

Altered Depression-Related Behaviors and Functional Changes in the Dorsal Raphe Nucleus of Serotonin Transporter-Deficient Mice

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Background: As a key regulator of serotonergic activity and target of many antidepressant treatments, the serotonin transporter (SERT) represents a potential mediator of anxiety- and depression-related behaviors. Using mice lacking the SERT (SERT KO), we examined the role of SERT function in anxiety- and depression-related behaviors and serotonergic neuron function.

Methods: Serotonin transporter knockout mice were evaluated in paradigms designed to assess anxiety-, depression-, and stress-related behaviors. Dorsal raphe nucleus (DRN) function was assessed by quantitative serotonergic cell counting and extracellular electrical recording of neuronal firing properties.

Results: Serotonin transporter knockout mice showed an increase in latency to feed in a novel situation, more immobility in a forced swim, increased escape latency in a shock escape paradigm, and decreased immobility in tail suspension. No differences in anxiety-related behaviors were seen in the open field and the elevated plus maze. Serotonin transporter knockout mice exhibit a 50% reduction in serotonergic cell number and a fourfold decrease in firing rate in the DRN.

Conclusions: Developmental loss of SERT produces altered behaviors in models of depression that are generally opposite to those produced by antidepressant treatment. The reduced serotonergic cell number and firing rate in the DRN of adult SERT KO mice suggest a mechanism for these altered behaviors. *Biol Psychiatry* 2003;54:960–971 © 2003 Society of Biological Psychiatry

Key Words: 5HTT, serotonin transporter, knockout, antidepressant, open field, learned helplessness, novelty suppressed feeding, tail suspension, immunohistochemistry, forced swim, shock avoidance, electrophysiology

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Introduction

Serotonin (5-hydroxytryptamine) is a key modulatory neurotransmitter in the central nervous system. It is implicated in the regulation of several developmental, behavioral, and physiologic processes, including anxiety and affective states in human and nonhuman species (Barchas et al 1973). At least 14 receptor subtypes mediate the effects of serotonin, but the activation of these receptors is regulated in large part by the function of the serotonin transporter (SERT)—a protein that acts to clear serotonin from extracellular regions through reuptake mechanisms. In the adult central nervous system, SERT is localized primarily on serotonergic neurons (Bengel et al 1997; Charnay et al 1996; Fujita et al 1993; McLaughlin et al 1996).

Several lines of evidence suggest that modulation of SERT activity in the central nervous system can have important effects on neuropsychiatric functioning. Pharmacologic inhibition of SERT via serotonin selective reuptake inhibitors (SSRIs), such as fluoxetine, enhances serotonergic transmission and decreases symptoms of depression and certain types of anxiety (for review, see Gorman and Kent 1999). Genetic modulation of SERT expression via a functional polymorphism in the promoter region of SERT is reported to decrease expression of the SERT in several tissues (Greenberg et al 1999; Lesch et al 1996). Contrary to the behavioral consequences of pharmacologic inhibition of SERT, in certain human populations, this 44-base-pair deletion in the SERT promoter (5HTTLPR) has been associated with elevated “neuroticism,” a factor mostly consisting of anxiety- and depression-related traits (Lesch et al 1996) and increased vulnerability to affective disorders (Collier et al 1996; Furlong et al 1998; Gutierrez et al 1998; Praschak-Rieder et al 2002). In addition, several imaging (Malison et al 1998; Willeit et al 2000) and postmortem studies (Arango et al 1995; Mann et al 2000) have demonstrated a reduced expression of the SERT in the brains of depressed individuals.

These observations suggest that depression and mood disorders might be associated with a reduced expression of SERT. This finding would seem at odds with the effects of SSRIs that reduce SERT function by pharmacologic inhibition. Thus, the relationship between transporter function and vulnerability to affective and anxiety disorders remains unclear. One hypothesis is that impairments of serotonin reuptake during development might be responsible for the increased vulnerability toward affective disorders. To assess this hypothesis, the SERT gene was disrupted in mice (SERT KO). In the present study, the alterations in anxiety- and depression-related behaviors in SERT KO mice were examined. In addition, the structure and electrical properties of dorsal raphe nucleus (DRN) neurons were assessed.

Methods and Materials

Subjects

Serotonin transporter knockout mice were generated by standard homologous recombination techniques with the use of a targeting construct in which the first coding exon of the transporter-coding region was partially deleted and replaced with a neomycin resistant cassette driven by the phosphoglycerate kinase promoter (pGK-NEO) in embryonic stem cells derived from a 129S6/SvEv background strain. Resulting chimeras were backcrossed to 129S6/SvEv mice (Taconic Farms, Germantown, New York) and maintained on this background for all subsequent generations. All subjects were derived from heterozygous crossings and genotyped with polymerase chain reaction of genomic deoxyribonucleic acid (DNA) isolated from tissue samples harvested at 18–21 days of age. Three independent groups of male subjects (14 wild-type [WT]/10 knockout [KO], 6–7 months old, weighing 25–35 g; 6 WT/7 KO, 4 months old, weighing 20–27 g; and 26 WT/23 KO, 3 months old, weighing 22–29 g) and two independent groups of female subjects (12 WT/12 KO, 4 months old; and 8 WT/13 KO, 3 months old, all weighing 16–22 g) were used in the experiments. Animals were group-housed, with both WT and KO present in each cage, maintained on a 12-hour light–dark cycle (8:00 AM/8:00 PM), and provided with food and water ad libitum (except as noted). Animal testing was conducted in accordance with the *Principles of Laboratory Animal Care*, National Institute of Health guidelines and with the approval of the Institutional Animal Care and Use Committee.

Autoradiography

Frozen coronal sections from WT and SERT KO mice were thaw-mounted on SuperFrost/Plus microscopic slides (Fisher-Brand, Atlanta, Georgia) and incubated with 5-iodo-2-[[2-2-[[dimethylamino)methyl]phenyl]thio]benzyl alcohol (125 I) DAM as previously described (Kung et al 1999). Coincubation of WT and SERT KO slices with 125 I) DAM and 10 μ mol/L fluoxetine yielded no detectable binding (data not shown).

