

A STUDY IN MALE AND FEMALE 5-HT TRANSPORTER KNOCKOUT RATS: AN ANIMAL MODEL FOR ANXIETY AND DEPRESSION DISORDERS

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Abstract—Human studies have shown that a reduction of 5-HT transporter (SERT) increases the vulnerability for anxiety and depression. Moreover, women are more vulnerable to develop depression and anxiety disorders than men. For that reason we hypothesized that homozygous 5-HT transporter knockout rat (SERT^{-/-}) models, especially female, are valuable and reliable animal models for humans with an increased vulnerability for anxiety- and depression-related disorders. As rats are extensively used in neuroscience research, we used the unique 5-HT transporter knockout rat, that was recently generated using N-ethyl-N-nitrosurea (ENU)-driven mutagenesis, to test this hypothesis. Behavioral testing revealed that male and female SERT^{-/-} rats spent less time in the center of the open field and spent less time on the open arm of the elevated plus maze compared with wild-type 5-HT transporter knockout rats (SERT^{+/+}). In the novelty suppressed feeding test, only male SERT^{-/-} rats showed a higher latency before starting to eat in a bright novel arena compared with SERT^{+/+} controls. Both male and female SERT^{-/-} rats showed a higher escape latency from their home cage than SERT^{+/+} littermates. Moreover, SERT^{-/-} rats were less mobile in the forced swim test, and sucrose consumption was reduced in SERT^{-/-} rats relative to SERT^{+/+} rats. Both effects were sex-independent. Neurochemically, basal extracellular 5-HT levels were elevated to a similar extent in male and female SERT^{-/-} rats, which was not influenced by the selective 5-HT reuptake inhibitor citalopram. 5-HT immunostaining revealed

no difference between SERT^{+/+} and SERT^{-/-} rats in the dorsal raphe nuclei, in both males and females. These findings demonstrate that SERT^{-/-} rats show anxiety and depression-related behavior, independent of sex. Genetic inactivation of the SERT has apparently such a great impact on behavior, that hardly any differences are found between male and female rats. This knockout rat model may provide a valuable model to study anxiety- and depression-related disorders in male and female rats. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: 5-HT transporter, knockout rat, anxiety, depression, sex.

5-HT is a key modulatory neurotransmitter and has been implicated in the pathophysiology and treatment of anxiety and mood disorders (Neumeister et al., 2002). It is widely accepted that disturbances in the 5-HT system are involved in the onset of depression (reviewed in Jans et al., 2007b). Several alterations in the 5-HT system have been reported in depression, including decreased plasma tryptophan levels (Coppen et al., 1973; Cowen et al., 1989) and decreased levels of 5-hydroxyindoleacetic acid (5-HIAA; metabolite of 5-HT) in cerebrospinal fluid (CSF) (Asberg et al., 1976a,b; Owens and Nemeroff, 1998), suggesting decreased 5-HT metabolism in the CNS. Moreover, brain imaging studies have reported a reduction in 5-HT_{1A} receptor binding, which failed to normalize after treatment for depression (Drevets et al., 1999; Sargent et al., 2000). The extracellular level of 5-HT is primarily regulated by the 5-HT transporter (Slc6a4; SERT), which reuptakes 5-HT from the extracellular space into the presynaptic neuron where it can be degraded or retained for future release (Blakely et al., 1991; Murphy et al., 1998). Due to this important role, it is not surprising that genetic alterations in the SERT are associated with multiple neuropsychiatric disorders (Murphy et al., 1999; Gingrich and Hen, 2001; Holmes et al., 2003b). For example, the human SERT gene transcription is modulated by a common polymorphism in its upstream regulatory region. Studies in reporter gene constructs and in human lymphoblastic cell lines found that the short variant of the polymorphism (*HTTLPR* s) reduces the transcriptional efficiency of the SERT gene (Lesch et al., 1996; Heils et al., 1996, 1997). Moreover, the long variant was associated with more rapid initial platelet 5-HT uptake (Greenberg et al., 1999). However, the *HTTLPR* genotype was not related to the level of SERT binding by autoradiography in the prefrontal cortex (Mann et al., 2000). It was even showed by van Dyck et al. (2004) that the SERT availability in the short-short ho-

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Abbreviations: CSF, cerebrospinal fluid; DAB, 3,3'-diaminobenzidine; DRN, dorsal raphe nuclei; ENU, N-ethyl-N-nitrosurea; PB, phosphate buffered; PBS, phosphate-buffered saline; SERT, 5-HT transporter; SERT^{-/-}, homozygous 5-HT transporter knockout rat; SERT^{+/+}, wild-type 5-HT transporter knockout; SSRI, selective 5-HT reuptake inhibitor; 5-HIAA, 5-hydroxyindoleacetic acid.

mozygotes was greater compared with long-short heterozygotes, in young aged subjects. Several 5-HTTLPR genetic studies have shown a linkage between the short variant and psychiatric conditions. For example carriers of the short version display significantly higher scores in neuroticism, an anxiety-related personality trait, and exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful events compared with individuals with the long version (Mann et al., 2000; Caspi et al., 2003; Schmitz et al., 2007; Dick et al., 2007; Canli and Lesch, 2007), although not every study has reached the same conclusions in this respect (e.g. Lang et al. (2004)).

Women experience depression about twice as often as men (Gorman, 2006), and several possible explanations for this phenomenon have been proposed, some of which are directly or indirectly related to 5-HT neurotransmission. In human, women have significantly higher concentrations of both 5-HIAA and HVA (homovanillic acid) in their CSF than men (Agren et al., 1986), however this provides no direct measure of 5-HT synthesis in the brain. *In vivo* measurements of 5-HT synthesis in the brain by positron emission tomography showed that whole brain rates of 5-HT synthesis is lower in women than in men (Nishizawa et al., 1997). Women also have lower SERT binding in the prefrontal cortex than men (Mann et al., 2000). Moreover, depressed women show a substantial decrease of SERT availability, while hardly any decrease in depressed men was reported (Staley et al., 2006). In view of these data, we hypothesized that rats lacking the SERT show increased vulnerability for anxiety- and depression-related behaviors, and that females are more vulnerable to develop anxiety- and depression-related behaviors after the SERT deletion than males.

Because numerous pharmacological and physiological studies on anxiety and depression have been done in rats, we recently generated a homozygous 5-HT transporter knockout rat (SERT^{-/-}) by N-ethyl-N-nitrosourea (ENU)-driven target-selected mutagenesis (Smits et al., 2004, 2006). This rat has a pre-mature stop-codon (TGC>TGA) introduced at position 3924 in the third exon encoding the second extracellular loop of the transporter protein. Northern blot analyses have revealed that the transversion from a C to an A results in a nonsense-mediated decay of the mutant SERT transcript. At a functional level, *d*-fenfluramine, a 5-HT releaser, and flesinoxan, a full 5-HT_{1A} agonist, are unable to elicit hypothermia in SERT^{-/-} rats (Homberg et al., 2007; Olivier et al., unpublished observations). Moreover, [³H]citalopram (selective 5-HT reuptake inhibitor (SSRI)) binding to brain slices is completely absent in SERT^{-/-} rats (Homberg et al., 2007).

