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Impaired hypothalamic-pituitary-adrenal axis and its feedback regulation in serotonin transporter knockout mice

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Summary

Our previous studies have demonstrated that mice with reduced or absent serotonin transporter (SERT+/− and SERT−/− mice, respectively) are more sensitive to stress relative to their SERT normal littermates (SERT+/+ mice). The aim of the present study was to test the hypothesis that the hypothalamic-pituitary-adrenal (HPA) axis and its feedback regulation are impaired in these mice. The function and gene expression of several components in the HPA axis and its feedback regulation in SERT+/+, +/− and −/− mice were studied under basal (non-stressed) and stressed conditions. The results showed that 1) under basal conditions, corticotrophin-releasing factor (CRF) mRNA levels in the paraventricular nucleus (PVN) of the hypothalamus was lower in both SERT+/− and −/− mice relative to SERT+/+ mice; 2) an increased response to CRF challenge was found in SERT−/− mice, suggesting that the function of CRF type 1 receptors (CRF R1) in the pituitary is increased. Consistent with these findings, ^{125}I-sauvagine (a CRF receptor antagonist) binding revealed an increased density of CRF R1 in the pituitary of SERT −/− under basal conditions. These data suggest that CRF R1 in the pituitary of SERT−/− mice is up-regulated. However, in the pituitary of SERT+/−mice, the function of CRF R1 was not changed and the density of CRF R1 was reduced relative to SERT+/+ mice; and 3). The expression of the glucocorticoid receptor (GR) in the hypothalamus, pituitary and adrenal cortex was significantly reduced in SERT+/−and −/− mice in comparison with SERT+/+ mice under basal conditions. Consistent with these findings, the corticosterone response to dexamethasone was blunted in SERT−/−mice relative to SERT+/+ and +/− mice. Furthermore, stress induces a rapid increase of the GR expression in the hypothalamus of SERT +/− and −/− mice relative to their basal levels. Together, the present results demonstrated that the HPA axis and its feedback regulation are altered in SERT knockout mice, which could account for the increased sensitivity to stress in these mice.

Keywords

CRF mRNA; CRF type 1 receptors; glucocorticoid receptors; stress; dexamethasone; Hypothalamus; Pituitary

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Introduction

The serotonin (5-HT) transporter (SERT, 5-HTT) functions as a 5-HT reuptake site to take extracellular 5-HT back into the nerve terminals, resulting in termination of 5-HT receptor stimulation. Thus, the function of SERT is critical to control 5-HT activity, which plays an important role in regulation of emotions. SERT is not only a target for selective serotonin reuptake inhibitors (SSRIs), the most widely used antidepressants, but also may be a genetic component for the pathogenesis of affective disorders. Several polymorphisms and mutations have been found in the SERT promoter and coding regions (Murphy et al. 2004), such as a polymorphism in the 5-HT transporter-linked promoter region (5-HTTLPR) (Hu et al. 2005; Nakamura et al. 2000; Lesch et al. 1996; Hu et al. 2006). Studies have demonstrated that individuals carrying lower SERT expression genotypes are more sensitive to stress. Several recent studies revealed that the number of stressful life events correlates to the severity and number of episodes of major depression in the individuals carrying lower SERT expression genotypes (Caspi et al. 2003; Zalsman et al. 2006; Roy et al. 2007). These data raise the question as to why individuals with lower SERT expression genotypes are more vulnerable to stressful events. A recent study reported that psychological stress, such as public speech, significantly increases plasma ACTH and cortisol levels in the individuals with lower SERT expression genotypes (Jabbi et al. 2007). These data suggest that the sensitivity of HPA axis response to stress is increased in the individuals with lower SERT expression genotypes. Since increased activity of HPA axis is known to be involved in the pathophysiology of affective disorders, the increased sensitivity of HPA axis response to stress could be a trigger for the development of affective disorders in these individuals when they suffer stressful events. Therefore, studying the mechanisms by which the reduction in SERT function during development influences the sensitivity of HPA axis response to stress will provide significant insight for our understanding the etiology of affective disorders and thus, may lead to develop better approaches to prevent and treat affective disorders.

SERT knockout mice were generated by homologous recombination that replaced the second exon of SERT with a neo-cassette (Bengel et al. 1998). In these mice, SERT is constitutively absent (SERT−/−) or reduced (SERT +/−). In several aspects, the SERT knockout mice, especially SERT+/− mice, are similar to humans with lower SERT expression genotypes (Li 2006; Kalueff et al. 2007; Murphy and Lesch 2008). For example, both SERT (+/−) and humans with lower SERT expression genotypes have about a 50% reduction in SERT expression during early development relative to the SERT+/+ mice and individuals with high expression genotype of 5-HTTLPR, respectively. In addition, the SERT uptake rate is slightly reduced in both SERT +/− (vs. SERT+/+) mice and individuals with lower expression genotype of 5-HTTLPR (vs. high expression genotype) (Greenberg et al. 1999). Behavioral studies reveal that the SERT knockout mice are more anxious compared to SERT+/+ mice (Holmes et al. 2003d). Furthermore, SERT knockout mice (both SERT +/− and −/− mice) are more sensitive to stress. Mild stress, such as handling and saline injection, significantly increases ACTH secretion in SERT+/− and −/− mice (Li et al. 1999). These data suggest that disruption of SERT function early in life may alter the development of the HPA axis, resulting in increased sensitivity to stress.

To test the hypothesis that the HPA axis and its feedback regulation are altered in SERT knockout mice, in the present study, we characterized the components of the HPA axis and its feedback regulation in SERT knockout mice. The HPA axis is a major system responsible for maintaining homeostasis in response to stress. Corticotrophin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus is the initial point of the HPA axis-stress system. Stress activates the CRF neurons through activation of numerous neurotransmitters and consequently increases the CRF secretion through the medial eminence to the anterior pituitary. The CRF then activates CRF type 1 receptors (CRF R1) in the anterior pituitary and

stimulates ACTH release into the circulation. The ACTH activates the secretor cells in the zona fasciculata of the adrenal cortex to release glucocorticoid, corticosterone (in rodent) or cortisol (in human). The glucocorticoids trigger gene transcription and alter protein synthesis through activation of mineralocorticoid (MR) and glucocorticoid receptors (GR). Activation of MR and GR also produces a negative feedback regulation for the HPA axis, which serves as a major control of the HPA axis. GR is expressed in the hypothalamus, pituitary, adrenal cortex and also in the hippocampus, as an upstream control of the HPA axis. On the other hand, MR is mainly expressed in the hippocampus and is responsible for controlling physiological and mild stress-related variation of the HPA axis (Pace and Spencer 2005). In the present study, the function and expression of components of the HPA axis, such as CRF and CRF R1, and its feedback regulation, such as GR, were examined in the hypothalamus, pituitary and adrenal gland of SERT+/+, +/− and −/− mice under basal (non-stressed) and stressed conditions.

Methods

Animals

SERT knockout mice were purchased from Taconic Farm Inc. (Hudson, NY) and bred in the University of Texas Medical Branch animal facility. In SERT knockout mice, the second exon of the SERT gene was replaced by a neo-cassette (Bengel et al. 1998). The mice were backcrossed on a congenital C57/BL6 genetic background for 14 generations. SERT+/− mice were used for mating to minimize potential effects of differences in maternal care behaviors. The genotypes of the mice were identified using a PCR procedure. Mouse genomic DNA was extracted from tail tissue (Bengel et al. 1998). The PCR was conducted with 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dTTP, dGTP, 0.1mM dCTP and 0.1mM 7-dezae-dCTP (GE Amersham, Piscataway, NJ), 0.5 μM each primers and premium Taq polymerase (Invitrogen, Carlsbad, CA) at 95°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 2 min, and 72°C for 10 min. Two primers used in the PCR were upstream exon 2 primer (encoding 3′end of intron 1): 5′-TCACGGCTGCAGTGATAAGT-3′ and downstream primer (encoding 672-652 bp of exon 2): 5′-TGCTGACTGGAGATCAGGC-3′. The wild type of SERT produced a 1.4 kb band, while SERT knockout presented a 2 kb size band.

Male mice were used in the studies. They were two to five months of age, with body weights of 20–25 g. The mice were housed in groups of four to five per cage in a light-(12 hour light/dark cycle, lights on at 6 a.m.), humidity- and temperature-controlled room. Food and water were available *ad lib*. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Procedure for animal studies

Characterization of HPA axis and its feedback regulation in SERT knockout mice under non-stressed and stressed conditions—Each genotype of SERT+/+, +/− and −/−mice was randomly divided into non-stressed and stressed subgroups. To avoid unexpected stress, two days before the experiment, mice were moved to a behavior room with an elevated plus maze (EPM) apparatus. The mice were housed two per cage; one mouse in a non-stressed group and the other one in a stressed group were housed in the same cage. During the experiment, mice in the stressed group underwent stress by placement on an elevated plus maze, while the non-stressed cage-mate was immediately decapitated in an adjacent room. The elevated plus maze was conducted as previously described by Holmes at al (Holmes et al. 2000; Holmes et al. 2003a). Briefly, mice were individually placed on the center square of the EPM facing an open arm and allowed to freely explore the apparatus for 5 min. Then, the mice were decapitated immediately after the EPM exposure in an adjacent room. The brain, pituitary and adrenal gland were collected. To determine GR levels in the hypothalamus using an immunoblot assay, the hypothalamus were dissected and then, homogenized immediately to

isolate non-nuclear (cytosol and membrane) and nuclear fractions, as described below. The brains used for autoradiography were frozen immediately with dry ice-cold 2-methylbutane followed by dry ice. The pituitaries and adrenal glands were frozen on dry ice. The brain, pituitary and adrenal gland were wrapped with plastic wrap, parafilm and aluminum foil and stored in −80°C until use.

Systemic CRF challenge test for the function of peripheral CRF R1—Male mice (SERT+/+, +/− and −/−, n=5–7) were injected with saline or CRF (human and rat (h/rCRF), 20 μg/kg, 5ml/kg, sc, Sigma, St. Louis, MO). 30 min after the injection, the mice were decapitated and the trunk blood was collected for plasma ACTH and corticosterone assays. The determinations of the dose of CRF and the time point for decapitation were based on the results from our preliminary study (data not shown).

Systemic dexamethasone challenge test to determine the function of GR in the peripheral tissue—Two experiments were conducted. To establish the optimal time point of the dexamethasone treatment, the time course of the effect of dexamethasone on ACTH and corticosterone secretion was first examined. Male C57/B6 mice were injected with saline or dexamethasone (water soluble, 20 μg/5ml/kg, ip, Sigma, St. Louis, MO) at 9:00 am. The mice were decapitated 1, 2, 4 and 6 hours after the dexamethasone injection or 2 and 6 hours after the saline injection. The trunk blood was collected for the plasma ACTH and corticosterone concentrations.

To determine the dexamethasone response in SERT knockout mice, mice (SERT+/+, +/− and −/−, n=6–8) were injected with dexamethasone (20 μg/5ml/kg, ip) or saline. Two hours after the injection, mice were decapitated and plasma was collected for a corticosterone assay. We chose the 2-hour time point because it was approximately the $t_{1/2}$ for the dexamethasone-induced suppression of corticosterone as determined by the time course above (see results and Fig. 8). This time point is more sensitive for detection of the alterations in response to dexamethasone.