Behavioral Testing

All animals were exposed to a series of behavioral paradigms designed to assess a range of anxiety-, depression-, and stress-related behaviors. The tests were administered in the following order: open field, elevated plus maze, novelty suppressed feeding, forced swim, shock avoidance, stress induced analgesia, and shock sensitivity, with a minimum of 4 days between each test. A separate group of mice was examined in the tail suspension test only. All behavioral testing took place between 10:00 AM and 2:00 PM, with the exception of novelty suppressed feeding (4:00 PM–7:00 PM) and shock avoidance (10:00 AM–6:00 PM). To eliminate odor cues, all testing equipment was thoroughly cleaned after each animal with benzalkonium chloride diluted 100-fold.

OPEN FIELD. Exploration and reactivity to a novel open field was assessed in Plexiglas activity chambers (model ENV-520; Med Associates, St. Albans, Vermont) (43.2 cm long \times 43.2 cm wide \times 30.5 cm high). Mice were placed into the center of the open field, and activity was recorded for 60 min. Testing took place under bright ambient light conditions to increase the anxiety component of the center areas of the field (defined as the central 15 cm \times 15 cm portion).

ELEVATED PLUS MAZE. The elevated plus maze task was performed as described previously (Gross et al 2002; Ramboz et al 1998; Santarelli et al 2001). Animals were placed into the central area facing one closed arm and allowed to explore the maze for 5 min. Mice were recorded by a video camera positioned above the maze and scored by an observer blind to mouse genotype.

NOVELTY SUPPRESSED FEEDING. After 24 hours of food deprivation (water available ad libitum), mice were placed in a brightly lit (60-W incandescent bulb 1.5 m above the arena) open arena (51 cm \times 35 cm) containing clean wood chip bedding. A white filter paper 125 mm in diameter was placed in the center of the arena, and one familiar food pellet weighing approximately 4 g was placed on the paper. Mice were removed from their home cage, placed in a holding cage for 30 min before the test, and then placed in one corner of the arena. The latency to begin a feeding episode was recorded (maximum time was 6 min for male mice and 15 min for female mice). Immediately after testing, mice were removed from the arena and placed into their home cage for 5 min, and food consumption was assessed.

FORCED SWIM TEST. Forced swim testing was performed according to methods previously described (Lucki et al 2001) with the use of a plastic cylinder (18 cm in diameter and 24 cm high) filled with water (25–27°C, 18 cm deep). Behavior was recorded by a video camera positioned above the cylinders, and duration of immobility occurring between minutes 3 and 6 was scored.

TAIL SUSPENSION TEST. Tail suspension testing was performed as previously described (Mayorga et al 2001; Steru et al

1985). Mice were recorded by a video camera and scored by a highly trained observer blind to the genotype of the mice.

SHOCK AVOIDANCE. Shock avoidance was performed in a Plexiglas box (model ENV 010 MD, Med Associates) located within a sound-attenuated chamber. The box was divided into two identical chambers (20 cm long \times 17 cm wide \times 20 cm high) and separated by an automated guillotine door. The apparatus was equipped with a grid floor made of a stainless steel, connected to a shock generator, and eight infrared beams for detecting the position and activity of the animal. Mice were placed into the chamber with the guillotine door raised and were allowed to freely explore both compartments for 5 min. At the beginning of each trial, the door was raised, and a mild scrambled foot shock (.2 mA; 10-sec duration) was delivered to the subject. The end of a trial was signaled by the closing of the guillotine door and was triggered either by a transition to the opposite chamber or after 10 sec had elapsed. Transitions were scored as “escapes,” and their latencies were recorded. “Escape failures” were scored if the subject failed to make a transition during the 10-sec duration of the foot shock, and a maximum latency of 10 sec was recorded. A session consisted of 30 trials separated by a 30-sec intertrial interval. Locomotor activity was assessed by counting total infrared beam interruptions during each session.

STRESS-INDUCED ANALGESIA. The apparatus consisted of four heated resistors (.5 cm wide) controlled by adjustable potentiometers. Each resistor was calibrated to different temperatures (42, 46, 50, and 54°C). The front paw was placed on the lowest-temperature bar, and the latency to withdraw was measured (20 sec maximum). The procedure was repeated with increasing temperatures. To induce stress, mice were placed in a cylinder filled with water (23°C) for a period of 10 min, and pain sensitivity measurements were repeated 2, 5, and 10 min later.

FOOT SHOCK SENSITIVITY. Foot shock sensitivity was measured with sound-attenuated startle response chambers (model PMH250, Med Associates) equipped with a Plexiglas cylindrical animal holder (9 cm in length, 3 cm in diameter) containing a grid shock floor mounted on a Plexiglas plate attached to a stabilimeter. An adjustable shock scrambler produced foot shock stimuli of 250 msec duration. For the shock threshold experiment, mice were habituated for 5 min in the apparatus before delivery of a series of foot shocks (.05, .1, .15, .2, .3, .4, .5, and .6 mA) with a 20-sec interstimulus interval. Immediately after the threshold test, mice were subjected to 10 stimuli of .2 mA, and startle response to each stimulus was measured.

Immunohistochemistry

Mice were deeply anesthetized (sodium pentobarbital, 100 mg/kg, IP) and perfused transcardially with 150 mL of .9% saline and 200 mL of buffered 4% paraformaldehyde (4°C). The brain was removed, postfixed for 1 hour, and infiltrated in 10%, 20%, and 30% sucrose over the next 5 days. Brains were sectioned frozen (MICROM HM 4405 sledge microtome [MICROM,

Portsmouth, New Hampshire] with Physitemp BFS-30TC Controller [Physitemp, Clifton, New Jersey]) at 40 μ m and collected into .5 mol/L phosphate buffer.

One set of sections was mounted onto glass slides and stained for Nissl with thionin. A second set of sections underwent immunocytochemistry for serotonin (antibody to serotonin from Proto Labs, Vector Laboratories (Burlingame, California) VECTASTAIN elite ABC kit). The immunocytochemical protocol is standard in our laboratory (Underwood et al 1999). Sections from pairs of WT and SERT KO mice were processed and reacted simultaneously to reduce assay variability. Antibody and reaction specificity was determined in control sections omitting the primary antibody.