Using this novel model, we here examined anxiety-related behaviors in several tests, including the open field test (Prut and Belzung, 2003), elevated plus maze test (Hogg, 1996), novelty suppressed feeding test (Bodnoff et al., 1988) and the home cage emergence task (Prickaerts et al., 1996). In addition, the knockout rats were tested in two depression-related tests, namely the sucrose consumption test (anhedonia) (Orsetti et al., 2007), and the

forced swim test (Porsolt et al., 1977). Male and female SERT^{-/-} and wild-type 5-HT transporter knockout (SERT^{+/+}) rats were tested to investigate genotype and sex differences in performance in these tasks. Moreover, we assessed the structure of the dorsal raphe nuclei (DRN) in male and female SERT^{-/-} and SERT^{+/+} rats. Mood and emotion are modulated by the serotonergic midbrain raphe system, which seems to be involved in the pathogenesis of psychiatric disorders like those of the affective spectrum (Gurwitz, 2000). Serotonergic neurons within the DRN give rise to serotonergic projections to forebrain systems involved in anxiety-related behavioral responses, such as the amygdala (Steinbusch, 1984; Maier et al., 1993; Graeff et al., 1996). Given these distributions and the knowledge that dysfunction of 5-HT neurons has been implicated in a wide variety of diseases, it is interesting to study whether SERT^{-/-} and SERT^{+/+} rats show differences in the number serotonergic neurons in this brain structure. Finally, we examined extracellular 5-HT levels in the hippocampus to explore how the genetic inactivation of the SERT affects extracellular 5-HT levels in male and female SERT^{-/-} rats, and studied the effect of the SSRI, citalopram on these levels.

EXPERIMENTAL PROCEDURES

Subjects

SERT knockout rats (Slc6a4^{1H^{ub}}) were generated by ENU-induced mutagenesis (Smits et al., 2004, 2006). All subjects were generated, bred and reared in the Central Animal Laboratory of the Radboud University of Nijmegen. Experimental animals were derived from crossing heterozygous 5-HT transporter knockout (SERT^{+/-}) rats that were outcrossed for four or five generations. In all experiments, male and female SERT^{+/+} and SERT^{-/-} littermates were compared. After weaning at the age of 21 days, ear cuts were taken for genotyping. All animals were housed two or three per cage in standard Macrolon[®] type 3 cages (42×26×20 cm, Nijmegen, Gelderland, The Netherlands) in temperature-controlled rooms (21 °C±1 °C) with standard 12-h light/dark cycle (lights on at 7:00 A.M.) and food (Sniff, long cut pellet, Bio Services, Uden, The Netherlands) and water available *ad libitum*. Seven groups of animals were used; the order of experiments was as follows: group 1: sucrose consumption+open field (3–4 months old, open field was performed 2 days after the sucrose consumption experiments ended); group 2: elevated plus maze (3–5 months old); group 3: novelty suppressed feeding (4–6 months old); group 4: microdialysis (2–2.5 months old); group 5: 5-HT immunohistochemistry (males 8–10 months old, females 3–4 months old); group 6: home cage emergence task (3–4 months old) and group 7: forced swim test (3–4 months old). The experiments were performed between 9:00 A.M. and 17:00 P.M. All experiments were approved by the Radboud University Nijmegen, according to the Dutch legal ethical guidelines. Experiments were designed to minimize the number of required animals and their suffering.

Anxiety-related tests

Open field. Twenty-four male ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) and 24 female rats ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) were isolated 24 h before testing. The open field test was conducted as reported in Jans et al. (2007a). In short, the open field is a square arena (100×100×40 cm), with an open top, dark walls (wood) and a dark floor (polyvinylchloride). The arena was subdivided in 'corner' (four squares each 16×16 cm), 'wall' (four rect-

angles each 16×64 cm) and 'center' (one square 64×64 cm) zones. Testing was carried out in dimmed white light. A camera was installed above the center of the field. Immediately after a rat was placed in the corner of the open field, the movements and position of the animals were recorded and registered automatically by a computerized system (EthoVision, Noldus Equipment, The Netherlands). Reported are the time (s) spent in the center of the open field and the total distance moved (cm). Testing was carried out on a 5-min trial. The floor of the open field was cleaned with 70% ethanol solution between trials to prevent transmission of olfactory cues.

Elevated plus maze. Twenty-four male ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) and 20 female rats ($n=10$ SERT^{+/+} and 10 SERT^{-/-}) were isolated 24 h before testing. The test was performed as described by de Jong et al. (2006). The apparatus was made of polyvinylchloride. It was elevated to a height of 50 cm with two open (50×10 cm) and two enclosed arms (50×10×40 cm) arranged such that the arms of the same type were opposite to each other. The illumination intensity measured in the open arms was 2.5 lux, and in the closed arm 0.2 lux. Rats were placed in the center of the maze, facing one of the open arms, for a free exploration period of 5 min. The movements and position of the animals were recorded and registered automatically by a computerized system (Plus Maze®, Nijmegen, The Netherlands). Results were expressed as the mean of time spent (s) in open arms, the mean time spent in closed arms, and the distance moved (cm) in both open and closed arms.

Novelty suppressed feeding. The novelty suppressed feeding test was performed as described by Lira et al. (2003). Twenty-two male ($n=11$ SERT^{+/+} and 11 SERT^{-/-}) and 20 female rats ($n=10$ SERT^{+/+} and 10 SERT^{-/-}) were isolated and food deprived. After 24 h of food deprivation (water available *ad libitum*), rats were placed in a brightly lit (60 W incandescent bulb 1.2 m above the arena) open arena (50×50 cm) containing clean wood chip bedding. A round white filter paper, with a radius of 6.25 cm, was placed in the center of the arena, and one home cage food pellet weighing approximately 2 g was placed on the paper. Rats were removed from their home cage, and then placed in one corner of the arena. The latency (s) to begin a feeding episode was recorded (maximum time was 600 s). Bodyweight (g) of the rats was measured before the 24 h of food deprivation.

Home cage emergence task. Twenty-four male ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) and 24 female rats ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) were housed socially (two per cage). The test was performed as described by Prickaerts et al. (1996). In short, the home cage was placed in an arena and the lid of the home cage was removed. During testing, the cage mate was placed in another cage for the duration of the trial. A grid was placed over the edge of the cage to make it easier for the rats to leave the home cage. Testing was carried out during the night period with red light. A stopwatch was used to measure the latency (s) to leave the cage. The latency of the rat to climb out of its cage into the arena was measured. The trial ended when all four paws of the rat were over the edge of the cage. If the rat did not emerge from its home cage within 600 s, the trial was ended, the home cage was closed again and the rat was given a score of 600 s.