Quantitative reverse transcription PCR for CRF mRNA in the hypothalamus

To determine the level of CRF mRNA in the hypothalamus of SERT knockout mice, the hypothalamus of SERT +/+, +/− and −/− mice were dissected and rapidly frozen in liquid nitrogen. The total RNA was extracted from the hypothalamus using Tri-Reagent (Sigma, St. Louis, MO) following the manufacturer's protocol. Briefly, the hypothalamus was homogenized in 0.3 ml Tri-Reagent by a homogenizer at speed scale 4 and 4°C for 10 seconds. After centrifugation, the supernatant was mixed with chloroform and the top phase was collected after centrifugation at 16,000 g for 5 min. The RNA extract was then mixed with an equal volume of isopropanol and centrifuged at 16,000 g for 5 min. The pellet was washed with 75% ethanol and dissolved in 50 μl define DEPC-treated H_2O after the pellet was dried briefly.

The total RNA from the hypothalamus was treated with DNase I (Invitrogen, Carlsbad, CA) before conducting the reverse transcription (RT) with superscript reverse transcriptase (Invitrogen, Carlsbad, CA). Briefly, 1 μg DNase I treated RNA was mixed with 1 μl oligo d $(T)_{20}$ and 2 μl 10mM dNTP and incubated at 65°C for 5 min. Then 4 μl 5x supertranscript RT buffer, 2 μl 0.1M DTT, 1 μl RNaseOut and 1ml superscript II RT were added into the mixture (for a total volume of 20 μl). The RT reaction then carried out at 42°C for 50min followed by 15 min incubation at 75°C. To degrade the RNA, 1 μl RNase H (2 units) was then added and incubated at 37°C for 20 min. The cDNA solution was stored at −20°C until use for the quantitative PCR.

The level of hypothalamic CRF mRNA was examined by using SYBR green real-time polymerase chain reaction (RT-PCR) with 7500 fast Real-Time PCR system (Applied Biosystems, Foster City, CA). In total volume of 20 μl, 2 μl of cDNA solution, 10 μl of SYBR green PCR Master mix (Applied Biosystems, Foster City, CA) and 200 nM of each primers was used for each RT-PCR reaction in a 96 well plate. According to manufacturer's suggested conditions, the real-time PCR was conducted at 50°C for 2 min, 95°C for 10 min to activate Taq polymerase and 50 cycles of 95°C for 0.15 min and 60°C for 1 min. An 83 bp fragment of CRF mRNA was amplified with a forward primer (5′-CACCTACCAAGGGAGGAGAA-3′, 143–162 bp in Accession NM_205769) that is located in the exon I of CRF gene, and a reverse primer (5′-CAGAGCCACCAGCAGCAT-3′, 225-208 bp in Accession NM_205769) that is located in the exon II of the CRF gene. To normalize the cDNA concentration in each sample, a RT-PCR for TATA box binding protein mRNA, i.e., a housekeeping gene, was conduced in parallel with the CRF mRNA. The sequences of TBP primers (Forward: 5′-GGCCTCTCAGAAGCATCACTA, Reverse: 5′-GCCAAGCCCTGAGCATAA) were obtained from RTPrimerDB (Pattyn et al. 2006; Pattyn et al. 2003). The reason for using TBP for normalization is that the TBP mRNA level is comparable to CRF mRNA levels in the brain (Vandesompele et al. 2002). The relative levels of CRF mRNA were calculated using comparative C_T ($\Delta\Delta C_T$) method by 7500 fast SDS software in the Applied Biosystems 7500 RT-PCR systems. The TBP mRNA was used as a normalizer and a sample in SERT+/+ group with medial value of ΔC_T was used as a calibrator. The mean of the C_T from three real-time PCR reactions for each sample was used for the calculation. The RT-PCR for CRF mRNA is linear within a three-log range of RNA concentrations. The coefficient variation of the real-time PCR is < 1%.

Autoradiography of receptor binding and In situ hybridization

Tissue preparation—Frozen brain, pituitary and adrenal gland were cut into 15 μm-thick coronal sections using a cryostat. Sections containing the hypothalamus [Bregma (−0.7) – (−1.0) mm] (Paxinos and Franklin 2001) were thaw-mounted on the slides and stored at −80° C until use. Pituitaries or adrenal glands from each group of mice were embed in one cryostat chuck and sectioned together, so that each section contained pituitary or adrenal gland slices of all the animals in the group. To limit the variation between the slides, each slide contained the brain sections from three mice (one per each genotype) or the pituitary and adrenal gland sections from the three groups (one per each genotype).

^{125}I-sauvagine binding for the density of CRF type1 receptors in the pituitary— Autoradiography of ^{125}I-sauvagine binding for the density of CRF R1 in the pituitary was conducted as described by Rominger et al with slight modification (Rominger et al. 1998). Briefly, pituitary sections were pre-incubated in pre-assay buffer (50 mM Tris-HCI, pH 7.4, containing 2 mM EDTA, 10 mM $MgCl_2$) for 30 min at room temperature. Then, the slides were incubated with 20 pM ^{125}I-sauvagine (2200 Ci/mmonl, Perkin Elmer, MA) in assay buffer (50 mM Tris-HCI, pH 7.4, containing 2 mM EDTA, 10 mM $MgCl_2$, 0.1% BSA and 0.25 TIU/ml aprotinin) for 2 hours at room temperature, in the presence of 20 nM antisauvagine-30 (a CRF R2 antagonist) to block CRF type2 receptors. Non-specific binding was defined by 1μM NBI 27914 (a CRF R1 antagonist, Sigma, St Louis, MO). The slides were washed 2 × 20min in 50 mM Tris-HCl (pH 7.4) buffer at 4°C, rapidly rinsed with ice-cold ddH_2O and dried. The slides were exposed on Kodak Biomax MR film at −80°C for 10 days. A set of ^{125}I microscales (Amersham, IL) was exposed with the slides for calibration of the optic density into nCi/mg tissue equivalent. The autoradiography of ^{125}I-sauvagine binding was analyzed using the MCID program (Research Image Inc. Ontario, Canada). Specific binding sites for each group of animals were calculated by subtracting the mean of non-specific binding sections from the mean of total binding sections in the group (S/N≥3).

In situ hybridization for mRNAs of CRF, CRF R1, and GR—The in situ hybridization procedures were carried out as previously described (Li et al. 2000). To generate probes, a cDNA fragment was amplified using PCR with specific primers as listed in Table 1. The PCR product was subcloned into a TOPO pCR II vector (Invetrogen, Carlsbad, CA). The generation of ^{35}S-UTP labeled ribo-probes and in situ hybridization were conduced as previously described (Li et al. 2000). The autoradiography of *in situ* hybridization was analyzed using the MCID program (Research Image Inc. Ontario, Canada). The mRNA level in each group of mice was calculated by subtracting the mean of sense binding from the mean of antisense binding.

Radioimmunoassay (RIA) for ACTH and corticosterone

RIAs for plasma ACTH and corticosterone were performed as previously described (Li et al. 1999). With the exception of the dexamethasone challenge test, 20 μl and 3 μl plasma were used for ACTH and corticosterone assays, respectively. In the dexamethasone challenge study, due to the reduction of hormone concentrations by dexamethasone treatment, 50 μl and 15 μl plasma were used in ACTH and corticosterone assays, respectively.

Immunoblot for GR in the nuclear and non-nuclear fraction of the hypothalamus

The nuclear and non-nuclear (cytosol and membrane) extractions of the hypothalamus were prepared according to the manufacturer's instructions for a nuclear extraction kit (Active motif Inc., Carlsbad, CA). Briefly, the hypothalamus was homogenized in 0.1 ml hypotonic buffer with a plastic pestle. After incubation in ice for 15 min and then centrifugation at 850 x g, 4° C for 10 min, the supernatant (cytosol) was collected and the pellet was resuspended in 0.1 ml hypotonic buffer. After incubating at 4°C for 15 min, 5 μl detergent was added to each vial and then vortexed for 10 sec at the highest setting. After centrifugation at 14,000 x g, 4°C for 30 sec, the supernatant (containing membrane proteins) was collected and combined with the previous supernatant (as non-nuclear extract). The pellet (nuclei) was then resuspended into 50 μl complete lysis buffer and incubated, vortexed and centrifuged as recommended by the manufacture's instruction. The supernatant was collected as nuclear extract. Both extracts were aliquotted and stored at −80°C freezer until use. The protein concentrations of the extractions were measured using a BCA protein assay kit (Pierce biotechnology Inc. Rockford, IL)

The GR levels in the nuclear and non-nuclear extraction of the hypothalamus (GR) were measured by using immunoblot as previously described with minor modification (Li et al. 2000). Briefly, after the proteins in the nuclear and non-nuclear extractions (~25μg protein/lane) were resolved by SDS polyacrylamide gel electrophoresis and the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, they were incubated with GR (1:200 dilution) monoclonal antibody (Affinity BioReagent Inc, Golden, CO) at 4°C overnight. After washes, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:10,000, Tropix, Bedford, MA) and reacted with chemiluminescence substrate solution, CDP-Star subtract (Tropix, Bedford, MA) as previously described (Li et al. 2000). The chemiluminescent bands in the membranes were captured by a Chemidoc XRS image system (Bio-Rad, Richmond, CA) and the density of the bands was analyzed using an image analysis program (Quantity One, Bio-Rad, Richmond, CA). Each blot contained 2–4 samples from each group of mice (SERT+/+, +/− and −/− mice with or without stress).

The samples were randomly arranged in the blot. The relative GR concentration of each sample was calculated as percent of the mean density of the samples from non-stressed SERT+/+ mice in the same blot. The data point for each mouse sample was the mean of the values on determined from three separate blots.

Statistical Analysis

The data were presented as mean ± SEM of 5–7 mice for autoradiographic and immunoblot studies and 8–10 mice for RIAs, unless mentioned in the experimental design section. The data were analyzed by two-way analysis of variance (ANOVA). In order to compare the difference between groups, a one way ANOVA was conduced followed by a Student Newman-Keuls post-hoc test. In the dexamethasone test, due to the time points differences between saline and dexamethasone treatments, a one-way ANOVA followed by a Student-Newman-Keuls post-hoc test was used to compare the difference among the time points within each treatment. A t-test was used for comparing the difference between saline and dexamethasone treatments 2 hours after the injection. All of the statistical analysis was conducted using StatView software (Abacus Concepts Inc, Berkeley, CA).

Results

Characterization of the HPA axis in SERT knockout mice

Alterations in CRF mRNA in the PVN—Using *in situ* hybridization, we detected high density of CRF mRNA in the PVN of of mice (Fig. 1A). The CRF mRNA level in the PVN of SERT+/− and −/− mice was significantly reduced in comparison to SERT +/+ mice under non-stressed (basal) conditions (Fig. 1B; One-Way ANOVA: $F_{(2,8)}$=14.57, P<0.01). The reductions of CRF mRNA in SERT +/− and −/− were 30% and 43%, respectively. To confirm the results, we further conduced a quantitative RT-PCR in the hypothalamus of SERT +/− and −/− mice. Consistently, the results from the real-time RT-PCR reveal that the CRF mRNA levels were reduced in the hypothalamus of SERT +/− and −/− mice (Figure 1C; 47% and 49% reduction for SERT +/− and −/− mice, respectively. One-Way ANOVA: $F_{(2, 9)}$ = 5.78, P = 0.024).