Estimation of the total number of DRN serotonergic neurons was determined with the fractionator (West et al 1991) under brightfield microscopy (Leitz Ergolux light microscope [Leitz, Oberkochen, Germany], with a Ludl motorized stage [Ludl, Hawthorne, New York]). The sampling was computer-assisted (Stereo Investigator v5.04.3; MicroBrightField, Inc., Williston, Vermont).

For each mouse, a sample of the cell density was ascertained to calculate the best-suited counting frame to provide an average of three neurons per dissector. The sampling grid size parameter provided an average of 69 sampling sites over all the sections from an individual animal. The counting frame ranged from 50 \times 50 μ m to 65 \times 65 μ m, whereas the sampling grid size ranged from 150 \times 150 μ m to 190 \times 190 μ m. There was a 3- μ m distance from top of section to the start of the counting frame, with a 15- μ m counting frame height. Neurons were counted at a total magnification of 400 \times . Neurons were identified by the presence of staining, and when possible by dendritic processes or nucleoli. Upon analyzing all the sections within a mouse, an estimated total neuron count was determined with the appropriate sample fractions in the fractionator.

Electrophysiology

Adult mice (age 18 weeks) were anesthetized with chloral hydrate (400 mg/kg, IP) and placed in a stereotaxic holder. Supplemental doses of chloral hydrate (100 mg/kg) were given so as to maintain an adequate level of anesthesia (no response to tail pinch). A tungsten wire electrode (5 M Ω , A-M Systems, Carlsborg, Washington) was positioned at 5.2–5.6 mm posterior to bregma and advanced into the DRN, typically found at a depth of 2.5–3 mm relative to bregma (Hof et al 2000). Putative serotonergic neurons were identified as described by Gobbi et al (2001): a slow (< 2.5 Hz) and regular firing rate and a long-duration (.8–1.2 msec) positive action potential. Extracellularly recorded spikes with characteristics of serotonergic neurons were readily obtained from both WT and KO mice. Nine units were recorded from two WT mice, and six units were recorded from two KO mice. Continuous recordings were obtained with a Cheetah data acquisition hardware and software package (Neuralynx, Tucson, Arizona). Spikes were extracted from these recordings by the setting of a threshold at the upper limit of the noise (typically 25–50 μ V). To ensure that recordings were obtained from individual units, identified spikes were sorted based on waveform parameters with SpikeSort software

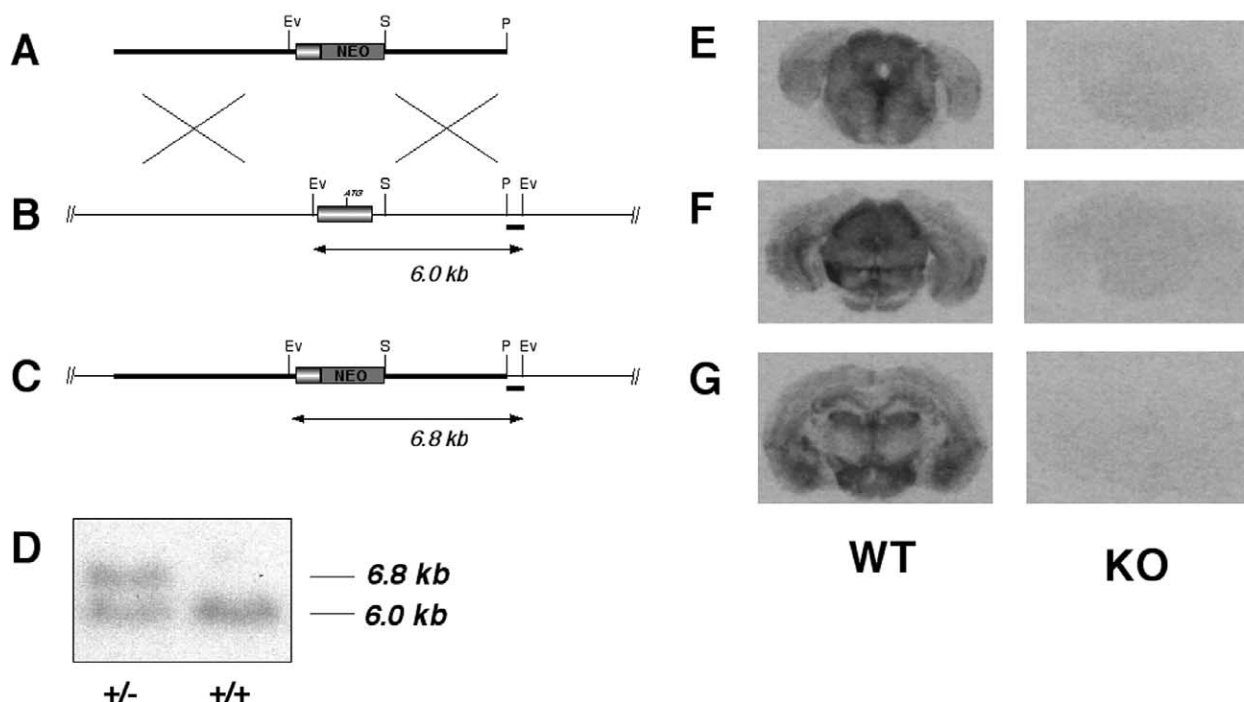


Figure 1. Generation of serotonin transporter knockout (SERT KO) mice. (A) Targeting construct in which the first coding exon of the SERT gene was replaced by a pGK-NEO minigene cassette. (B) Depiction of the endogenous locus of the SERT gene in mice. The double-sided arrow indicates the expected size of EcoRV digested deoxyribonucleic acid (DNA) probed by the external probe—indicated by the short black line (EcoRV/PstI restriction fragment). (C) Representation of the endogenous locus modified by homologous recombination. The double-sided arrow indicates the expected size of the EcoRV restriction fragment from this modified locus. (D) Autoradiogram of a Southern blot obtained from tail DNA from chimera offspring. (E–G) Autoradiographic images obtained from three different coronally sliced brain areas incubated with [¹²⁵I] DAM from wild-type (WT) and SERT KO mice. Images are representative of those obtained from three mice of each genotype. Ev, EcoRV; S, SacI; P, PstI; ATG, putative start codon of the SERT gene; [¹²⁵I] DAM, 5-iodo-2[[2-2-[dimethylamino)methyl]phenyl]thio]benzyl alcohol.