Depression-related tests

Sucrose consumption. The procedure was performed as described by van der Kam (2006). Twenty-four male ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) and 24 female rats ($n=12$ SERT^{+/+} and 12 SERT^{-/-}) were housed individually and habituated to the two-bottle paradigm by offering them water in two plastic drinking cylinders on top of the cage, one on each side, for a total of 5 days. Immediately after this 5 day period, the two bottles, free-choice, 24 h sucrose vs. water paradigm started. In short, animals were

presented either with water in both bottles or, on alternating days, with water and increasing sucrose percentages (2% to 10%). Bottles were switched on sucrose days to prevent spatial bias. Fluid consumption (g) and bodyweight (g) were measured daily and used to calculate two measurements, namely the preference of sucrose above water (sucrose intake in ml divided by total intake×100%) and the intake in grams of a 100% sucrose solution per kg bodyweight (intake in ml corrected for the voluminal weight of sucrose and recalculated toward a 100% solution divided by bodyweight in kg).

Forced swim test. Forty male ($n=20$ SERT^{+/+} and 20 SERT^{-/-}) and 36 female rats ($n=17$ SERT^{+/+} and 19 SERT^{-/-}) were isolated 24 h before testing. The forced swim test was conducted as reported in Porsolt et al. (1977). In short, cylindrical glass tanks (50 cm tall×18 cm diameter), filled to a depth of 30 cm with 22 (±1) °C water, were used in the forced swimming test. Testing consisted of two phases, the induction phase and the test phase. During the induction phase animals were placed in the water for 15 min. After 24 h the rats are placed in the same tanks for 5 min. The movements of the rats were videotaped for off-line measurement of the duration of immobility (s). The behavioral variable 'immobility' was defined as follows: making no movements for at least 2 s or making only those movements that were necessary to keep the nose above the water. The rats were allowed to slightly move their forepaws or support themselves by pressing their paws against the wall of the cylinder. Active climbing, diving and swimming along the wall were scored as mobility (s).

Microdialysis

For the microdialysis studies 10 male ($n=5$ SERT^{+/+} and 5 SERT^{-/-}) and 12 female rats ($n=6$ SERT^{+/+} and 6 SERT^{-/-}) were used. Microdialysis was performed as described by Homberg et al. (2007).

Surgery and microdialysis. Rats were anesthetized using isoflurane (2.5%, 400 ml/min N₂O, 600 ml/min O₂). Lidocaine (10% m/v) was used for local anesthesia. The animals were fixed in a stereotaxic frame (Kopf Instruments, USA) and I-shaped probes (dialyzable surface 4 mm, hospital AN 69, Brainlink, The Netherlands) were inserted into the ventral hippocampus according to the following coordinates: bregma -5.3 mm, lateral to midline +4.8 mm and ventral to dura -8.0 mm (for detailed description see Cremers et al. (2004)) and then secured with dental cement and screws. Experiments were performed 24–48 h after surgery. On the day of the experiment, animals were connected with a flexible PEEK tubing to a microperfusion pump (CMA 102, Microdialysis AB, Sweden) and perfused with an artificial CSF, comprising 147 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂, and 1.2 mM MgCl₂ at a flow rate of 1.5 μl/min. Fifteen minute microdialysis samples were collected in HPLC vials containing 7.5 μl of 0.02 M formic acid and kept at -80 °C until analyzed. Baseline samples were taken after a habituation of 150 min, for 75 min (six samples).

Drug administration. Citalopram HBr was dissolved in saline. Drugs were separately injected s.c. with a volume of 1 ml/kg. Citalopram was injected at a dose of 3 mg/kg directly after baseline measurements.

5-HT analysis. Samples of 20 μl microdialysate were injected via an autoinjector (Gilson 231, France) onto a 100×2.0 mm C18 Hypersil 3 μm diameter column (Bestar, Amstelveen, The Netherlands) and separated using a mobile phase consisting of 4.2 g/l sodium acetate, 500 mg/l EDTA, 50 mg/l heptane sulfonic acid, 4% methanol v/v, and 30 μl/l of triethylamine, pH was set at 4.75 at a flow rate of 0.4 ml/min (Shimadzu LC-10 AD). 5-HT was detected amperometrically using a glassy carbon electrode at 500 mV vs. Ag/AgCl reference electrode

(Antec Leyden, Leiden, The Netherlands). The detection limit was 0.5 fmol 5-HT per 20 μ l sample (signal to noise ratio of 3).

5-HT immunohistochemistry

Immunohistochemistry was performed as described by de Jong et al. (2005). Twelve male ($n=6$ SERT^{+/+} and 6 SERT^{-/-}) and 12 female rats ($n=6$ SERT^{+/+} and 6 SERT^{-/-}) were deeply anesthetized with a mixture of N₂O/O₂ (1:2) and isoflurane (2.5%; Rhodia Organique Fine Limited, Bristol, UK). Then, they were perfused transcardially with 0.1 M phosphate-buffered saline (PBS), pH 7.3, followed by 400 ml 4% paraformaldehyde dissolved in 0.1 M phosphate buffered (PB), pH 7.2. Subsequently, the brains were removed from the skull and postfixed overnight in 4% paraformaldehyde at 4 °C. Before sectioning, the brains were cryoprotected with 30% sucrose in 0.1 M PB. Brain sections were cut on a freezing microtome, thickness 40 μ m, and collected in six parallel series (six vials per rat brain with each 1/6 part of the DRN) in 0.1 M PBS containing 0.1% azide. One vial of each rat was used for the staining. The free-floating sections were washed three times in PBS and preincubated with 1% perhydrol (30% H₂O₂, Merck) for 30 min. After washing three times in PBS the sections were presoaked for 30 min in an incubation medium consisting of: PBS with 0.1% bovine serum albumin and 0.5% Triton X-100. Then the sections were incubated overnight at room temperature, on a shaker with a polyclonal anti-5-HT antiserum raised in rabbit (batch 3–9, gift from prof. Dr. H. Steinbusch) diluted 1:10,000 in the incubation medium. The sections were incubated for 90 min at room temperature in donkey anti-rabbit (1:1500 in incubation medium, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and for 90 min at room temperature in ABC-elite (Vector Elite 1:800 in PBS). In between incubations, sections were rinsed three times with PBS. The 5-HT-antibody peroxidase complex was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining. Sections were incubated for 10 min in a chromogen solution consisting of 0.02% DAB and 0.03% Ni-ammonium sulfate in 0.05 M Tris-buffer (pH 7.6), and subsequently for 10 min in chromogen solution containing 0.006% H₂O₂. This resulted in a blue-black staining. Following the immunohistochemical staining procedures, the sections were rinsed three times in PBS and mounted on gelatin chrome alum-coated glass slides, dried overnight in a stove at 37 °C, dehydrated in an increased series of ethanol, cleared in xylene, embedded with Entellan (Merck), and coverslipped.

Quantification. Numbers of 5-HT-immunopositive cells were quantified using the software program Neurolucida (MicroBrightfield Inc., Williston, VT, USA). The number of 5-HT-positive cells was counted in the same level of the DRN in homologous square fields (using a grid overlay with a size of 100 \times 100 μ m) that displayed a representative density of stained cells.

