Published in final edited form as:

Neuropharmacology. 2015 February ; 89: 185–192. doi:10.1016/j.neuropharm.2014.09.017.

ENHANCED 5-HT_{1A} RECEPTOR-DEPENDENT FEEDBACK CONTROL OVER DORSAL RAPHE SEROTONIN NEURONS IN THE SERT KNOCKOUT MOUSE

Mariano Soiza-Reilly^{1,2}, Nathalie M. Goodfellow³, Evelyn K. Lambe^{3,4}, and Kathryn G. Commons^{1,2,*}

¹Department of Anesthesiology, Perioperative, and Pain Medicine, Boston Children's Hospital, 300 Longwood Ave., Boston, MA 02115, USA.

²Department of Anaesthesia, Harvard Medical School, Boston, MA 02115, USA.

³ Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

⁴ Department of Obstetrics and Gynaecology, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

Abstract

5-HT_{1A} receptors are widely expressed in the brain and play a critical role in feedback inhibition of serotonin (5-HT) neurons through multiple mechanisms. Yet, it remains poorly understood how these feedback mechanisms, particularly those involving long-range projections, adapt in mood disorders. Here, we examined several aspects of 5-HT_{1A} receptor function in the 5-HT transporter knockout mouse (SERT-KO), a model of vulnerability to stress and mood disorders. We found that in comparison to wild-type (WT) mice, SERT-KO mice had more passive coping in response to acute swim stress and this was accompanied by hypo-activation of medial prefrontal cortex (mPFC) Fos expression. Both of these effects were reversed by systemically blocking 5-HT_{1A} receptors. *Ex-vivo* electrophysiological experiments showed that 5-HT exerted greater 5-HT_{1A}-mediated inhibitory effects in the mPFC of SERT-KO mice compared to WT. Since 5-HT_{1A} receptors in the mPFC provide a key feedback regulation of the dorsal raphe nucleus (DRN), we used a disinhibition strategy to examine endogenous feedback control of 5-HT neurons. Blocking 5-HT_{1A} receptors disinhibited several fold more 5-HT neurons in the DRN of SERT-KO than in WT mice, revealing the presence of enhanced feedback inhibition of 5-HT neurons in the SERT-KO. Taken together our results indicate that increased stress sensitivity in the SERT-KO is associated with the enhanced capacity of 5-HT_{1A} receptors to inhibit neurons in the mPFC as well as to exert feedback inhibition of DRN 5-HT neurons.

© 2014 Elsevier Ltd. All rights reserved.

*Correspondence to: kathryn.common@childrens.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure: Authors declare no conflict of interests.

Keywords

Serotonin Transporter; Feedback Inhibition; Dorsal Raphe Nucleus; Swim Stress; Depressive-like Behavior; Mood Disorders

INTRODUCTION

Serotonin (5-HT) neurons in the dorsal raphe nucleus (DRN) are a major source of 5-HT to the forebrain, which influences arousal, mood and motivated behavior (Amat et al., 2005; Nakamura et al., 2008; Takahashi et al., 2010; Bruchas et al., 2011; Warden et al., 2012). The activity of 5-HT neurons in the DRN is influenced by feedback mechanisms involving 5-HT_{1A} receptors. Specifically, 5-HT_{1A} receptors can be found as autoreceptors on 5-HT-producing neurons in the DRN and as heteroreceptors on neurons in other brain regions (Celada et al., 2001; Sharp et al., 2007; Sprouse and Aghajanian, 1987). In particular, 5-HT_{1A} heteroreceptors in the medial prefrontal cortex (mPFC) have been suggested to play a key role in long-range feedback to the DRN (Celada et al., 2001; Sharp et al., 2007; Hajós et al., 1999). Despite the importance of 5-HT_{1A} receptors in the pathophysiology of mood disorders and their treatment (Reviewed by (Savitz et al., 2009), it is poorly understood how these different 5-HT_{1A}-mediated feedback pathways adapt in concert to regulate 5-HT neuron activity in pathological conditions.