Alterations in CRF R1 receptors in the pituitary—To determine the function of CRF R1 in SERT knockout mice, a systemic CRF challenge study was conducted. Consistent with our previous observations, saline injection significantly increased plasma ACTH concentration in both SERT+/− and −/−mice (Fig. 2 top panel), suggesting an increased sensitivity to stress. ACTH responses to CRF were increased in a genotype-dependent manner in SERT knockout mice. The CRF-induced increase in ACTH secretion was significantly higher in SERT−/− mice than that in SERT+/+mice (Fig. 2 top panel, Two-way ANOVA: main effect of genotype, $F_{(2, 27)}$ = 5.98, P = 0.01; main effect of CRF, $F_{(1, 27)}$ = 40.58, P < 0.001; and interaction between genotype and CRF, $F_{(2, 27)}$ = 1.39, P = 0.27). An increased corticosterone concentration was observed in SERT−/− mice 30 min after saline injection (Fig 2 bottom panel). Administration of CRF increased plasma corticosterone concentrations in SERT +/+ and +/− mice, but did not further increase corticosterone levels in SERT −/− mice. The CRF-induced increase of corticosterone concentration was not significantly different among three genotypes of the mice (Fig. 2 bottom panel, Two-way ANOVA: main effect of genotype, $F_{(2, 33)}$ = 9.01, P<0.001; main effect of CRF, $F_{(1, 33)}$ = 26.5, P<0.001; and interaction between genotype and CRF, $F_{(2, 33)}$ = 5.39, P < 0.01).

To determine whether the increased ACTH response to CRF is due to an increase in the binding sites of CRF R1, the density of CRF R1 in the pituitary was examined using ^{125}I-sauvagine binding. As shown in fig. 3, the density of CRF R1 in the pituitary was differentially altered in SERT +/− and −/− mice. Under non-stressed conditions, the density of CRF R1 was reduced in SERT+/− mice, but increased in SERT−/− mice relative to SERT+/+ mice. Stress with EPM for 5 min significantly increased the density of CRF R1 in the pituitary of all three genotypes of mice (Fig. 3B, Two-way ANOVA: main effect of genotype, $F_{(2, 133)}$ = 26.808, P<0.001; main effect of stress, $F_{(1,133)}$ = 37.044, P<0.001; and interaction between genotype and stress, $F_{(2, 133)}$ = 0.214, P=0.807).

To determine whether the changes in the binding sites of CRF R1 are due to alterations in the expression of CRF R1, the CRF R1 mRNA in the pituitary of SERT knockout mice was examined by in situ hybridization. As shown in Fig 4A, although there is visible binding of the sense probe (non-specific binding), the density of the antisense probe binding was significantly higher than that of sense probe (S/N ≈ 2; 78 ±1.9 (antisense) vs. 39 ±1.2 (sense) nCi/g tissue in non-stressed SERT+/+ mice). Therefore, the difference between the antisense and sense is sufficient to represent the specific binding for CRF R1 mRNA. Although a trend of genotype-dependent reduction in CRF R1 mRNA levels was observed in the pituitary, there were no statistically significant differences among SERT +/+, +/− and −/− mice under non-stressed conditions. Stress with EPM for 5 min did not alter CRF R1 mRNA in the pituitary of SERT+/+ and −/− mice, but significantly decreased CRF R1 mRNA in SERT+/− mice. The CRF R1 mRNA in both SERT+/− and −/− mice were significantly lower than SERT +/+ mice under stressed conditions (Fig. 4B, Two-way ANOVA: main effect of genotype, $F_{(2, 62)}$ =7.92, P<0.001; main effect of stress, $F_{(1, 62)}$ =10.351, P< 0.05; and interaction between genotype and stress, $F_{(2, 62)}$ = 0.66, P = 0.52).

Alterations in the feedback regulation of the HPA axis

The activity of the HPA axis is controlled by glucocorticoid receptors (GR). GRs are nuclear receptors. Before binding to ligand, the Grs are located in the cytoplasm. When activated by corticosterone, these receptors move to the nucleus and alter the gene transcription. Therefore, the amount of GR in the cytoplasm represents the basal level of the receptors, whereas the GR in the nucleus indicate the activated receptors. In order to distinguish the basal and activated receptors, we examined the GR in the nuclear and non-nuclear fractions of the hypothalamic extracts.

GR in the hypothalamus—As shown in Fig. 5A, the GR monoclonal antibody detected a ~90 Kd band in both fractions of the hypothalamus. Under non-stressed conditions, the GR levels in both non-nuclear and nuclear fractions of the hypothalamus were significantly lower in SERT+/− and −/− mice than that in SERT+/+ mice (Fig. 5B). The nuclear GR level in the hypothalamus of SERT+/− mice was increased to normal levels after stress with EPM for 5 min. This stress-induced increase in GR level was not observed in SERT−/− mice (Two-way ANOVA for non-nuclear fraction: main effect of genotype, $F_{(2, 24)}$ = 12.75, P<0.001; main effect of stress, $F_{(1, 24)}$ = 3.45, P = 0.076; and interaction between genotype and stress, $F_{(2, 24)}$ = 0.396, P = 0.676. Two-way ANOVA for nuclear fraction: main effect of genotype, $F_{(2, 26)}$ = 10.212, P<0.001; main effect of stress, $F_{(1, 26)}$ = 13.595, P < 0.05; and interaction between genotype and stress, $F_{(2, 26)}$ = 6.403, P<0.05).

GR mRNA in the hypothalamus—To determine whether the changes in the GR protein level are due to the alterations in expression of GR, GR mRNA levels in the hypothalamus were examined using in situ hybridization. As shown in Fig 6A, high levels of GR mRNA were detected in the PVN. In comparison to SERT+/+ mice, GR mRNA in the PVN of the hypothalamus is reduced in the SERT+/− and −/− mice under non-stressed conditions (Fig. 6B). Stress for 5 min did not significantly alter the levels of GR mRNA in the PVN of any genotype of the mice relative to those under the non-stressed conditions (Fig. 6B). Two-way ANOVA for the GR mRNA in the PVN: main effect of genotype, $F_{(2, 24)}$ = 10.9, P<0.001; main effect of stress, $F_{(1, 24)}$ = 3.1, P = 0.09; and interaction between genotype and stress, $F_{(2, 24)}$ = 1.1, P=0.33.

GR mRNA in the pituitary and adrenal cortex—To determine whether GR expression was altered in the pituitary and adrenal cortex, GR mRNA levels were determined using *in situ* hybridization. The GR mRNA was distributed in the anterior pituitary and adrenal cortex (Fig. 7A). In comparison to the pituitary, a relatively high sense probe binding (non-specific

binding) was observed in the adrenal gland. Since the concentration of the probe and other assay conditions were identical for both tissues, the high non-specific binding in the adrenal cortex may be due to different tissue structures between the pituitary and adrenal cortex. However, the density of antisense probe binding was significantly higher than that of sense probe. The signal to noise ratio is about 2 in non-stressed SERT+/+ mice (324.8 ± 20.7 vs 154.6 ± 10.3). Therefore, the difference between the antisense and sense is sufficient to represent the specific binding for CRF R1 mRNA.

Under non-stressed conditions, the GR mRNA levels were significantly reduced in the pituitary of SERT+/− and −/− mice relative to SERT+/+ mice. Stress with EPM for 5 min significantly reduced pituitary GR mRNA in SERT+/+ mice (Fig 7B). Although there was a slight but significant reduction of GR mRNA in SERT−/− mice after stress, the stress-induced reduction of GR mRNA was not observed in SERT+/− mice (two-way ANOVA: main effect of genotype, $F_{(2, 137)} = 2.027$, $P = 0.136$; main effect of stress, $F_{(1, 137)} = 21.47$, $P<0.001$; and interaction between genotype and stress, $F_{(2, 137)} = 14.113$, $P < 0.001$).

In the non-stressed mice, the GR mRNA levels in the adrenal cortex were significantly reduced in SERT +/− and −/− mice relative to SERT+/+ mice (Fig. 7C). The reduction was in a genotype-dependent manner. The level of GR mRNA in the SERT−/−mice was only about one fourth of that in SERT+/+ mice. Stress with EPM for 5 min significantly reduced GR mRNA in SERT+/+ and +/− mice, but not in SERT−/− mice (two-way ANOVA: main effect of genotype, $F_{(2, 107)} = 33.952$, $P<0.001$; main effect of stress, $F_{(1, 107)} = 46.63$, $P<0.001$; and interaction between genotype and stress, $F_{(2, 107)} = 13.905$, $P<0.001$).

Systemic dexamethasone challenge study to examine peripheral GR negative feedback regulation of the HPA axis—To determine the optimal time point for the actions of dexamethasone, a time course of effect of dexamethasone on suppression of ACTH and corticosterone secretion was conducted in normal C57/B6 male mice. As shown in Fig. 8, the basal corticosterone levels (saline injected mice) were significantly altered between 11:00am – 3:00pm o'clock (2–6 hours after the injection, one-way ANOVA: $F_{(1,10)}=8.26$, $P < 0.05$). Dexamethasone reduced ACTH secretion one hour after the injection. The peak effect of dexamethasone on ACTH secretion was observed 2 hours after the injection. The ACTH concentration returned to normal four hours after the injection (Fig. 8, One-way ANOVA: $F_{(3,20)}=5.13$, $P < 0.05$). Corticosterone concentrations were reduced two hours after the injection and peaked 4 hours after the injection of dexamethasone. The corticosterone concentration returned to near normal 6 hours after the dexamethasone injection (Fig. 8, One-way ANOVA: $F_{(3,20)}=9.84$, $P < 0.05$). Therefore, we chose two hours as the time point for the dexamethasone challenge test in SERT knockout mice.

Consistent with our previous observation (Li et al. 1999), the basal corticosterone levels in SERT knockout mice were reduced in a genotype-dependent manner (Fig. 9). The dexamethasone injection (20 μg/kg, ip) significantly reduced plasma corticosterone concentrations in SERT +/+ and +/− mice comparing to the saline-treated mice. However, the dexamethasone-induced suppression of corticosterone secretion was blunted in SERT−/− mice (Fig. 9, two-way ANOVA: main effect of genotype, $F_{(2, 25)} = 2.598$, $P=0.0944$; main effect of dexamethasone, $F_{(1, 25)} = 27.713$, $P<0.001$; and interaction between genotype and stress, $F_{(2, 25)} = 7.545$, $P < 0.05$).

Discussion

In the present studies, we determined the function and expression of several components of the HPA axis and its negative feedback regulation in SERT knockout mice under basal and stressed conditions. As summarized in Table 2, our results demonstrated that the HPA axis in

SERT +/− and −/− mice is impaired. The overall basal activity of the HPA axis in the SERT knockout mice was reduced, as demonstrated by the decreased CRF expression (Fig 1) and low basal plasma corticosterone concentrations (Li et al. 1999;Lanfumey et al. 2000) (Fig 9). The reduction of CRF may induce an up-regulation of CRF type 1 receptor (CRF R1), resulting in an increase in the function and the density of CRF R1 in the pituitary of SERT −/− mice. However, the reduced CRF expression did not increase, but indeed, reduced CRF R1 in the pituitary of SERT+/− mice. These data suggest that the CRF R1 is differentially regulated between SERT+/− and −/− mice. Under stressed conditions, the HPA axis in SERT +/−and −/− mice is super-activated, consistent with our previous reports that minor stress, such as saline injection, produces a significant increase of ACTH release in SERT +/−and −/− mice (Fig 2) (Li et al. 2004;Li et al. 1999). The increased sensitivity to stress may be due to a reduction in the glucocorticoid receptor-mediated feedback regulation. GR expression was significantly reduced in all three organs of the HPA axis (the hypothalamus, pituitary and adrenal cortex) of SERT+/− and −/− mice. These results support our hypothesis that the HPA axis and its feedback regulation are impaired in SERT +/− and −/− mice, which could account for the increased sensitivity to stress in these mice.