Data analysis

Behavioral data and immunohistochemistry data were analyzed with two-way ANOVA. Genotype and sex were assessed as independent variables. Where appropriate, post hoc comparisons were conducted using one-way ANOVA. For the sucrose consumption test, effects were evaluated using three-way ANOVA with genotype, sex and concentration as repeated measures. If an interaction was found, effects were further determined with a two-way ANOVA, followed by a one-way ANOVA where appropriate. For microdialysis studies, effects were evaluated using three-way ANOVA with genotype, sex and time as repeated measures. Differences between citalopram treatment were analyzed using two-way ANOVA for repeated measurements followed by independent sample *t*-test analysis where appropriate. Level of significance was set at $P<0.05$. All statistical analyses were performed using the Statistical Package for the Social Sciences version 12.0.1 for windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Anxiety-related tests

Open field. To determine whether SERT^{-/-} rats have an increased level of anxiety, they were subjected to the open field test. This test is based on an exploration-conflict and an increase in time spent in the central part of the open field is considered to be an indication of anxiolytic behavior (Walsh and Cummins, 1976; Prut and Belzung, 2003). A genotype effect was found in the open field (Fig. 1A), with SERT^{-/-} rats spending significantly less time in the center compared with SERT^{+/+} rats ($F_{(1,43)}=13.362$; $P<0.001$). There was no genotype effect found in the total distance moved (Fig. 1B; $F_{(1,43)}=1.187$; $P=0.282$). However, there was a significant sex effect (Fig. 1C; $F_{(1,43)}=10.503$; $P<0.002$), with female rats moving a greater distance than male rats. However there were no significant genotype \times sex interactions.

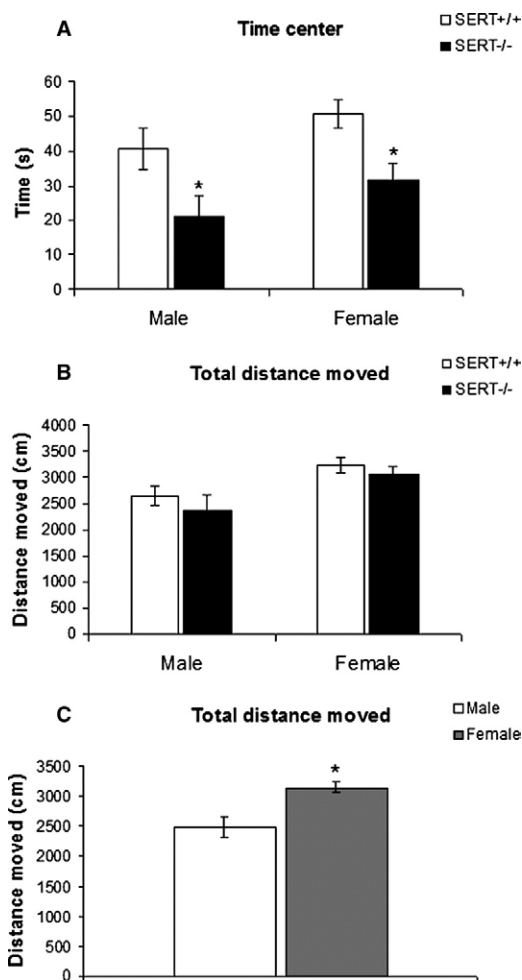


Fig. 1. Open field test. Behavior was recorded for 5 min in SERT^{+/+} and SERT^{-/-} male and female rats. (A) Mean \pm S.E.M. time (s) spent in the center of the open field. (B) Mean \pm S.E.M. distance moved (cm). (C) Mean \pm S.E.M. distance moved (cm) in male and female rats (males: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; females: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; * $P<0.05$).

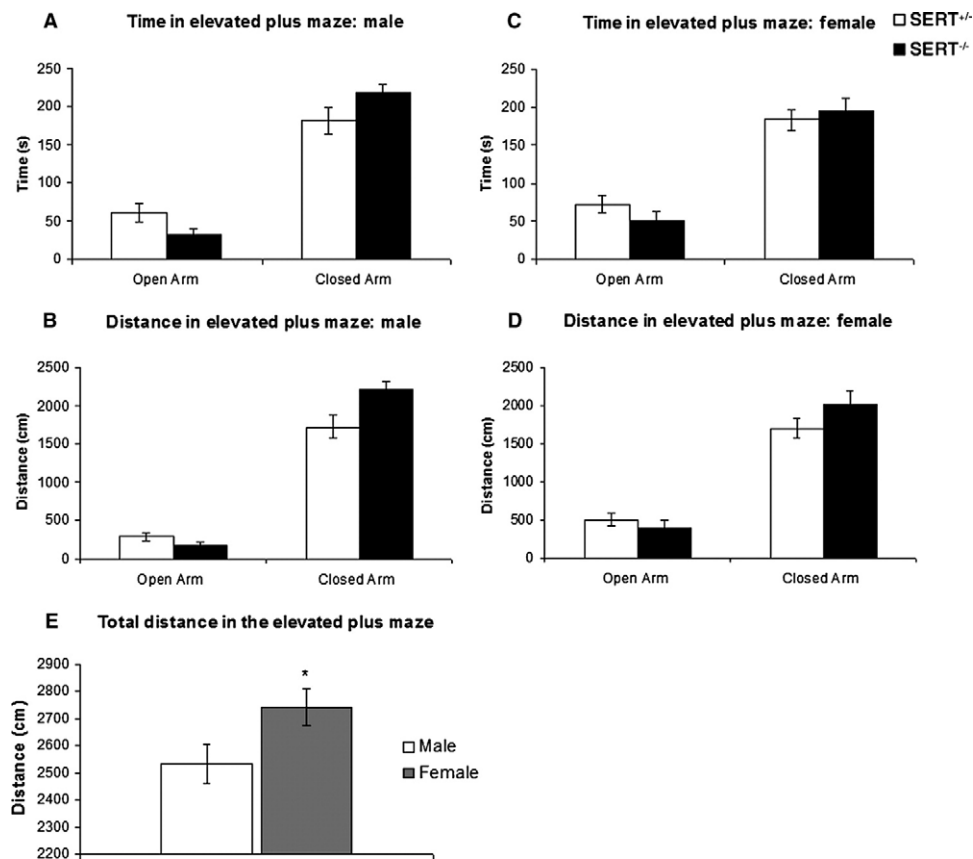


Fig. 2. Elevated plus maze test. Behavior was recorded for 5 min in SERT^{+/+} and SERT^{-/-} male and female rats. (A) Mean±S.E.M. time (s) spent in the open or closed arm in males. (B) Mean±S.E.M. distance moved (cm) in the open or closed arm of the maze in males. (C) Mean±S.E.M. time (s) spent in the open or closed arm in females. (D) Mean±S.E.M. distance moved (cm) in the open or closed arm of the maze in females. (E) Mean±S.E.M. total distance moved (cm) in males and females (males: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; females: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; * $P<0.05$). Note: SERT^{-/-} rats spend significant less time in the open arm [$F_{(1,40)}=5.194$; $P<0.028$] and moved a greater distance in the closed arms [$F_{(1,40)}=8.407$; $P<0.006$] compared with SERT^{+/+} rats.