(Neuralynx), although in most cases single units stood out clearly from the background noise. Each unit was recorded for at least 1 minute, and frequency was calculated as the inverse of the average interspike interval. At the conclusion of the recordings, electrolytic lesions (5 μ A, 5 sec) were placed at the recording site. Animals were euthanized with an overdose of chloral hydrate and perfused with 10% formalin. The location of the lesions and/or electrode tracks were confirmed in Nissl-stained sections.

Data Analysis

Data were analyzed with either Student *t* test or one-way analysis of variance (ANOVA) with repeated measures, as appropriate. Genotype was assessed as the independent variable. The criterion for significance for all analyses was $p < .05$. When applicable, post hoc comparisons were conducted with Newman-Keuls test.

Results

Generation of SERT KO Mice

To inactivate the SERT gene, a construct comprising approximately 9 kilobases of genomic DNA was engi-

neered to delete the first coding exon of the SERT gene (Figure 1A). The structure of this region in the endogenous locus is shown in Figure 1B. Electroporation of the deletion construct into embryonic stem cells derived from 129S6/SvEv mice resulted in three positive homologously recombined clones (Figure 1C) from approximately 300 colonies screened (targeting efficiency approximately 1%). Injection of one of these clones into developing blastocysts resulted in six transmitting chimeras. These chimeras were crossed with female mice of the same origin as the embryonic stem cells (129S6/SvEv) to produce a mixture of heterozygous offspring on a homogenous genetic background. The expected Southern blot digestion products of tail DNA from a representative offspring of these chimeras is shown in Figure 1D.

Autoradiography of coronal sections of the brain with [¹²⁵I] DAM revealed that SERT KO mice had undetectable levels of serotonin transporter expression compared with WT littermates (Figure 1E–G).

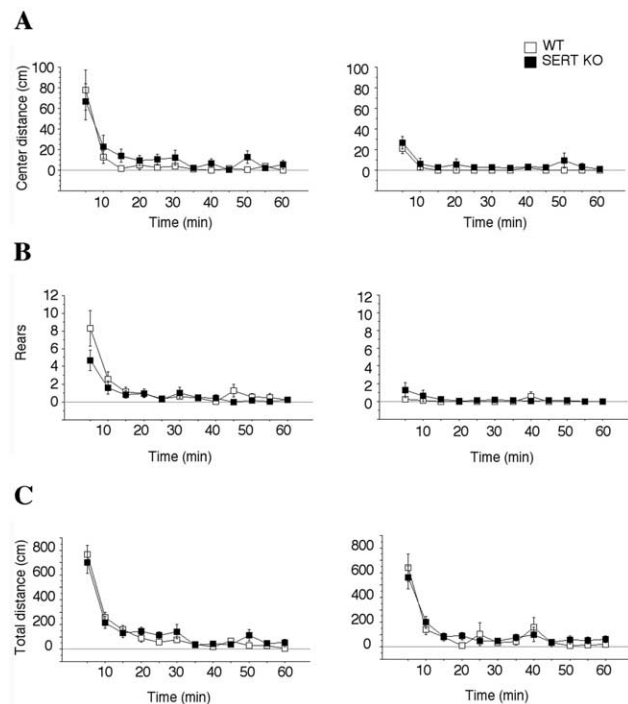


Figure 2. Open field. Behavior was recorded for 60 min in male (left) and female (right) mice and binned into 5-min intervals. The following parameters were scored: center distance (A), rearing (B), and total distance (C). WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 20$ WT and 17 SERT KO male mice, and 6 WT and 10 SERT KO female mice.)

Open Field

No significant effect of genotype was found on the center activity, an index of anxiety-like behavior (Figure 2A), or the total distance traveled (Figure 2C). In addition, rearing activity was comparable in WT and SERT KO mice (Figure 2B). Females exhibited less rearing activity and center activity during the initial 5 minutes of the test than males ($p < .05$), but overall similar behaviors were observed in both male and female groups.

Elevated Plus Maze

There was no effect of genotype on time spent in the open or closed arms (Figure 3A) or percent of entries to the open and closed arms (Figure 3B); however, activity differed between the genotypes, with SERT KO mice showing less total arm entries ($p < .05$ for male, $p < .01$ for female mice) and fewer head dips ($p < .05$ for males, $p < .05$ for females) than their WT littermates (Figure 3C).

Novelty Suppressed Feeding

Serotonin transporter knockout mice exhibited longer latency to begin feeding in a novel environment than WT

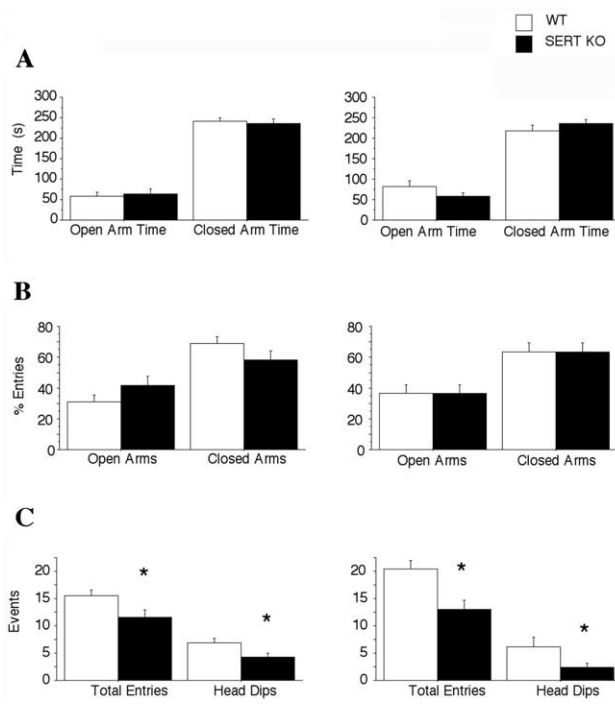


Figure 3. Elevated plus maze. Behavior was recorded for 5 min in male (left) and female (right) mice, and time spent in the open or closed portion of the maze was recorded in seconds (A). The number of entries to open and closed arms was recorded and expressed as percentage of all arm entries (B). Total (open + closed) entries and head dips are shown (C). WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 20$ WT and 17 SERT KO male mice, and 6 WT and 10 SERT KO female mice; * $p < .05$, Student t test.)

mice (Figure 4A; $p < .05$ for male, $p < .05$ for female mice). Female mice exhibited longer latencies than male mice ($p < .05$), but there was no sex \times genotype interaction. To examine a possible genotype difference in the effects of food deprivation, body weights (Figure 4C) and amount of food consumed in the home cage after the test were compared (Figure 4B), and no differences between WT and SERT KO mice were found.