An elevated plus maze (EPM) was used as a stressor in the present study. We have previously found that minor stress, such as handling and saline injection, increases ACTH secretion in SERT+/− and −/− mice relative to SERT+/+ mice (Li et al. 1999). However, handling and saline injection are not standard stressors. Tjurmina et al did not observe the differences in ACTH response to restraint stress between the SERT knockout mice and their SERT normal littermates (Tjurmina et al. 2004), which could be due to the fact that restraint stress produced a maximal ACTH response in all three genotypes. Therefore, it is necessary to find a stressor that can distinguish the stress response between SERT+/+ mice and SERT knockout mice. EPM is a test for anxiety-like behavior, which tests the fear of the height and novelty (Rodgers et al. 1997). Thus, EPM is a psychological stressor, a “stimuli which threaten the individual’s current or anticipated state” (Dayas et al. 2001). We have found that the ACTH response to EPM was more extensive in SERT+/− and −/− mice than that in SERT+/+ mice (Li 2006). Therefore, EPM is an ideal stressor for the studies on increased sensitivity to stress in SERT knockout mice. Since the anxiety-like behaviors of SERT knockout mice examined by EPM have been reported by Holmes et al (Holmes et al. 2003b; Holmes et al. 2003c), we only used EPM as a stressor in the present study.

Our present results showed that under non-stressed conditions CRF mRNA levels in the PVN of SERT+/− and −/− mice were significantly lower than that of SERT+/+ mice. The data suggest that the increased sensitivity of HPA axis to stress is not due to increase in the CRF expression, but may result from the increase in the CRF release. However, further studies are required to determine the mechanisms underlying the reduction of CRF mRNA in basal conditions. One possible explanation is that the reduction of CRF mRNA in the PVN of SERT knockout mice could be an adaptive change resulting from increased sensitivity of the HPA axis to stress. Studies have found that chronic stress reduced CRF mRNA levels in the PVN (Lightman 2008). Although activation of GR is the major inhibitor for the activity of the HPA axis, the reduced GR expression in the SERT+/− and −/− mice (Fig. 6 and 7) suggests that other transcription factors may be more important in suppression of CRF expression. This is consistent with evidence that manipulating corticosterone levels does not affect the kinetics of stress-induced alterations in the phosphorylation of the transcription factor cAMP-response element-binding protein (CREB) in CRF neurons in 5 –15 min after stress, which is paralleled with increases in the CRF heteronuclear RNA (hnRNA) (Kovacs and Sawchenko 1996; Kovacs et al. 2000). An alternative mechanism could be that the reduction in the CRF mRNA expression in SERT knockout mice is mediated by epigenetic regulation during early development of these animals. It will be interesting to study the mechanisms underlying the regulation of CRF expression in the PVN of SERT knockout mice.

Studies have found that the resting level of corticosterone in SERT−/− mice is reduced, whereas the resting level of ACTH remains normal (Li et al. 1999; Lanfumey et al. 2000). Consistent with these results, we observed a significant reduction in the corticosterone concentration 2 hours after a saline injection in SERT −/− mice (i.e resting level, Fig 9). On the other hand, as a results of an increase in the sensitivity to stress, minor stressors, such as handling and saline injection, increased HPA axis activity in SERT knockout mice (Li et al. 1999). The increased sensitivity of the HPA axis to stress could account for the increased corticosterone levels 30 minutes after the saline injection in SERT−/− mice (Fig. 2). Interestingly, in SERT+/− mice, minor stress (such as a saline injection) induces a significant increase in the ACTH response, whereas the increase in the corticosterone responses to stress is not as remarkable as the ACTH responses (fig.2). These observations suggest dissociation in ACTH-induced increase of corticosterone secretion in SERT knockout mice. Dissociation between ACTH and corticosterone secretions has been reported in both physiological and pathological conditions, which could be due to the dysregulation of adrenal cortex (Ahrens et al. 2008; Bornstein et al. 2008). For example, children of low-income women with maternal depressive symptoms have a lower basal cortisol level and a blunted stress response (Fernald et al. 2008). Depressive symptoms in elderly are associated to hypo- or hyper-cortisol levels (Penninx et al. 2007). Therefore, the dissociation between ACTH and corticosterone secretions in SERT knockout mice could be related to the increased sensitivity of the HPA axis to stress and altered behaviors. However, the mechanism underlying the dissociation is still unknown.

The present results demonstrate that the basal GR expression in all three organs of the HPA axis system are reduced in SERT+/− and −/− mice relative to SERT +/+ mice. The reductions of GR in the hypothalamus are to a similar levels in SERT+/− and −/−mice. However, in the pituitary and adrenal cortex, the reduction of GR mRNA is more extensive in SERT−/− mice than that in SERT+/− mice. Differences between SERT+/−and −/− mice are further confirmed by the dexamethasone challenge test. Dexamethasone is a GR agonist and does not cross blood brain barrier. Also, GR in the adrenal cortex has the most direct effect on the inhibition of corticosterone secretion. Therefore, the effect of dexamethasone on the reduction of corticosterone secretion mainly measures the function of GR in the adrenal cortex. In the present study, we observed a blunted corticosterone response to dexamethasone in SERT−/− mice, but an almost normal response in SERT+/− mice. Considering about 30–40% reduction of the GR mRNA in the adrenal cortex of SERT+/− mice, these results suggest that in the adrenal cortex and pituitary there is receptor reserve. It is interesting that the alterations in the HPA axis observed in the SERT knockout mice are similar with those in transgenic mice that express GR antisense and have a 50% reduction of GR (Dijkstra et al. 1998). Both transgenic mice and SERT knockout mice showed decreased basal CRF mRNA and basal corticosterone levels (Dijkstra et al. 1998; Li et al. 1999; Lanfumey et al. 2000). This similarity suggests that the alterations in the HPA axis in SERT knockout mice could result from the reduction of GR expression. However, it does not explain why the decrease of the GR expression produces a reduction in the basal level of the HPA axis. Besides GR in the hypothalamus, pituitary and adrenal cortex, two corticoid receptors, MR and GR, in the hippocampus also play an important role in regulating the HPA axis activity. However, since the present manuscript is focused on alterations in the HPA axis, we will not further discuss the possible impact of these receptors on alterations of the HPA axis.

In the hypothalamus, basal levels of both GR mRNA and protein are significantly reduced in SERT+/− and −/− mice (Fig 5 and 6) relative to SERT+/+ mice, suggesting that the reduction of GR in the PVN of SERT knockout mice may be mediated by an alteration at the transcriptional level. However, the mechanisms underlying the reduction of the GR expression in SERT knockout mice are still unknown. Evidence has demonstrated that the transcription of GR can be epigenetically programmed by early environment (Szyf et al. 2005;Weaver et al. 2004;Weaver et al. 2002;Weaver et al. 2005;Weaver et al. 2007). For example, alterations in

the maternal care can alter the methylation status of GR promoter and change GR expression in adulthood (Weaver et al. 2004). One can expect that increased 5-HT concentrations during early life of SERT knockout mice may cause epigenetic alterations that affect GR expression later in life. Currently, no data is available regarding the regulation of GR expression in the PVN of mice. It will be interesting to investigate the mechanisms that mediate the reduction of GR expression in the hypothalamus of SERT knockout mice.

As a commonly accepted concept, transcriptional regulation in the brain usually takes hours. Most studies observe alterations in mRNA levels more than 30 minutes after initiation of stress. No data are available regarding transcriptional regulation of the HPA axis elements immediately after 5 min of stress. As expected, our results showed that stress with EPM for 5 minutes did not alter the levels of GR mRNA in the PVN of SERT+/+ mice (Fig 6). However, the GR mRNAs levels in the pituitary and adrenal cortex were altered by stress for only 5 min in SERT +/+ mice (Fig. 7). Similarly, after stress for 5 min, expression of CRF R1 is increased in the pituitary and adrenal cortex of SERT knockout mice (Fig 4). Since we have carefully controlled animal housing and experimental conditions, it is unlikely that the alterations observed were due to stimulation by additional stressors. Because the stress-induced alterations mainly occurred in the pituitary and adrenal cortex, these data suggest that the regulation of GR and CRF R1 expression in the pituitary and adrenal cortex may be more sensitive than that in the brain. Currently, little data is available regarding the regulation of GR and CRF R1 expression in the pituitary and adrenal cortex during acute stress within 30 minutes. However, several studies demonstrated that the CRF R1 in the pituitary is more sensitive to CRF or corticosterone injections or longer stress (Aguilera et al. 1997;Aguilera et al. 2001;Nikodemova et al. 2002;Rabadan-Diehl et al. 1996;Rabadan-Diehl et al. 1997;Luo et al. 1995). For example, a transient reduction of CRF R1 mRNA in the pituitary was detected 2 hours after injection with dexamethasone (Luo et al. 1995). Consistent with this finding, we have also observed that stress with EPM for 5 minutes produced a reduction of CRF R1 mRNA in the pituitary of SERT knockout mice. Thus, it is possible that stress-induced decreases in CRF R1 expression in the pituitary are a transient response induced by the increased CRF secretion. However, future studies are required to understand the rapid alteration of gene-expression in the pituitary and adrenal gland.

Several behavioral, cellular and molecular alterations have been observed in SERT −/− mice, such as increased anxiety-like behaviors, reduced aggression, reduced 5-HT_{1A} receptors and increased 5-HT_{2C} receptors (Holmes et al. 2003c; Murphy et al. 1999; Li 2006). However, most of these alterations were not statistically significant between SERT+/+ and SERT+/− mice. The most remarkable change in SERT +/− mice is the increase in sensitivity to stress. This is specifically interesting because it is similar to human individuals with lower SERT expression genotypes, who have anxiety-related personality traits and increased sensitivity to stress. In the present studies, we demonstrated that the alterations of the CRF and GR in the PVN of SERT +/− mice are similar with that in SERT −/− mice, which is consistent with the increased sensitivity to stress in both SERT+/− and −/− mice. However, in the pituitary and adrenal cortex, the alterations in the SERT +/− mice are not always similar with that in the SERT−/− mice. For example, the density of CRF R1 in the pituitary is increased in the SERT −/− mice, but reduced in SERT +/− mice (Fig. 3). This is consistent with the results observed from CRF challenge study, in which SERT−/−, but not SERT+/−, mice showed an increased response to CRF injection (Fig. 2). On the other hand, although the expression of GR was reduced in the pituitary and adrenal cortex of both SERT+/− and −/− mice, the SERT+/− mice showed a normal response to dexamathasone challenge (Fig. 9). Furthermore, the basal level of plasma corticosterone is reduced in SERT−/− mice, but not in SERT+/− mice. These data suggest that the impairment of the HPA axis in SERT+/− and −/− mice may not be mediated by similar mechanisms. Since the phenotypes of SERT+/− mice are closer to humans with lower SERT expression genotype, studying in the mechanisms mediating the increased HPA

axis sensitivity to stress in SERT+/− mice will provide an important insight for us to understanding the etiology of affective disorders and developing better therapeutic approaches for these diseases.

In summary, the present studies demonstrate, for the first time, that the HPA axis and its feedback regulation are impaired in SERT +/− and −/− mice, which may account for the increased sensitivity to stress in these mice. These data are important because they provide insight for the hypersensitivity to stress observed in human individuals with lower SERT expression genotype. The alterations of the HPA axis and its feedback regulation in SERT knockout mice could occur during development because 5-HT functions as a neurotrophic factor during development. One can expect that alterations in extracellular 5-HT concentrations will cause numerous changes in the neuronal development. Another possible cause for alterations in the HPA axis is that SERT knockout mice are more anxious due to the alterations of 5-HT receptors (Holmes et al. 2003d). Long-term stress could cause changes in the HPA axis in these mice. Understanding these mechanisms will have an important impact on understanding the etiology of affective disorders, especially for the individuals with lower SERT expression genotypes or individuals whose SERT function is altered during development due to other circumstances, such as administration of selective serotonin reuptake inhibitors (SSRIs) during pregnancy and maternal stress.