Elevated plus maze. To determine anxiety in a different assay, SERT^{-/-} rats were subjected to the elevated plus maze. Elevated open alleys arouse greater avoidance responses than elevated closed alleys. Voluntary passage onto the open arms of an elevated, plus-shaped maze is associated with neurobiological changes indicative of a decreased anxiety (Handley and Mithani, 1984; Hogg, 1996). As shown in Fig. 2, a significant genotype effect was found in the elevated plus maze with SERT^{-/-} rats spending less time in the open arms (Fig. 2A, C; $F_{(1,40)}=5.194$; $P<0.028$) and moving a greater distance in the closed arms compared with SERT^{+/+} rats (Fig. 2B, D; $F_{(1,40)}=8.407$; $P<0.006$). In addition, we found a significant sex effect on the total distance moved (Fig. 2E; $F_{(1,40)}=7.337$; $P<0.010$), with females moving a greater distance compared with males. Moreover, females moved a greater distance in the open arms than males ($F_{(1,40)}=9.706$; $P<0.003$). Again, as with the open field test, no significant genotype×sex interactions were found on any of the parameters in the elevated plus maze.

Novelty suppressed feeding. We subjected our SERT^{-/-} rats to a third anxiety test, the novelty sup-

pressed feeding assay. The latency to approach the bright lit center and start eating is considered to be an indication of anxiety (Shephard and Broadhurst, 1982). A significant genotype effect was found for the latency to start eating in a novel environment ($F_{(1,41)}=17.344$; $P<0.001$). As shown in Fig. 3, SERT^{-/-} rats exhibited a longer latency to start eating compared with SERT^{+/+} rats. This was completely due to the male SERT^{-/-} rats, although there was no sex effect, a



Fig. 3. Novelty suppressed feeding test. Behavior was recorded for 10 min for male (left) and female (right) SERT^{+/+} and SERT^{-/-} rats. Data are expressed as mean±S.E.M. latency (s) to start eating (males: $n=11$ SERT^{+/+} and $n=11$ SERT^{-/-} rats; females: $n=10$ SERT^{+/+} and $n=10$ SERT^{-/-} rats; * $P<0.05$).

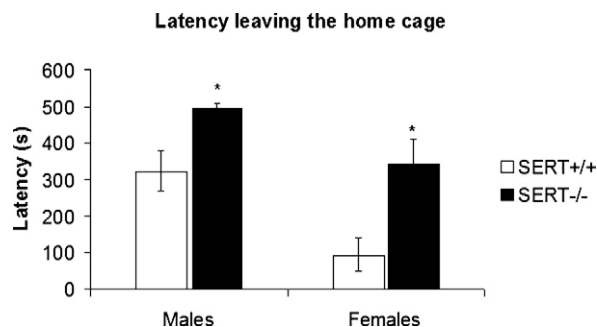


Fig. 4. Home cage emergence task. Behavior was recorded for 10 min for male (left) and female (right) SERT^{+/+} and SERT^{-/-} rats. Latency (s) leaving the home cage is shown as mean±S.E.M. (male: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; female: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; * $P<0.05$).

genotype×sex interaction was found ($F_{(1,41)}=11.858$; $P<0.001$). Male SERT^{-/-} rats exhibited a longer latency to start eating (Fig. 3) in a novel environment than male SERT^{+/+} rats ($F_{(1,19)}=27.684$; $P<0.001$), whereas this was not significantly different between female SERT^{-/-} rats and SERT^{+/+} rats (Fig. 3; $F_{(1,22)}=0.274$; $P=0.606$). The body-weight of all rats was measured before the 24 h deprivation. There was a significant effect in sex; males weighed more compared with females ($F_{(1,41)}=166.109$; $P<0.001$). There was no genotype or genotype×sex effect found in weight.

Home cage emergence task. The home cage emergence test was used as a last assay to assess the level of

anxiety in the SERT^{-/-} rats. An increase in anxiety results in an increased escape latency to leave the home cage (Prickaerts et al., 1996). As shown in Fig. 4, a significant genotype effect was found in the home cage emergence task, with SERT^{-/-} rats having a longer latency leaving their home cage than SERT^{+/+} rats ($F_{(1,44)}=18.025$; $P<0.001$). In addition, a significant sex effect ($F_{(1,44)}=14.912$; $P<0.001$) was found with females emerging faster from their home cage than males. However, as with the previous tests, we did not find a significant genotype×sex interaction.

Depression-related tests

Sucrose consumption. Loss of interest or pleasure in events that are usually enjoyed (anhedonia) is a core symptom of depression. In animal studies a decreased consumption of palatable solutions is used to measure anhedonia. This decreased consumption can be prevented by antidepressants (Willner et al., 1987; Muscat et al., 1992). Moreover, chronic exposure to mild unpredictable stress has been found to depress the consumption of palatable sweet solutions (Willner et al., 1987; Orsetti et al., 2007). The preference of sucrose above water and the intake in grams of a 100% sucrose solution per kg body-weight were measured in the sucrose consumption test as described separately below.

Sucrose preference. A significant genotype effect was observed for the sucrose preference (Fig. 5; $F_{(1,33)}=4.625$; $P<0.039$), with SERT^{-/-} rats drinking less sucrose