Forced Swim

In the forced swim test paradigm, both sexes of SERT KO mice exhibited significant increases of immobility compared to WT mice ($p < .05$ for male, $p < .01$ for female mice) (Figure 5A, B).

Tail Suspension

Wild-type and SERT KO mice were examined in the tail suspension test. Serotonin transporter knockout mice exhibited a reduction in immobility compared with WT mice ($p < .05$) (Figure 6).

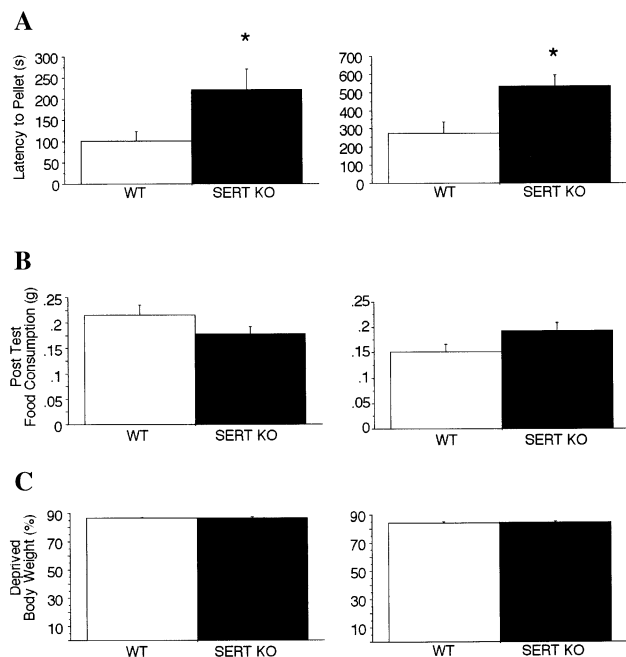


Figure 4. Novelty suppressed feeding. Behavior was recorded for male (left) and female (right) mice. Latency to begin feeding is shown in seconds (A). Posttest, mice were placed into their home cage and allowed to feed for 5 min. Amount of food consumed during this period is shown in grams (B). Body weight was measured and percentage of average free-feeding body weight calculated (C). WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 14$ WT and 10 SERT KO male mice, and 6 WT and 10 SERT KO female mice; $* p < .05$, Student t test.)

Shock Avoidance Test

Serotonin transporter knockout mice exhibited longer latencies to escape foot shock (genotype effect $p < .001$ for male and female mice; Figure 7A) as compared with WT mice. Serotonin transporter knockout mice also had a higher rate of escape failures (genotype effect $p < .01$ for male and female mice; Figure 7B). This higher rate of escape failures in SERT KO mice necessarily translated

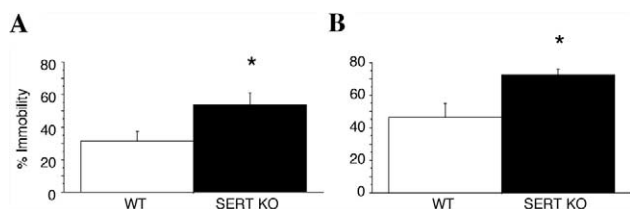


Figure 5. Forced swim test. Performance was scored during minutes 3–6 of the test in male (A) and female (B) mice. WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 8$ WT and 7 SERT KO male mice, and 8 WT and 13 SERT KO female mice; $* p < .05$, analysis of variance.)

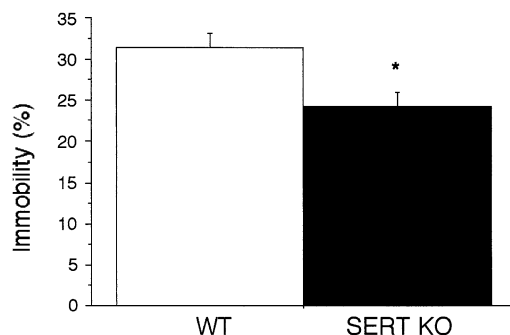


Figure 6. Tail suspension test. Behavior was scored during minutes 1–6 after suspension. Immobility duration expressed as a percentage of total time is plotted. WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 26$ WT and 23 SERT KO male mice; $* p < .05$, analysis of variance.)

into fewer escapes as compared with their WT littermates (Figure 7B; $p < .05$ for male, $p < .01$ for female mice). The activity during the test was measured by the number of beam interruptions (Figure 7C), and no differences between the genotypes in either sexual group were found.

Foot Shock Sensitivity

No genotype differences in foot shock sensitivity were observed (Figure 8A). Mice were also exposed to a series of foot shocks of the same intensity as was used in shock avoidance; however, no differences in startle response over time were observed in either genotype (Figure 8B).

Stress-Induced Analgesia

At baseline, WT and SERT KO mice demonstrated comparable levels of pain sensitivity, as measured by paw withdrawal latency from heated resistors (effect of temperature on latency, $p < .0001$). Both genotypes exhibited significant stress-induced analgesia in response to a 23°C swim stress ($p < .0001$ compared with baseline analgesic response), with no significant differences between the genotypes (Figure 9).

Serotonergic Neuron Number in the DRN

Serotonergic cell number in the DRN was quantified by immunohistochemistry and stereologic counting techniques in WT and SERT KO mice. Serotonin transporter knockout mice were found to have significantly fewer 5-HT immunopositive neurons than WT mice ($p < .05$; Figure 10E). Representative sections at low and high power are shown for WT (Figure 10A, C) and SERT KO mice (Figure 10B, D).