Reference List

Aguilera G, Jessop DS, Harbuz MS, Kiss A, Lightman SL. Differential regulation of hypothalamic pituitary corticotropin releasing hormone receptors during development of adjuvant-induced arthritis in the rat. J Endocrinol 1997;153:185–191. [PubMed: 9166107]

Aguilera G, Rabadan-Diehl C, Nikodemova M. Regulation of pituitary corticotropin releasing hormone receptors. Peptides 2001;22:769–774. [PubMed: 11337090]

Ahrens T, Deuschle M, Krumm B, Van Der PG, den Boer JA, Lederbogen F. Pituitary-Adrenal and Sympathetic Nervous System Responses to Stress in Women Remitted From Recurrent Major Depression. Psychosom Med. 2008

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, et al. Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymethamphetamine (“ecstasy”) in serotonin transporter-deficient mice. Molecular Pharmacology 1998;53:649–655. [PubMed: 9547354]

Bornstein SR, Engeland WC, Ehrhart-Bornstein M, Herman JP. Dissociation of ACTH and glucocorticoids. Trends Endocrinol Metab. 2008

Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, et al. Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. Science 2003;301:386–389. [PubMed: 12869766]

Dayas CV, Buller KM, Crane JW, Xu Y, Day TA. Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. Eur J Neurosci 2001;14:1143–1152. [PubMed: 11683906]

Dijkstra I, Tilders FJH, Aguilera G, Kiss A, Rabadan-Diehl C, Barden N, et al. Reduced activity of hypothalamic corticotropin-releasing hormone neurons in transgenic mice with impaired glucocorticoid receptor function. Journal of Neuroscience 1998;18:3909–3918. [PubMed: 9570818]

Fernald LC, Burke HM, Gunnar MR. Salivary cortisol levels in children of low-income women with high depressive symptomatology. Dev Psychopathol 2008;20:423–436. [PubMed: 18423087]

Greenberg BD, Tolliver TJ, Huang SJ, Li Q, Bengel D, Murphy DL. Genetic variation in the serotonin transporter promoter region affects serotonin uptake in human blood platelets. American Journal Of Medical Genetics 1999;88:83–87. [PubMed: 10050973]

Herman JP, Schafer MK, THOMPSON RC, Watson SJ. Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. Mol Endocrinol 1992;6:1061–1069. [PubMed: 1324419]

Holmes A, Kinney JW, Wrenn CC, Li Q, Yang RJ, Ma L, et al. Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze. Neuropsychopharmacology 2003a;28:1031–1044. [PubMed: 12700679]

Holmes A, Li Q, Murphy DL, Gold E, Crawley JN. Abnormal anxiety-related behaviour in serotonin transporter null mutant mice: the influence of genetic background. Genes Brain and Behavior 2003b; 2:365–380.

Holmes A, Murphy DL, Crawley JN. Abnormal behavioral phenotypes of serotonin transporter knockout mice: Parallels with human anxiety and depression. Biological Psychiatry 2003c;54:953–959. [PubMed: 14625137]

Holmes A, Parmigiani S, Ferrari PF, Palanza P, Rodgers RJ. Behavioral profile of wild mice in the elevated plus-maze test for anxiety. Physiology & Behavior 2000;71:509–516. [PubMed: 11239669]

Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL. Mice lacking the serotonin transporter exhibit 5-HT1A receptor-mediated abnormalities in tests for anxiety-like behavior. Neuropsychopharmacology 2003d;28:2077–2088. [PubMed: 12968128]

Hu X, Oroszi G, Chun J, Smith TL, Goldman D, Schuckit MA. An expanded evaluation of the relationship of four alleles to the level of response to alcohol and the alcoholism risk. Alcohol Clin Exp Res 2005;29:8–16. [PubMed: 15654286]

Hu XZ, Lipsky RH, Zhu G, Akhtar LA, Taubman J, Greenberg BD, et al. Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder. Am J Hum Genet 2006;78:815–826. [PubMed: 16642437]

Jabbi M, Korf J, Kema IP, Hartman C, Van Der PG, Minderaa RB, et al. Convergent genetic modulation of the endocrine stress response involves polymorphic variations of 5-HTT, COMT and MAOA. Mol Psychiatry 2007;12:483–490. [PubMed: 17453062]

Kalueff AV, Ren-Patterson RF, Murphy DL. The developing use of heterozygous mutant mouse models in brain monoamine transporter research. Trends Pharmacol Sci 2007;28:122–127. [PubMed: 17275930]

Kovacs KJ, Foldes A, Sawchenko PE. Glucocorticoid negative feedback selectively targets vasopressin transcription in parvocellular neurosecretory neurons. J Neurosci 2000;20:3843–3852. [PubMed: 10804224]

Kovacs KJ, Sawchenko PE. Sequence of stress-induced alterations in indices of synaptic and transcriptional activation in parvocellular neurosecretory neurons. J Neurosci 1996;16:262–273. [PubMed: 8613792]

Lanfumey L, La Cour CM, Froger N, Hamon M. 5-HT-HPA interactions in two models of transgenic mice relevant to major depression. Neurochemical Research 2000;25:1199–1206. [PubMed: 11059794]

Lesch KP, Bengel D, Heils A, Sabol SZ, Greenberg BD, Petri S, et al. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 1996;274:1527–1531. [PubMed: 8929413]

Li Q. Cellular and molecular alterations in mice with deficient and reduced serotonin transporters. Mol Neurobiol 2006;34:51–66. [PubMed: 17003521]

Li Q, Holmes A, Ma L, Van de Kar LD, Garcia F, Murphy DL. Medial hypothalamic 5-hydroxytryptamine (5-HT)1A receptors regulate neuroendocrine responses to stress and exploratory locomotor activity: application of recombinant adenovirus containing 5-HT1A sequences. J Neurosci 2004;24:10868–10877. [PubMed: 15574737]

Li Q, Wichems C, Heils A, Lesch KP, Murphy DL. Reduction in the density and expression, but not G-protein coupling, of serotonin receptors (5-HT1A) in 5-HT transporter knock-out mice: Gender and brain region differences. Journal of Neuroscience 2000;20:7888–7895. [PubMed: 11050108]

Li Q, Wichems C, Heils A, Van de Kar LD, Lesch KP, Murphy DL. Reduction of 5-Hydroxytryptamine (5-HT)(1A)-Mediated Temperature and Neuroendocrine Responses and 5-HT(1A) Binding Sites in 5-HT Transporter Knockout Mice. J Pharmacol Exp Ther 1999;291:999–1007. [PubMed: 10565817]

Lightman SL. The neuroendocrinology of stress: a never ending story. J Neuroendocrinol 2008;20:880–884. [PubMed: 18601712]

Luo X, Kiss A, Rabadan-Diehl C, Aguilera G. Regulation of hypothalamic and pituitary corticotropin-releasing hormone receptor messenger ribonucleic acid by adrenalectomy and glucocorticoids. Endocrinology 1995;136:3877–3883. [PubMed: 7649095]

Ma XM, Camacho C, Aguilera G. Regulation of corticotropin-releasing hormone (CRH) transcription and CRH mRNA stability by glucocorticoids. Cell Mol Neurobiol 2001;21:465–475. [PubMed: 11860185]

Murphy DL, Lerner A, Rudnick G, Lesch KP. Serotonin transporter: Gene, genetic disorders, and pharmacogenetics. Molecular Interventions 2004;4:109–123. [PubMed: 15087484]

Murphy DL, Lesch KP. Targeting the murine serotonin transporter: insights into human neurobiology. Nat Rev Neurosci 2008;9:85–96. [PubMed: 18209729]

Murphy DL, Wichems C, Li Q, Heils A. Molecular manipulations as tools for enhancing our understanding of 5-HT neurotransmission. Trends In Pharmacological Sciences 1999;20:246–252. [PubMed: 10366867]

Nakamura M, Ueno S, Sano A, Tanabe H. The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. Mol Psychiatry 2000;5:32–38. [PubMed: 10673766]

Nikodemova M, Diehl CR, Aguilera G. Multiple sites of control of type-1 corticotropin releasing hormone receptor levels in the pituitary. Arch Physiol Biochem 2002;110:123–128. [PubMed: 11935409]

Pace TW, Spencer RL. Disruption of mineralocorticoid receptor function increases corticosterone responding to a mild, but not moderate, psychological stressor. Am J Physiol Endocrinol Metab 2005;288:E1082–E1088. [PubMed: 15671079]

Pattyn F, Robbrecht P, De PA, Speleman F, Vandesompele J. RTPrimerDB: the real-time PCR primer and probe database, major update 2006. Nucleic Acids Res 2006;34:D684–D688. [PubMed: 16381959]

Pattyn F, Speleman F, De PA, Vandesompele J. RTPrimerDB: the real-time PCR primer and probe database. Nucleic Acids Res 2003;31:122–123. [PubMed: 12519963]

Paxinos, G.; Franklin, KBJ. The mouse brain in stereotaxic coordinates. Academic Press Inc; 2001.

Penninx BW, Beekman AT, Bandinelli S, Corsi AM, Bremmer M, Hoogendijk WJ, et al. Late-life depressive symptoms are associated with both hyperactivity and hypoactivity of the hypothalamo-pituitary-adrenal axis. Am J Geriatr Psychiatry 2007;15:522–529. [PubMed: 17545451]

Rabadan-Diehl C, Kiss A, Camacho C, Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptor in the pituitary during stress. Endocrinology 1996;137:3808–3814. [PubMed: 8756551]

Rabadan-Diehl C, Makara G, Kiss A, Zelena D, Aguilera G. Regulation of pituitary corticotropin releasing hormone (CRH) receptor mRNA and CRH binding during adrenalectomy: role of glucocorticoids and hypothalamic factors. J Neuroendocrinol 1997;9:689–697. [PubMed: 9355036]

Rodgers RJ, Cao BJ, Dalvi A, Holmes A. Animal models of anxiety: An ethological perspective. Brazilian Journal Of Medical And Biological Research 1997;30:289–304. [PubMed: 9246227]

Rominger DH, Rominger CM, Fitzgerald LW, GRZANNA R, Largent BL, Zaczek R. Characterization of [125I]sauvagine binding to CRH2 receptors: membrane homogenate and autoradiographic studies. J Pharmacol Exp Ther 1998;286:459–468. [PubMed: 9655891]

Roy A, Hu XZ, Janal MN, Goldman D. Interaction between childhood trauma and serotonin transporter gene variation in suicide. Neuropsychopharmacology 2007;32:2046–2052. [PubMed: 17356577]

Szyf M, Weaver IC, Champagne FA, Diorio J, Meaney MJ. Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. Front Neuroendocrinol 2005;26:139–162. [PubMed: 16303171]

Tjurmina OA, Armando I, Saavedra JM, Li Q, Murphy DL. Life-long serotonin reuptake deficiency results in complex alterations in adrenomedullary responses to stress. Ann N Y Acad Sci 2004;1018:99–104. [PubMed: 15240357]

Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:RESEARCH0034. [PubMed: 12184808]

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nat Neurosci 2004;7:847–854. [PubMed: 15220929]

Weaver IC, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ, et al. Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 2005;25:11045–11054. [PubMed: 16306417]