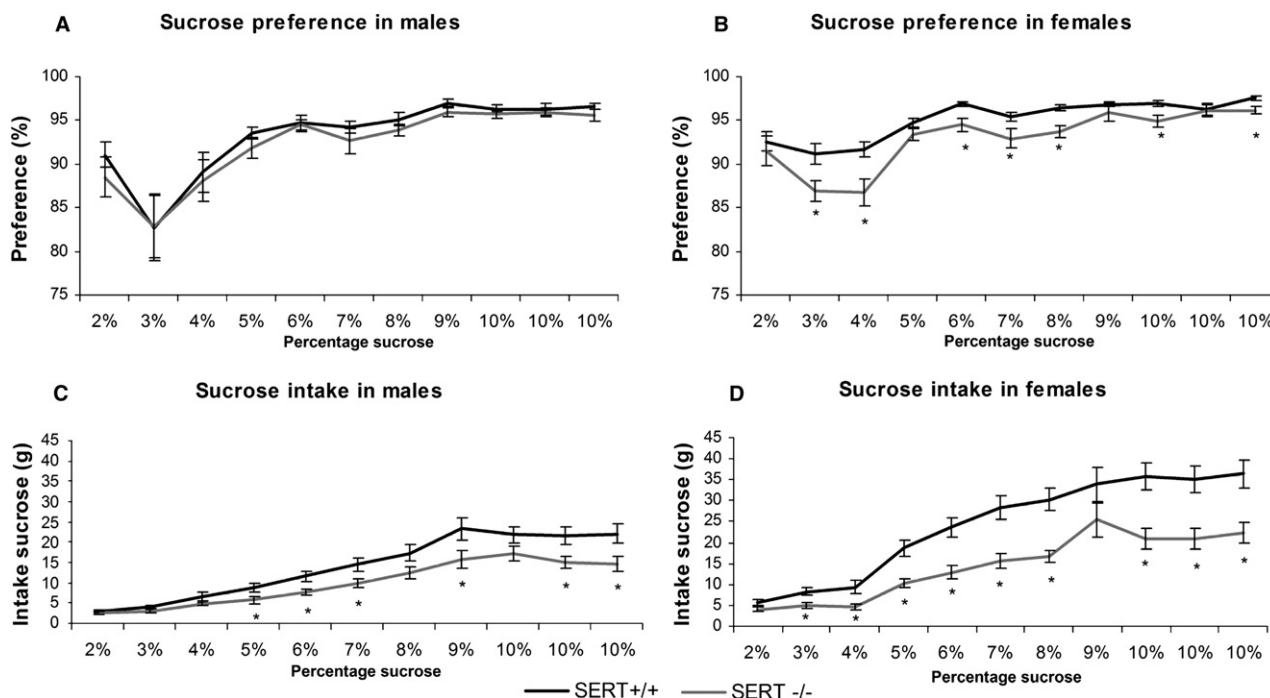


Fig. 5. Sucrose consumption test. In a free-choice two-bottle paradigm male and female SERT^{+/+} and SERT^{-/-} rats were allowed to consume increasing sucrose solutions (2–10%) on alternating days. Data are expressed as mean±S.E.M. sucrose preference (sucrose intake/total fluid intake×100%) in female (A) and male (B) rats, and as mean±S.E.M. total sucrose intake (g) in female (C) and male (D) rats (male: $n=12$ SERT^{+/+} and $n=12$ SERT^{-/-} rats; female: $n=12$ SERT^{+/+} and $n=11$ SERT^{-/-} rats; * $P<0.05$).

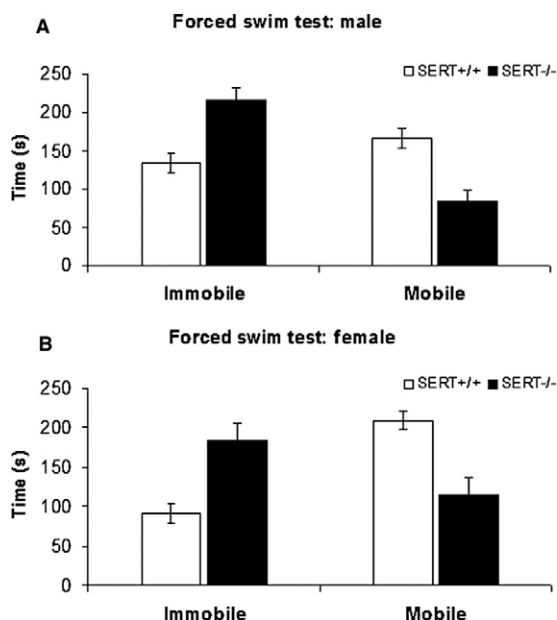


Fig. 6. Forced swim test. Behavior was scored for 5 min in male (A) and female (B) SERT^{+/+} and SERT^{-/-} rats on the 2nd day of the test, and expressed as mean \pm S.E.M. time spent (s) on mobility and immobility (male: $n=20$ SERT^{+/+} and $n=20$ SERT^{-/-} rats; female: $n=17$ SERT^{+/+} and $n=19$ SERT^{-/-} rats). Note: SERT^{-/-} rats were significantly less mobile [$F_{(1,72)}=22.461$; $P<0.001$] and more immobile [$F_{(1,72)}=22.521$; $P<0.001$] in the forced swim test compared with SERT^{+/+} rats.

solution compared with SERT^{+/+} rats. This effect was independent of the concentration, since no genotype \times concentration interaction was found. Although there was no significant sex effect, a significant sex \times concentration interaction was found ($F_{(10,330)}=2.411$; $P<0.009$). Females showed a higher preference to sucrose at lower concentration of sucrose (Fig. 5) compared with males. At higher concentration, the preference is similar in males and females. We did not find any significant genotype \times sex interactions.

Sucrose intake. As for sucrose preference, there was also a significant genotype effect for sucrose intake ($F_{(1,39)}=19.779$; $P<0.001$), the sucrose intake being significantly lower in SERT^{-/-} rats compared with SERT^{+/+} rats (Fig. 5). Moreover a genotype \times concentration interaction for sucrose intake was found ($F_{(10,390)}=6.703$; $P<0.001$). The sucrose intake was lower in SERT^{-/-} rats compared with SERT^{+/+} rats especially at higher concentration of sucrose (Fig. 5). In addition a significant sex effect ($F_{(1,39)}=21.845$; $P<0.001$) as well as a significant sex \times concentration interaction was found ($F_{(10,390)}=6.719$; $P<0.001$) with male rats taking less sucrose compared with female rats, especially at higher concentration of sucrose. However, we again did not observe any significant genotype \times sex interactions. These data reveal that SERT^{-/-} rats have a decreased consumption of sucrose compared with SERT^{+/+} rats, indicating a more depressive-like phenotype.

Forced swim test. When rats are forced to swim in an inescapable situation, they typically display an immobile posture, which is considered to reflect a state of behavioral despair. Antidepressant treatments are known to reduce immobility time in the forced swim test (Porsolt et al., 1977; Connor et al., 2000), while chronic mild stress and maternal separation increase immobility in the rat (Molina et al., 1994; Huang and Lin, 2006). Therefore increased immobility is considered as 'depression'-like behavior. A significant genotype effect was found in the forced swim test (Fig. 6A, B; $F_{(1,72)}=22.521$; $P<0.001$), with SERT^{-/-} rats spending significantly less time in a mobile state compared with SERT^{+/+} rats. Moreover SERT^{-/-} rats spent a longer time in the immobile phase than SERT^{+/+} rats ($F_{(1,72)}=22.461$; $P<0.001$). No significant sex or genotype \times sex interactions were found. The results of the forced swim test are therefore in line with the data on sucrose consumption and indicate that SERT^{-/-} rats have an enhanced level of depressive-like behavior compared with SERT^{+/+} rats, independent of the sex.

Microdialysis

We previously reported that the lack of SERT led to a ninefold higher level of extracellular 5-HT in male SERT^{-/-} rats (Homberg et al., 2007). To determine whether this is sex dependent we here investigated the 5-HT levels in male and female SERT^{-/-} rats. As shown in Fig. 7, 5-HT levels were sevenfold elevated in the SERT^{-/-} rats com-

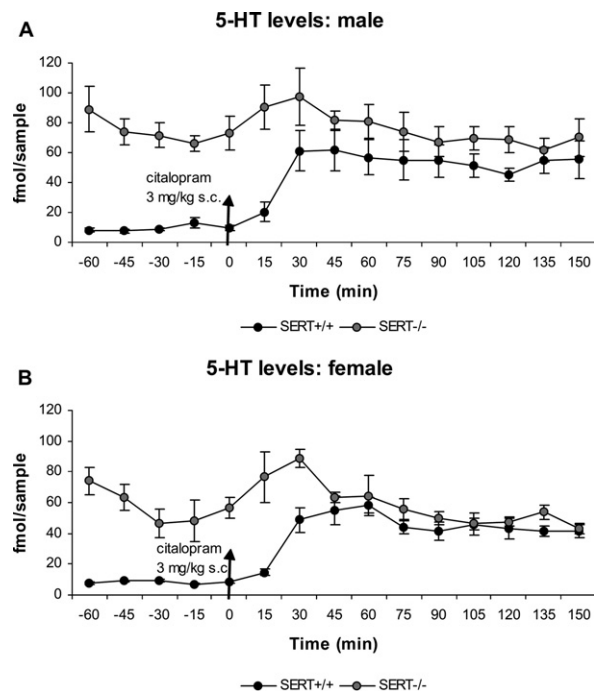


Fig. 7. Microdialysis. 5-HT levels were measured in the hippocampus under basal condition and after administration of citalopram in male (A) and female (B) SERT^{+/+} and SERT^{-/-} rats. Data are expressed as mean \pm S.E.M. fmol 5-HT per sample (male: $n=5$ SERT^{+/+} and $n=5$ SERT^{-/-} rats; female: $n=6$ SERT^{+/+} and $n=6$ SERT^{-/-} rats). Note: SERT^{-/-} have significantly higher basal 5-HT levels [$F_{(1,18)}=50.227$; $P<0.001$] compared with SERT^{+/+} rats.