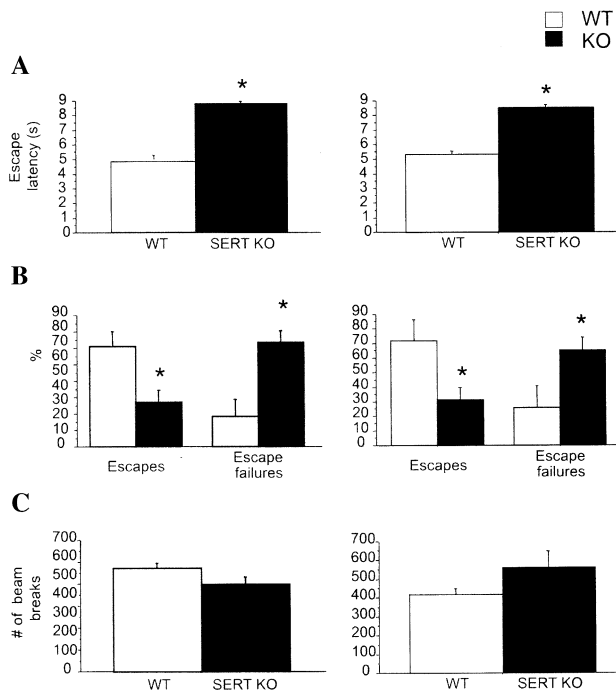


Figure 7. Shock avoidance. Behavior was assessed in male (left) and female (right) mice. (A) Escape latency in seconds. (B) Escapes and escape failures, expressed as percentage of total number of completed trials. (C) Activity as measured by number of infrared beam interruptions. Asterisks indicate significant genotype effects. WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 6$ WT and 7 SERT KO male mice, and 6 WT and 10 SERT KO female mice; * $p < .05$, analysis of variance.)

Extracellular Recording of Serotonergic Cell Activity in the DRN

The activity of putative serotonergic neurons from the DRN was recorded in WT and SERT KO mice. There were no differences in waveform shape across genotypes (Figure 11A, C). A representative trace of continuous

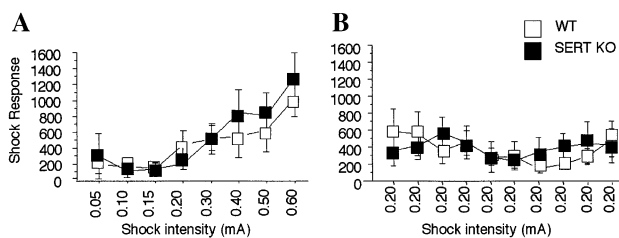


Figure 8. (A) Shock sensitivity is measured as the startle amplitude (arbitrary units) for each group at the shock intensities shown (.05–.6 mA). (B) Sensitization to shock of .2 mA intensity is also shown. WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 7$ WT and 9 SERT KO male mice.)

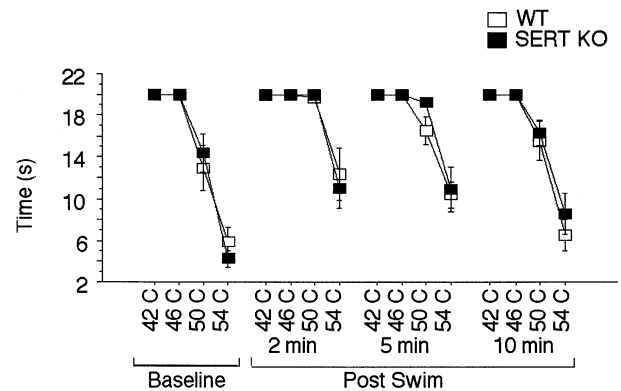


Figure 9. Stress-induced analgesia. The sensitivity to a painful stimulus was measured as latency (s) to withdraw the front paw from the heated resistor. Latency is measured at baseline conditions and after a 10-min, 23°C swim stress (2 min, 5 min, and 10 min post-swim). WT, wild-type mice; SERT KO, serotonin transporter knockout mice. ($n = 7$ WT and 9 SERT KO male mice; $p < .0001$ analysis of variance effect of temperature and stress on latency; no stress \times genotype interactions.)

firing for each genotype is shown in Figure 11B, D. The average firing rate of these dorsal raphe neurons (Figure 11E) was reduced approximately fourfold in the SERT KO mice ($p < .05$).

Discussion

The present study examined the impact of SERT mutation on anxiety- and depression-related behaviors in mice. No effect of this mutation was observed on viability, health, gross morphology, or locomotor behavior. No differences in anxiety-like behaviors in the open field or the elevated plus maze were observed. On the other hand, a significant genotype effect was observed in several behavioral models sensitive to antidepressants (forced swim, shock avoidance, novelty suppressed feeding, and tail suspension), suggesting that the SERT mutation produces alterations in depression-like behaviors.

The two exploration–conflict anxiety tests used in this study, the novel open field and the elevated plus maze, are generally sensitive to benzodiazepine anxiolytics and insensitive to antidepressant treatments (Johnston and File 1988; Lister 1987). In these two tests, anxiety, exploratory, and locomotor-related measures are highly correlated (Ramos et al 1997). Although open field locomotor activity was comparable between genotypes, total arm entries in the elevated plus maze demonstrated that the SERT KO mice were less active than WT mice in this paradigm. This difference appears to be due to the tendency of SERT KO mice to remain in the center portion of the maze (data not shown) rather than explore the entire

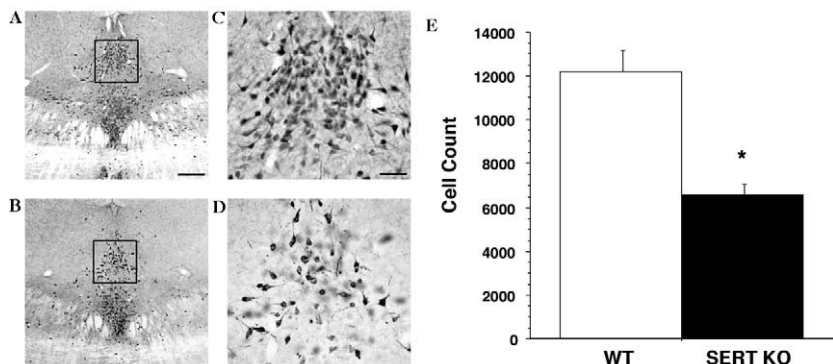


Figure 10. Serotonergic neuron number in the dorsal raphe nucleus (DRN). Serotonin immunoreactivity in the DRN in wild-type (WT) (A, C) and serotonin transporter knockout (SERT KO) mice (B, D) from representative sections. Stereologic techniques reveal fewer serotonin neurons in the SERT KO compared with WT mice (E). Low-power magnification (A, B), bar = 200 μ m; high-power magnification (C, D), bar = 50 μ m. ($n = 3$ WT and 3 SERT KO mice; $*p < .05$.)