Weaver IC, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S, et al. The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. J Neurosci 2007;27:1756–1768. [PubMed: 17301183]

Weaver IC, Szyf M, Meaney MJ. From maternal care to gene expression: DNA methylation and the maternal programming of stress responses. Endocr Res 2002;28:699. [PubMed: 12530685]

Zalsman G, Huang YY, Oquendo MA, Burke AK, Hu XZ, Brent DA, et al. Association of a triallelic serotonin transporter gene promoter region (5-HTTLPR) polymorphism with stressful life events and severity of depression. Am J Psychiatry 2006;163:1588–1593. [PubMed: 16946185]

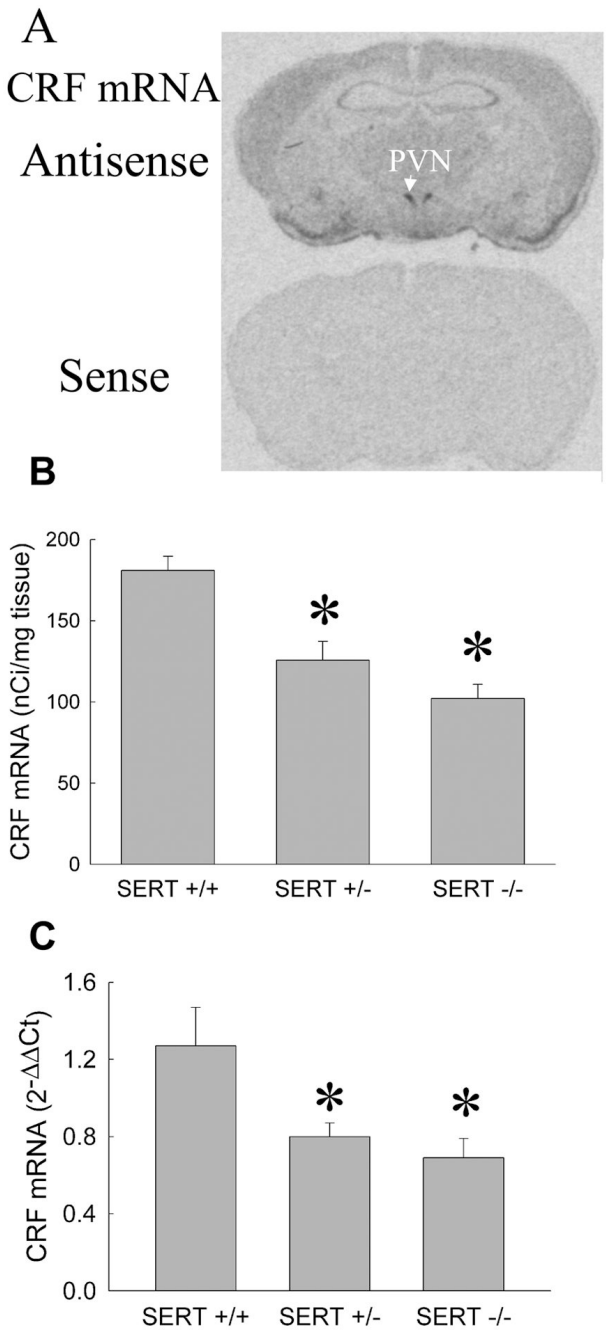

Fig. 1. Reduction of the CRF mRNA in the PVN of SERT knockout mice under non-stressed condition

A: Example of autoradiography of the CRF mRNA *in situ* hybridization in the paraventricular nucleus (PVN); B: Reduction of the CRF mRNA in the PVN of SERT knockout mice examined by *in situ* hybridization. C: Reduction of the CRF mRNA in the hypothalamus of SERT knockout mice determined by quantitative RT-PCR. The data were represented as mean ± SEM (n = 4–5 mice). * Significantly different from SERT+/+ mice, $P<0.05$ (Student-Newman-Keuls post hoc test).

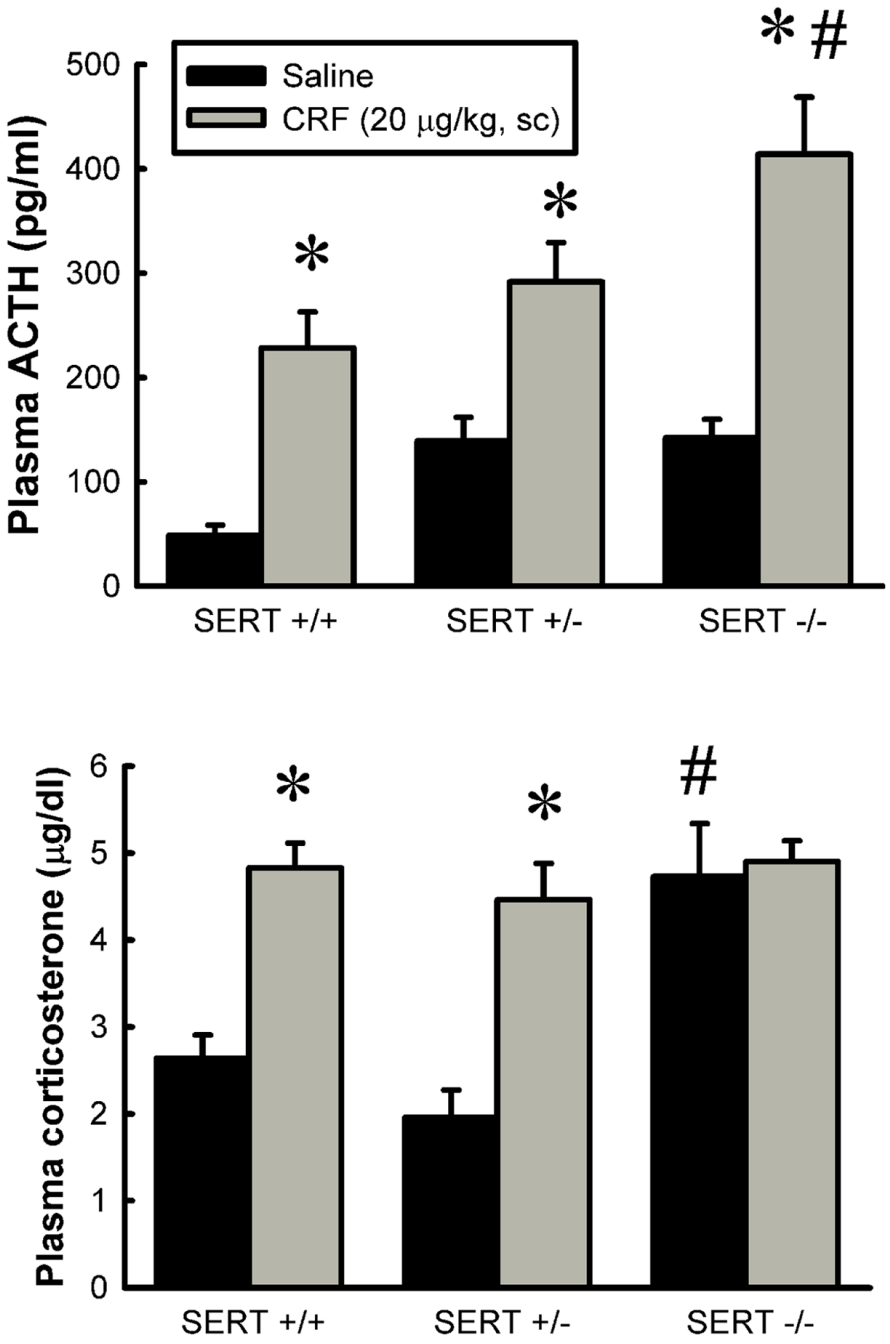

Fig. 2. Hormonal responses to systemic injection of the CRF in SERT knockout mice

SERT knockout mice were injected with CRF (20μg/kg, sc) or saline 30 min before decapitation. The data were represented as mean ± SEM (n = 5–7 mice). Top panel: ACTH response to CRF; Bottom panel: Corticosterone response to CRF. *: Significantly different from saline group of same genotype mice, $P<0.05$. #: significantly different from SERT+/+ mice with same stress treatment $P<0.05$ (Student-Newman-Keuls post hoc test).

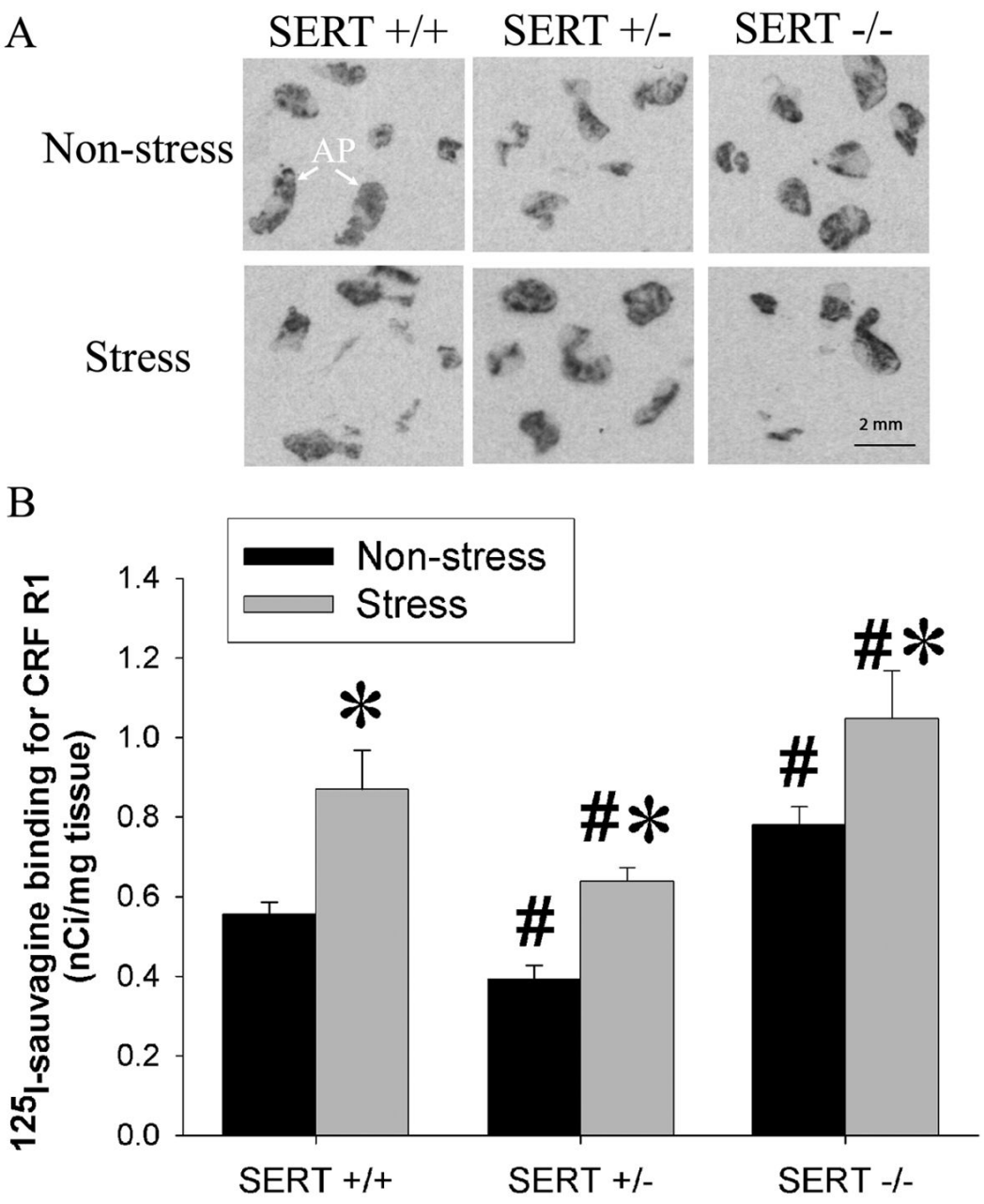

Fig. 3.