pared with SERT^{+/+} rats ($F_{(1,18)}=50.227$; $P<0.001$). These differences were found for male (Fig. 7A) and female (Fig. 7B) rats. The percentage rise in 5-HT after citalopram treatment was significantly different between SERT^{+/+} and SERT^{-/-} rats ($F_{(1,17)}=19.593$; $P<0.001$), because the high basal levels of 5-HT in the SERT^{-/-} rats did not increase, whereas 5-HT levels in the SERT^{+/+} rats were significantly increased after citalopram administration. The difference in rise of 5-HT after citalopram administration was independent of the sex of the rat.

5-HT immunohistochemistry

5-HT neurons within the DRN project to forebrain systems involved in anxiety-related behavioral responses (Steinbusch, 1984; Maier et al., 1993; Graeff et al., 1996). We investigated the DRN of SERT^{-/-} and SERT^{+/+} rats to reveal possible neural differences. Serotonergic cell numbers in the DRN were quantified as shown in Fig. 8. The number of 5-HT immunopositive cells was not significantly different between SERT^{+/+} and SERT^{-/-} rats. Moreover there was not any significant sex or genotype \times sex effect.

DISCUSSION

In the present study, we analyzed anxiety- and depression-like behaviors in the SERT knockout rat model, with additional focus on possible sex-specific effects. The results showed that SERT^{-/-} rats consistently displayed increased levels of anxiety- and depression-like behaviors, independent of sex and independent of the specific test used.

Compared with SERT^{+/+} rats, SERT^{-/-} rats spent less time in the center part of the open field as well as on the open arm of the plus maze, suggestive of an enhanced level of anxiety in the SERT^{-/-} rats. In this respect, it is important to realize that the total distance moved in the open field did not differ between SERT^{+/+} and SERT^{-/-} rats, indicating that the observed differences are unlikely to be due to differences in exploratory drive per se. The novelty suppressed feeding paradigm, similar to the open field and elevated plus maze, can also be considered as a conflict paradigm, such that hunger becomes the primary drive above exploration (Bodnoff et al., 1988). In this paradigm an increased latency to start eating was found in the

SERT^{-/-} rats. This effect was only found in male rats. A latency difference was found between male SERT^{+/+} and female SERT^{+/+} rats (data not shown). This difference might have arisen by the difference in start body weight of the animals. Moreover the loss of body weight after 24 h was higher in SERT^{+/+} and in SERT^{-/-} females compared with SERT^{+/+} and in SERT^{-/-} males (data not shown). This may also have influenced the feeding behavior. Nevertheless the increased latency in male SERT^{-/-} rats indicates a higher level of anxiety compared with male SERT^{+/+} rats. Finally, the home cage emergence test was performed to measure anxiety-like behavior in the SERT^{-/-} rats. Again SERT^{-/-} rats showed higher levels of anxiety-like behavior since the latency for escaping from their home cage was higher compared with SERT^{+/+} rats. Taken together these results show that loss of SERT induces anxiety-like behavior in all tests conducted here.

Previous research showed that higher anxiety-like behaviors were also found in several SERT^{-/-} mouse models. Both male and female SERT^{-/-} mice with a 129S6/SvEv background did show an increased anxiety like effect in the novelty suppressed feeding test, but did not display an increased anxiety-like behavior in the open field and elevated plus maze (Lira et al., 2003). On the other hand, male and female SERT^{-/-} mice on a Swiss albino CD-1 strain showed reduced locomotor activity in a novel environment (Alexandre et al., 2006). Moreover, male and female SERT^{-/-} mice with a C57BL/6J background strain exhibited increased anxiety-like behavior in the open field, elevated plus maze, activity patterns and emergence test (Holmes, 2003; Holmes et al., 2003a; Zhao et al., 2006; Kalueff et al., 2007). However, opposite results exist in SERT^{-/-} mice on a C57BL/6J. For example, male SERT^{-/-} mice were only more anxious when they were exposed to predator odor exposure and not on basal levels in the EPM and light/dark box (Adamec et al., 2006). The SERT^{-/-} rats on a Wistar background showed a general increase in anxiety-like behavior, as evident in the open field, the elevated plus maze, the home cage emergence test, and the novelty suppressed feeding. Humans are genetically heterogenous, and in that respect the outbred Wistar background of SERT^{-/-} rats could be an advantage as opposed to inbred mouse strains. However, we did not test the effect of SERT^{-/-} in other rat strains, and as in mice, rat strain background may affect the phenotype.

SERT^{-/-} rats displayed alterations for all depression-like behaviors tested in this study. First SERT^{-/-} rats consumed less sucrose in the two-bottle paradigm compared with SERT^{+/+} rats, being indicative for anhedonia-like. And second, SERT^{-/-} rats showed increased immobility in the forced swim test compared with SERT^{+/+} rats. Taken together, these results indicate a higher 'depression'-like state in SERT^{-/-} rats.

In some SERT^{-/-} mice, depression-like behavior was also present. For example mice with a 129S6 background (male and female) showed an increased immobility in the forced swim test (Holmes, 2002; Lira et al., 2003), but SERT^{-/-} mice generated on a C57/6J genetic background only showed an increased immobility when they were re-

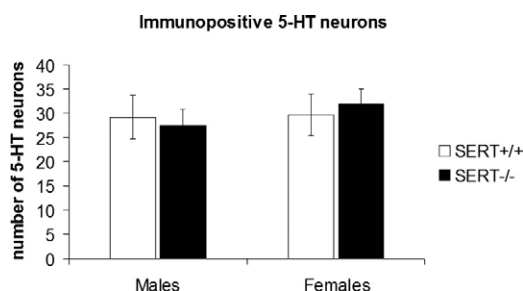


Fig. 8. 5-HT immunoreactivity in the DRN of male and female SERT^{+/+} and SERT^{-/-} rats. Data represent mean \pm S.E.M. number of 5-HT immunoreactive neurons (male $n=6$ SERT^{+/+} and $n=6$ SERT^{-/-} rats; female $n=6$ SERT^{+/+} and $n=6$ SERT^{-/-} rats).

peatedly exposed to the forced swim test (Wellman et al., 2007). In the tail suspension, a test used to measure depressive-like behavior in mice, the immobility of SERT^{-/-} mice on a Swiss albino CD-1 strain (Alexandre et al., 2006) and of SERT^{-/-} mice on a C57BL/6J background (Zhao et al., 2006) was increased. The sucrose consumption test was only performed in male SERT^{-/-} mice with a C57BL/6 background. These mice were not different from the SERT^{+/+} mice in this test (Kalueff et al., 2006), suggesting that genetic ablation of the SERT with this specific genetic background does not induce anhedonia, in line with the unaltered forced swim test performance found in these mice (Holmes, 2002). However, it does not rule out that SERT^{-/-} mice with other background do not show this behavior. Thus, SERT ablation leads to either predominantly anxiety-like behavior in mice (on a C57BL/6 background) or depression-like behavior (on a 129S6 background). In rats on a Wistar background, SERT ablation induces both anxiety and depression-like behavior, although it remains to be established whether this is a species or a strain dependent phenomenon. The similarities found between SERT^{-/-} rats and mice indicate that gene function is conserved among species and underlines the value that SERT^{-/-} animals represent an interesting model for the anxiety/depression co-morbidity state in humans.

