (A) Distance traveled in the open field. WT and SERT Ala56 animals did not differ in their baseline activity ($t = 0.86$, $P = 0.40$; WT, $n = 15$; Ala56, $n = 22$). (B) A histogram of the number of arm entries on the elevated plus-maze is shown. A significant majority of mice, shown in white, had minimal entries, remaining largely stationary during the test. These mice significantly increased the variability of the test, as some of them remained in a single open or closed arm for the duration of the test. The number of entries for each mouse followed an apparently bimodal distribution, and therefore, only mice with more than four entries were included in the primary analysis shown in Fig. S5D. (C) A histogram of the number of chamber entries on the Crawley sociability test is shown. A substantial minority of mice, shown in white, had four or fewer entries, typically entering only one or none of the stimulus chambers, and remaining largely stationary during the test. The number of entries for each mouse on this test also followed an apparently bimodal distribution, with a cluster of mice with four or fewer entries and an extended range of mice with more than four entries that were included in the primary analysis shown in Fig. 2E. (D) The ratio of time in the open arm divided by total time in either open or closed arms is shown. Mice with four or fewer total entries were excluded from the analysis because of inactivity (Fig. S5A and B). WT and SERT Ala56 animals did not differ in their anxiety-like behavior on the elevated plus-maze ($t = 0.11$, $P = 0.92$; WT, $n = 10$; Ala56, $n = 15$). (E) The total number of entries to the open arms of the elevated plus-maze is shown. No difference was seen between genotypes ($t = 0.52$, $P = 0.61$). (F) The total number of entries to the open or closed arms is shown. No difference was seen between genotypes ($t = 0.19$, $P = 0.85$). (G) The ratio of time in the open arm divided by total time in either open or closed arms is shown for active and inactive mice ($t = 1.22$, $P = 0.18$; WT, $n = 15$; Ala56, $n = 21$). (H) Time in each chamber of the three-chamber Crawley sociability test is shown for both active and inactive mice. Two-way repeated-measures ANOVA revealed a trend for a main effect for chamber ($F = 2.90$, $P = 0.095$), a significant effect of genotype for total time spent in a side chamber ($F = 5.49$, $P = 0.023$), and no interaction between genotype and stimulus ($F = 0.37$, $P = 0.86$; WT, $n = 22$; Ala56, $n = 31$). Bonferroni post-test revealed no significant preference for the social chamber in the WT ($P > 0.05$) or SERT Ala56 animals ($P > 0.05$) when active and inactive mice are analyzed together.
Fig. S7. No change in spatial memory on the Morris water maze, motor memory on the accelerating rotarod, or immobility in the forced swim test in SERT Ala56 knock-in mice. (A) Acquisition of the platform location in the Morris water maze is shown to the left of the vertical line and to platform reversal is shown to the right. Both genotypes successfully acquired the initial platform location and reversal location (two-way repeated-measures ANOVA, main effect of day, \( F = 20.55, P < 0.0001 \); main effect of genotype, \( F = 0.68, P = 0.41 \); genotype–day interaction, \( F = 0.57, P = 0.80 \); genotype \( n = 10 \) per genotype). (B) The time spent in the platform quadrant during the 1-min probe trial following acquisition of the initial platform location on the Morris water maze is shown. No difference was seen between genotypes (\( t = 0.12, P = 0.90 \); WT, \( n = 10 \); Ala56, \( n = 10 \)). (C) The time spent in the reversal platform quadrant during the 1-min probe trial following acquisition of the reversal platform location on the Morris water maze is shown. No difference was seen between genotypes (\( t = 1.25, P = 0.23 \)). (D) Latency to fall off the rotarod apparatus is shown for each genotype. No significant difference was seen between the genotypes (two-way repeated-measures ANOVA, main effect of trial, \( F = 21.57, P < 0.0001 \); main effect of genotype, \( F = 1.32, P = 0.26 \); genotype–trial interaction, \( F = 0.45, P = 0.89 \); WT, \( n = 15 \); Ala56, \( n = 13 \)). (E) Immobility in the last 4 min of the 6-min forced swim test is shown for each genotype for both the saline solution and fluoxetine 10 mg/kg treated animals. No significant difference was seen between the genotypes at baseline (\( t = 1.32, P = 0.21 \)). A significant increase in immobility was seen with fluoxetine, but no significant interaction was seen between drug and genotype (two-way ANOVA, main effect of drug, \( F = 5.34, P = 0.029 \); main effect of genotype, \( F = 0.74, P = 0.40 \); drug–genotype interaction, \( F = 0.76, P = 0.39 \); Bonferroni post-test revealed a significant effect of fluoxetine in Ala56 mice (\( P < 0.05 \)) but not in WT controls (\( P > 0.05 \); WT saline solution, \( n = 8 \); WT fluoxetine, \( n = 8 \); Ala56 saline solution, \( n = 9 \); Ala56 fluoxetine, \( n = 9 \)).

Fig. S8. Alteration in prepulse inhibition in SERT Ala56 knock-in mice. (A) Acoustic startle response to the 120-dB startle cue across a range of prepulse amplitudes is shown, including the baseline acoustic startle with no prepulse (0 dB). Mice that showed negative prepulse inhibition at three or more prepulse amplitudes were excluded (\( n = 1 \) WT, \( n = 2 \) Ala56). A two-way, repeated-measures ANOVA revealed a significant interaction between genotype and prepulse amplitude (\( F = 3.47, P = 0.009 \)). (B) Prepulse inhibition across a range of prepulse amplitudes is shown. A two-way, repeated-measures ANOVA revealed a significant interaction between genotype and prepulse amplitude (\( F = 2.91, P = 0.036 \); WT, \( n = 26 \); Ala56, \( n = 30 \)).
Fig. S9. No change in marble burying in SERT Ala56 knock-in mice. The number of marbles buried during 30 min is shown. No significant difference was seen between the genotypes. As a result of unequal variances between the groups ($F = 4.22$, $P = 0.043$), the Mann–Whitney test was used ($U = 45.0$, $P = 0.73$).