^{125}I-sauvagine binding for the density of CRF R1 in pituitary of SERT knockout mice.

A: Example of ^{125}I-sauvagine binding in the pituitary. Arrows indicate the anterior pituitary regions (AP); B: Alteration in the density of CRF R1 in the pituitary of SERT knockout mice. The data were represented as mean ± SEM (n = 5–7, mice). * Significantly different from basal level of same genotype mice $P<0.05$. #: significantly different from SERT+/+ mice with same stress treatment, $P<0.05$ (Student-Newman-Keuls post hoc test).

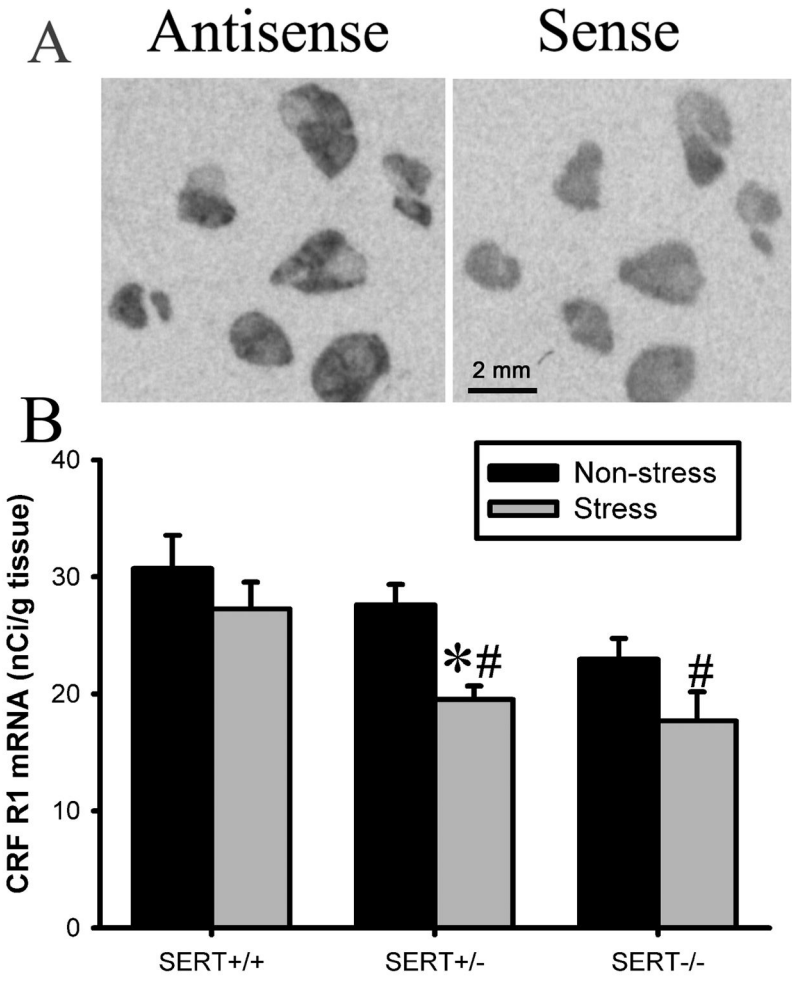

Fig. 4. CRF R1 mRNA in the pituitary of SERT knockout mice

A: Example of autoradiography of CRF R1 mRNA *in situ* hybridization in the pituitary. Each picture contains seven pituitaries. The regions measured were same as shown in Fig. 3; B: Alteration of the CRF R1 mRNA in the pituitary of SERT knockout mice during basal and stressed conditions. The data was represented as mean ± SEM (n=5–7, mice). * Significantly different from basal level of same genotype mice, $P<0.05$. #: significantly different from SERT +/+ mice with same stress treatment $P<0.05$ (Student-Newman-Keuls post hoc test).

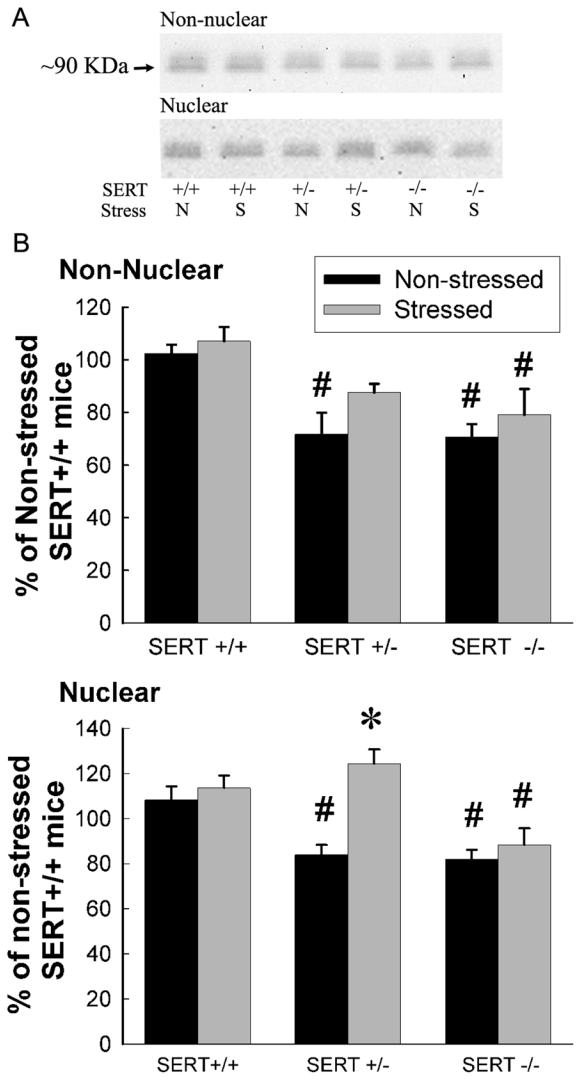

Fig.5. Alteration of the GR level in the hypothalamus of SERT knockout mice under non-stressed and stressed conditions

A: Example of immunoblot of the GR in the hypothalamic subcellular extractions. N: Non-stressed; S: Stressed; B: The GR protein levels in the non-nuclear (top, cytosol/membrane) and nuclear (bottom) fractions of the hypothalamus of SERT knockout mice were examined using immunoblots. The data were calculated as percent of non-stressed SERT+/+ mice and presented as mean ± SEM (n = 5–6, mice). * Significantly different from non-stressed mice with same genotype, $P<0.05$. #: significantly different from SERT+/+ mice with same stress treatment, $P<0.05$ (Student-Newman-Keuls post hoc test).

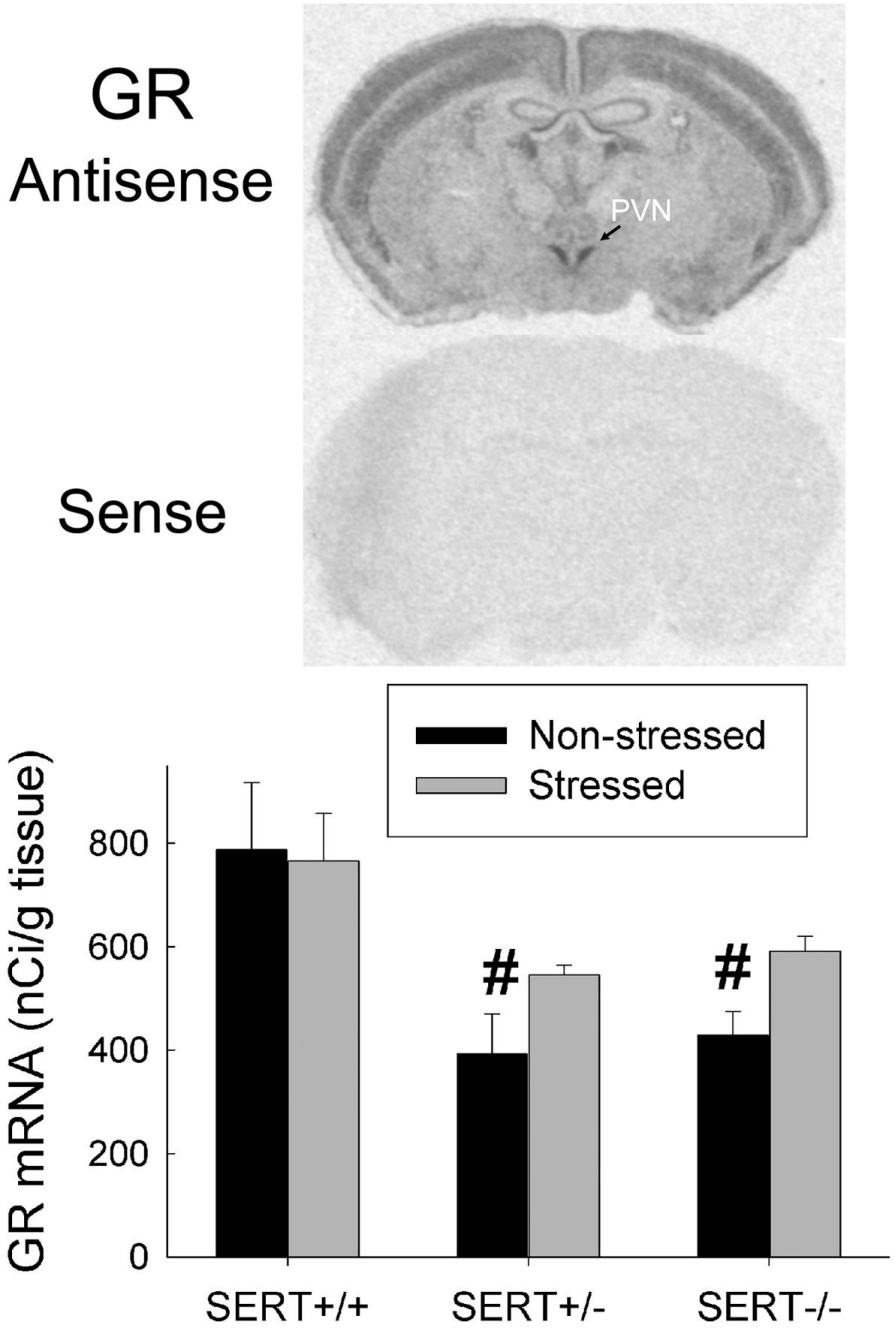

Fig 6. Alterations of GR mRNA in the PVN of SERT knockout mice under non-stressed and stressed conditions

A: Example of autoradiography of in situ hybridization for GR mRNA in the PVN; B: Alteration in the GR mRNA in the PVN of SERT knockout mice. The data were represented as mean ± SEM (n = 5–6). #: significantly different from SERT+/+ mice with same stress treatment $P<0.05$ (Student-Newman-Keuls post hoc test).