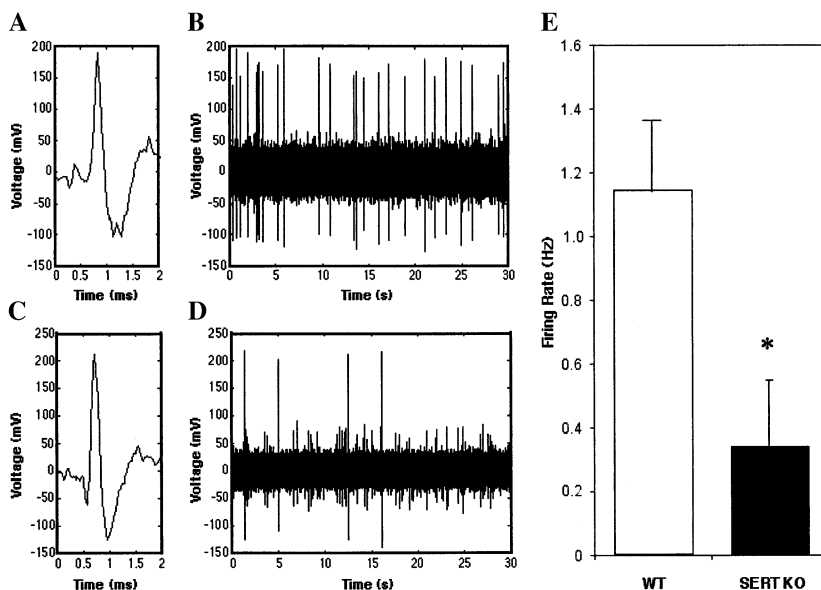
maze. Factor analysis of elevated plus maze behavior in mice has shown that behavior in the maze is largely accounted for by three factors: a total activity factor, an anxiety factor, and a center-time factor (Rodgers and Johnson 1995). Increased time spent in the center portion of the elevated plus maze has been interpreted as representing a decisional component to the task representing approach/avoidance conflict (Rodgers and Johnson 1995). Although controversy exists as to the usefulness of center measures (Wall and Messier 2000), this finding suggests that SERT KO mice exhibit increased levels of indecision and risk assessment in this task.

The novelty suppressed feeding paradigm shares many similarities to exploration–conflict tests, except that hunger becomes the primary drive rather than exploration (Bodnoff et al 1988). The latency to approach the center and begin feeding is sensitive to anxiolytic medications, such as benzodiazepines, but unlike the other anxiety tests used here, it is also sensitive to antidepressant treatment (Bodnoff et al 1988). Both antidepressants and anxiolytics

have been shown to reduce the latency to begin feeding in the 129S6/SvEv strain used here (R. Hen, L. Santarelli, unpublished observation). In contrast, SERT KO mice exhibited significantly longer latencies to begin feeding than WT mice. The difference between WT and SERT KO mice is unlikely to be explained by different effects of food deprivation because both genotypes were of comparable weight before the test, showed similar weight reductions after food deprivation, and consumed similar amounts of food in their home cage (familiar environment) after the test. It also seems unlikely that locomotor differences could account for these findings because no differences in locomotion were found in the open field, a setting that most closely resembles the structure of the novel chamber where feeding latency is assessed.

In the forced swim test, acute antidepressant treatment has been shown to decrease immobility in a forced swim test in mice (Lucki et al 2001; Porsolt et al 1977; Thiebot et al 1992); however, SERT KO mice exhibited increased immobility compared with their WT littermates. The

Figure 11. Firing rates of serotonergic neurons in the DRN. Single extracellularly recorded spikes in wild-type (WT) (A) and serotonin transporter knockout (SERT KO) mice (C). Continuously recorded spike trains from WT (B) and SERT KO (D) mice. Comparison of firing rates in SERT KO and WT mice (E). ($n = 9$ and 6 recordings from two WT and two SERT KO mice, respectively; $*p < .05$.)



results of this test could be affected by differences in activity or fatigability; however, no genotypic differences in locomotor activity in the open field and no differences in grip strength testing were observed in the SERT KO mice tested here (data not shown). Moreover, there was no significant increase in immobility during the 4-min observation period, making fatigue an unlikely factor in these findings. Thus, it seems likely that the increased immobility in the forced swim test is related to the behavioral vulnerability of the SERT KO mice to this form of inescapable stress.

To further explore the response of the SERT KO mice to stress, we examined their behavior in response to foot shock in a two-chamber design. We observed significantly longer escape latency and a higher rate of escape failures in SERT KO mice. Normally, high rates of escape failures do not occur spontaneously and are induced by exposing the animal to a series of inescapable foot shocks—a paradigm termed “learned helplessness” (Seligman 1972). In the case of the SERT KO mice, no prior training was required to produce deficits in escape latency and elevated numbers of escape failures. This finding suggests that SERT KO mice exhibit an inherent response to stress that is similar to that produced by repeated inescapable shock in WT mice (Caldarone et al 2000; Vaugeois et al 1996).

In the learned helplessness paradigm, antidepressant treatment reduces escape failures (Leshner et al 1979; Petty and Sherman 1979; Telner et al 1981), and serotonin might play an important role in this phenomenon (Brown et al 1982). The escape deficit of the SERT KO mice in shock avoidance does not seem to be related to nonspecific effects, such as reduced shock sensitivity, enhanced stress-induced analgesia, or reduced activity. Thus, the performance in shock avoidance might be additional evidence of a spontaneous behavior in SERT KO mice that is paradoxically opposite to that seen with acute antidepressant treatment.