Despite, the higher level of activity of females in the open field, elevated plus maze and home cage emergence task, no differences were found in vulnerability to the deletion of SERT between male and female rats. In fact, if anything, male rats showed a slightly increased vulnerability for the deletion, since the latency to initiate food consumption in the novelty suppressed feeding test was increased in male SERT^{-/-} rats, while in females there is no difference in latency between SERT^{-/-} and SERT^{+/+} rats. Thus, despite of basal 5-HT differences between males and females found before (Watts and Stanley, 1984; Carlsson and Carlsson, 1988; Haleem et al., 1990; Dominguez et al., 2003) our data show that lifelong absence of SERT in rats, leads to a sex-independent increase of anxiety- or depression-like behavior. This is in line with data from human studies showing an increased risk for both male and female individuals with the short version of the SERT gene promoter polymorphism to develop depression (Mann et al., 2000). No big differences were found between male and female SERT^{-/-} mice in anxiety and depression-like behavior (Holmes, 2002, 2003; Lira et al., 2003; Holmes et al., 2003a).

The increase in anxiety- and depression-like behavior is most likely due to alterations in serotonergic neurotransmission in SERT^{-/-} rats. As previously shown for male rats (Homborg et al., 2007) we found that besides males, also female SERT^{-/-} rats have increased 5-HT levels. Increased extracellular 5-HT is expected to cause excess activity at postsynaptic 5-HT receptors which, in turn, could underlie increased anxiety-like behaviors in SERT^{-/-} rats (Iversen, 1984; Graeff et al., 1996). Also in the SERT^{-/-} mice a fourfold to sixfold increase in basal levels of forebrain extracellular 5-HT has been found (Montanez et al.,

2003; Mathews et al., 2004). We can therefore conclude that a lifelong absence of the SERT in SERT^{-/-} rats causes alterations in the serotonergic neurotransmission, independent of the sex.

As a first attempt to explain the observed effects on anxiety and depression in our SERT^{-/-} rats, we studied the number of serotonergic cells in the DRN in SERT^{+/+} and SERT^{-/-} rats. The DRN contains the largest number of serotonergic cell bodies and hence is a crucial structure within the 5-HT network related to anxiety and depression. It contains over 50% of all 5-HT neurons projecting to the forebrain (Steinbusch, 1984). We used different ages for male and female rats, however a comparison between the groups is possible since aging has no effect on the number of serotonergic cells in the DRN (van Lujtelaar et al., 1992). Somewhat surprisingly, we found no significant differences in the number of immunopositive 5-HT neurons in the DRN between SERT^{-/-} and SERT^{+/+} rats, indicating that the absence of SERT has no consequences for the number of serotonergic cells. Obviously, this does not imply that the sensitivity of the 5-HT neurons may not be altered. It would be useful to further assess this for example by electrical recording of neural firing properties. In addition, we have found reduced levels of 5-HT and 5-HIAA tissue levels in SERT^{-/-} rats (Homborg et al., 2007), presumably reflecting an adaptive reduction of intracellular concentrations. In contrast to SERT^{-/-} rats, SERT^{-/-} mice on a 129S6 background showed an approximately 50% reduction in 5-HT neuron number in the DRN (Lira et al., 2003), indicating another clear difference between rats and mice. Since the SERT^{-/-} mice on a 129S6 background, in contrast to the SERT^{-/-} rats, did not display any anxiety like behavior in the elevated plus maze and open field, it is tempting to speculate that the adaptive mechanism at the level of the DRN may somehow protect the SERT^{-/-} mice. However, further research is needed to substantiate this suggestion. Nonetheless, it is important to realize that in postmortem studies of human individuals suffering from depression no reduction in the number of serotonergic neurons in the DRN was found (Rajkowska, 2000; Hendricksen et al., 2004), suggesting that the SERT^{-/-} rat represents a good animal model for affective disorders in human.

It is paradoxical that SSRIs are effective in the treatment of depression in adults, while neonatal SSRI treatment (Ansorge et al., 2004) or genetic inactivation of the SERT (present study) induces depression-like symptoms. In addition, down-regulation of the SERT with *in vivo* RNAi method (knockdown gene expression) in adult BALB/c mice, results into a reduction in immobility in the forced swim test (Thakker et al., 2005). A possible explanation for this paradox is that either underactivity or overactivity of a neurotransmitter system causes anxiety and mood disorders. This means that the relationship between 5-HT and 5-HT disorders operates according to an 'inverted-U' function, since high 5-HT levels in SERT^{-/-} rats and low 5-HT levels in human patients show similar symptoms (Calabrèse and Baldwin, 2001). Chamberlain et al. (2006) used this theory to explain their results in a cognition experiment

in which either underactivity or overactivity of the 5-HT system impaired cognition (Chamberlain et al., 2006). Alternatively, a more likely explanation is that the effects of low SERT gene function are present from conception on, also during critical periods of development. Several studies have shown that 5-HT is implicated during the development and organization of the CNS (Zhang, 2003). The ontogenic role of monoamines can have a huge impact on the appearance of normal emotional behavior (Meaney et al., 2000; Caspi et al., 2002). In SERT^{-/-} mice, it is already known that postnatal absence of the SERT gene can lead to disorganization of certain cortical regions (Persico et al., 2001; Salichon et al., 2001). Moreover, a greater spine density in the pyramidal neurons in the basolateral amygdala was found, and the length of apical dendritic branches of infralimbic cortex pyramidal neurons of SERT^{-/-} mice was increased compared with SERT^{+/+} mice (Wellman et al., 2007). In SERT^{-/-} rats it is likely that a similar disorganization, or perhaps more widespread neurodevelopment abnormalities, has taken place and it is important to further elucidate this in the near future.

CONCLUSION

In conclusion, SERT^{-/-} rats showed increased anxiety and depression-like behavior in a variety of tests. To understand how genetic reduction in SERT contributes to the vulnerability to develop mood or anxiety disorders later in life, more research is needed. Moreover, it is relevant to know if disorganization of brain regions, important for these disorders, takes place during a critical period of development. The SERT^{-/-} rat will be a valuable model for unraveling developmental origins of mood and anxiety disorders. Moreover, since SERT^{-/-} rats do not respond to SSRIs like citalopram, this animal model can be used to develop novel therapies especially useful for the relatively large group of patients that are not responding to current SSRI treatment.

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