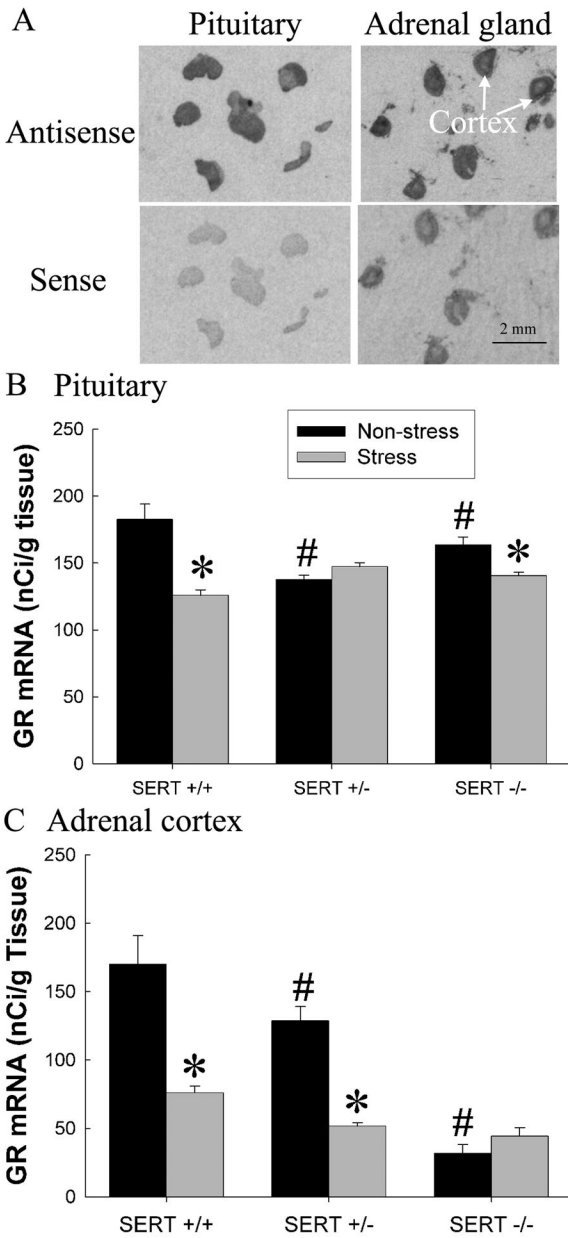

Fig 7. Alterations of GR mRNA in the pituitary and adrenal cortex of SERT knockout mice under non-stressed and stressed conditions

A: Example of autoradiography of in situ hybridization for GR mRNA in the pituitary and adrenal cortex. Arrow indicates the adrenal cortex. Each picture contained six pituitaries or adrenal glands. B: Alteration in the GR mRNA in the pituitary of SERT knockout mice. C: Alteration in the GR mRNA in the adrenal cortex of SERT knockout mice. The data were represented as mean ± SEM (n = 5–7). * Significantly different from non-stressed mice with same genotype, $P<0.05$. #: significantly different from SERT+/+ mice with same stress treatment, $P<0.05$ (Student-Newman-Keuls post hoc test).

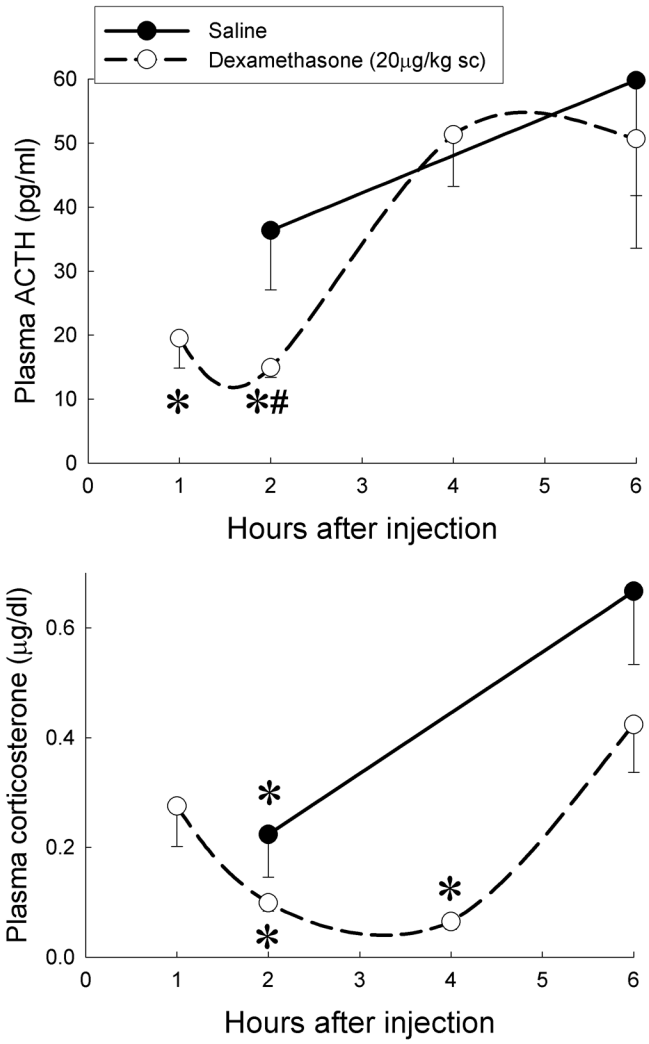

Fig 8. Time course of the effect of dexamethasone on ACTH and corticosterone

Mice (C57/B6) were injected with dexamethasone (20 μg/kg, ip) at 9:00am and discapited 1, 2, 4 and 6 hours after the injection. Trunk blood was collected for determination of plasma ACTH and corticosterone levels. The data were represent as mean ± SEM (n = 6–7 mice). *: Significant difference from 6 hours after same injection, $P<0.05$. #: Significant difference from saline injected group at same time-point, $P<0.05$ (Student-Newman-Keuls post hoc test).

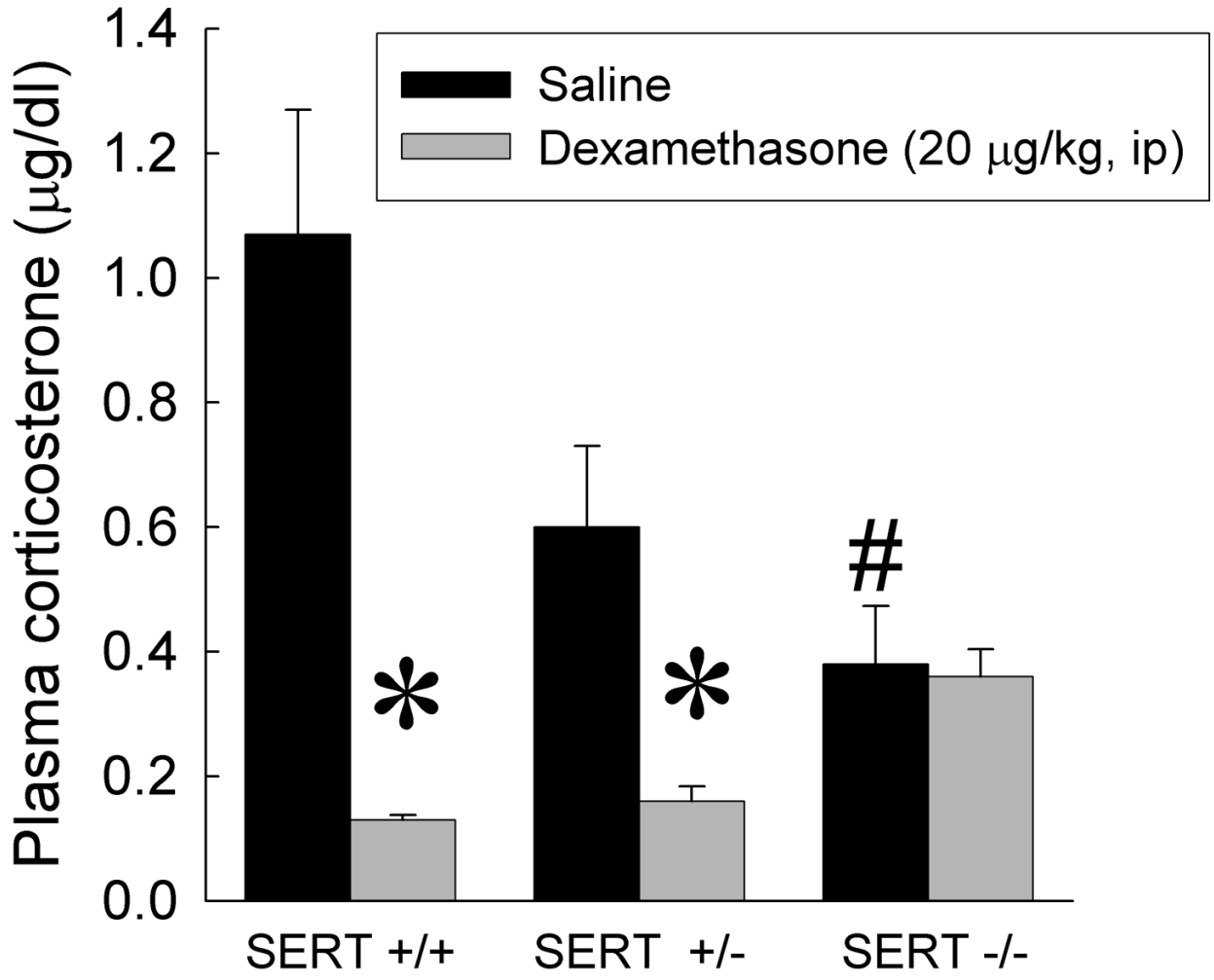

Fig 9. Systemic dexamethasone challenge study in SERT knockout mice

Dexamethasone or saline were injected into SERT knockout mice. The mice were decapitated 2 hours after the injection. The data were represent as Mean ± SEM (n = 6–8 mice). * Significantly different from saline injected same genotype mice $P<0.05$. #: significantly different from SERT+/+ mice with same treatment $P<0.05$ (Student-Newman-Keuls post hoc test).

Table 1

Design of ribo-probes for in situ hybridization

mRNA	cDNA fragment	Accession No.	Primers	References
CRF mRNA	118–692	NM_205769	5′-CTC TGC AGA GGC AGC AGT G-3′ 5′-GCT AAC TGC TCT GCC CGG-3′	(Schmidt et al., 2003)
GR mRAN	1203–1674	NM-008173	5′-CAC CCC ACC AAT TCC TGT TGGTTC-3′ 5′-CAG AAG TGT CTT GTG AGA CTC C-3′	(Schmidt et al.,2003)
CRF R1 mRNA	672–1277	NM-007762	5′-CAC CGC TTT CAT CCT GCG CAA C-3′ 5′-GAA CAC AGA CAC GAA GAA GCC-3′	(Luo et al., 1995)

Table 2

Summary of alterations in HPA axis of SERT knockout mice

Treatment SERT	Non-stressed			Stressed		
	+/+	+/−	−/−	+/+	+/−	−/−
PVN						
CRF mRNA	C	↓	↓	~	↑	↑
GR mRNA	C	↓	↓	~	~	~
GR	C	↓	↓	~	↑*	~
Pituitary						
CRF R1 binding	C	↓	↑	↑	↑	↑
CRF R1 mRNA	C	~	~	~	↓	↓
GR mRNA	C	↓	↓	↓	~	↓
ACTH response to CRF	↑	↑	↑↑			
Adrenal Cortex						
GR mRNA	C	↓	↓	↓	↓	~
Cort response to Dex	↓	↓	↑↓			

C: control;

~indicates no change;

↑ *and* ↓indicate increase and decrease, respectively. The basal level (Non-stressed) of SERT+/− and −/− were compared with the basal level of SERT+/+ mice (C). The data from stressed groups were compared with the basal levels in same genotype mice,

*the increase is observed in the nucleus but not the cytoplasm.

#the decrease is in the cytoplasm but not the nucleus. The ACTH response to CRF and Cort response to Dex were presented as changes relative to saline group.

Abbreviation: Cort: corticosterone; Dex: dexamethasome.