To better understand the neural mechanisms underlying these behavioral changes, the function of the DRN was examined. Two major abnormalities were found that might partly account for the behavioral changes observed in this study: an approximately 50% reduction in serotonin neuron number in the DRN, and approximately fourfold decrease in raphé firing rate. The former result is supported by a recent *ex vivo* study, where approximately 50% fewer serotonergic neurons are found in primary cell cultures derived from late-stage SERT KO embryos (M.C. Miquel, D. Verge, personal communication) and is also consistent with the approximately 60% reduction in brain serotonin content reported in SERT KO mice (Bengel et al 1998). The latter finding of reduced firing frequency of putative serotonergic neurons in the DRN seen here is consistent with previous findings in SERT KO mice of the

C57/BL6 strain (Gobbi et al 2001). Taken together, the SERT KO mice seem to have significant impairments in the DRN that would lead to reduced serotonergic function. This might partially explain some of the abnormal behaviors seen in these mice. Reduced raphe firing has been hypothesized to be a causative factor in depression because this physiologic state would reduce serotonergic transmission (Blier and de Montigny 1994).

Other alterations in the brains of SERT KO mice have also been reported. For example, serotonin 1A, 1B, and 2A receptors have been shown to be down-regulated (Fabre et al 2000; la Cour et al 2001; Li et al 2000; Rioux et al 1999), but similar changes are also seen with chronic antidepressant treatment (Blier and de Montigny 1994), making it less likely that these alterations are related to the increased depression- and anxiety-related behaviors of SERT KO mice.

The behavioral impact of the SERT mutation seems to interact significantly with the background strain of the mice (Gingrich and Hen 2000). For this study, the SERT mutation has been maintained on the 129S6/SvEv strain, whereas in recent reports the behavior of a similar SERT mutation on a C57/BL6 strain has been described (Holmes et al, *in press*, 2002a). The background strain seems to affect the phenotype of the SERT KO mice in certain respects. Specifically, in the 129S6/SvEv strain used in this study, no differences in locomotor activity in the novel open field were observed, whereas on the C57/BL6 background, reduced locomotor behavior in the open field and in the home cage was noted (Holmes et al 2002a). Furthermore, the SERT mutation on the 129S6/SvEv background studied here demonstrated no differences in anxiety-related measures in the open field and plus maze, whereas the SERT mutation on the C57/BL6 background was reported to increase anxiety-related behaviors in these paradigms (Holmes et al, *in press*). To evaluate these seeming discrepancies, our principal findings were replicated in four independent groups of SERT KO and WT mice, with the same results.

Recently, the behavior of SERT KO mice on the 129S6/SvEv and C57/BL6 in forced swim and tail suspension paradigms was described (Holmes et al 2002b), and strain differences were observed in these tests as well. Specifically, SERT KO mice on the C57/BL6 background exhibited no differences in forced swim, whereas the same mutation on a 129S6/SvEv background exhibited the same finding described here—increased immobility. In the tail suspension test, the SERT mutation on the C57/BL6 background showed no differences in immobility (Holmes et al 2002b), whereas on the 129S6/SvEv background small but significant decreases in immobility were seen both by us and others (Holmes et al 2002b). In our study, the tail suspension test was complicated by both age

effects and clasping behaviors seen in SERT KO mice (unpublished data). Thus far, the tail suspension test is the only behavioral test that seems confounded by this issue. Further investigation will be required to determine whether the tail suspension test is a useful measure of depression-related behaviors in SERT KO mice.

These findings highlight the importance of background strain in the manifestation of the phenotypic changes seen with single-gene mutations (Gingrich and Hen 2000). As the findings reported here indicate, the SERT KO mutation on the 129S6/SvEv background might prove to be a more robust model of depression-related behaviors, whereas the SERT mutation on the C57/BL6 background might better model anxiety-related phenotypes associated with transporter function.

Of potential clinical relevance, a common variant of the SERT promoter region (5HTTLPR deletion) has been shown by some investigators to reduce transporter expression by approximately 50% in human subjects (Greenberg et al 1999; Heinz et al 2000; Lesch et al 1996; Little et al 1998). In certain populations, carriers of the 5HTTLPR deletion have been shown to exhibit higher rates of affective disorders, such as depression (Collier et al 1996; Furlong et al 1998; Gutierrez et al 1998; Praschak-Rieder et al 2002). This raises the possibility that genetic reduction of SERT function during early development might have opposing effects on affective function than pharmacologic inhibition of SERT in a mature organism. The SERT KO mice might represent a useful animal model of the behavioral syndrome produced by the 5HTTLPR deletion. Although the SERT KO mice represent a more exaggerated version of the 5HTTLPR deletion, mice, because of their abbreviated developmental time course, often require more extreme versions of a human mutation to mimic the same disease phenotype (Mangiarini et al 1996; Watase et al 2002; White et al 1997).

These findings also have implications for the use of antidepressants during pregnancy and during childhood. It is likely that pharmacologic inhibition of the SERT function during critical periods of development might mimic the phenotype of the SERT KO mice. For example, rats treated with SSRIs during the early postnatal period exhibit increased immobility in the forced swim test later in life (Hansen et al 1997; Velazquez-Moctezuma and Diaz Ruiz 1992) but no differences in anxiety-like behaviors (File and Tucker 1983; Hansen et al 1997). Evidence for a critical developmental window of serotonin function has also been demonstrated with the use of genetic techniques. For example, the elevated levels of anxiety seen in serotonin 1A receptor-deficient mice can be mimicked by reducing receptor expression only during early development (Gross et al 2002). Thus, it is possible that either genetic or pharmacologic inhibition of seroto-

nin function during critical periods of development might increase the vulnerability to affective/anxiety disorders later in life.

In summary, SERT KO mice exhibit several abnormalities in depression- and stress-related behaviors. Most of these alterations are found in tests that are responsive to antidepressants, and the changes are largely in the opposite direction normally produced by antidepressants. Additional work will be required to understand how genetic reductions in SERT produce elevated susceptibility to depression-related disorders later in life or whether a critical period of development requires SERT function. To address these important questions, the SERT KO mice should prove to be a valuable experimental tool for delineating the possible developmental origins of affective disorders.

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