The 5-HT transporter knockout mouse (SERT-KO) represents a model of vulnerability to stress and mood disorders, suitable for investigating adaptations in 5-HT_{1A} receptor feedback control of the DRN. The SERT-KO mouse is highly stress-sensitive, showing depressive and anxiety-like phenotypes (Reviewed by (Murphy and Lesch, 2008; Haenisch and Bönisch, 2011). Of note, 5-HT_{1A} autoreceptors are strongly desensitized and their expression down-regulated in the SERT-KO (Li et al., 1999, 2000; Fabre et al., 2000; Mannoury la Cour et al., 2001). Yet, studies using systemic drug administration show that agonists of 5-HT_{1A} receptors can still inhibit 5-HT neuron firing (Gobbi et al., 2001) and antagonists of 5-HT_{1A} receptors can normalize some of the behavioral deficits of the SERT-KO mouse (Holmes et al., 2003). Together, these findings suggest a potential adaptation in 5-HT_{1A} receptors in regulating the activity of 5-HT neurons in this stress-sensitive mouse model.

In this study, a combination of approaches was used to study the effects of 5-HT_{1A} receptors and their feedback regulation of 5-HT neurons in WT and SERT-KO mice. Behavioral pharmacology and Fos studies were combined with *ex-vivo* electrophysiology in the mPFC, an essential cortical region responsible for multisynaptic or 'long range' 5-HT_{1A} receptor-mediated feedback inhibition of DRN neurons (Hajós et al., 1999; Celada et al., 2001; Sharp et al., 2007). A disinhibition strategy was used to assess the effects of 5-HT_{1A} receptors on 5-HT neurons. That is, 5-HT_{1A} receptors were pharmacologically blocked and Fos expression was assessed, under the premise that novel activation would provide evidence of prior inhibition (Commons, 2008).

MATERIALS AND METHODS

Fos expression and behavior

Animals—All anatomy and behavioral procedures were reviewed and approved by the Institutional Care and Use Committee at Boston Children's Hospital, in agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. SERT knockout mice (SERT-KO) (Bengel et al., 1998) and C57BL/6J mice (wild-type, WT) were obtained from The Jackson Laboratory (Bar Harbor, ME). SERT-KO mice were bred from heterozygote females and either heterozygote or homozygote males and group housed. Only homozygous WT or SERT-KO male mice of 8 weeks of age (23–30 g) were used in this study. In all experiments, treatments were randomly assigned and animals were number coded to conduct all measurements blind to experimental groups.

Swim Behavior—SERT-KO and WT mice were injected subcutaneously with either saline (0.9% NaCl) or WAY-100635 at 0.4 mg/kg (Fletcher et al., 1996; Fornal et al., 1996) using a solution concentration of 0.1 mg/ml in saline. Mice were returned to their home cage, and 5 minutes after injection, animals were subjected to a modified Porsolt swim test (Porsolt et al., 1978; Detke et al., 1995; Cryan et al., 2005). In this test, mice were placed in a cylindrical glass tank (46 cm high $\times$ 20 cm diameter) filled with water ($25 \pm 1^\circ\text{C}$) to a depth of 20 cm for 15 minutes. Behaviors in the tank were videotaped for subsequent analysis. Swimming, immobility and climbing behaviors were scored in a cumulative manner, using a 5 seconds interval sampling method previously described (Cryan et al., 2005). Swimming behavior was defined as movements throughout the tank (typically horizontal), while immobility was scored when minimal movements were required to keep the head above the water. Climbing behavior was determined when both forepaws were in contact with the tank's wall. Immediately after the swim, mice were removed from the tank, towel dried, and returned to their home cage and rejoined previous cage-mates that were similarly manipulated. Group housing as well as handling associated with s.c. injections could influence the appearance of Fos, however these were kept constant between groups. Perfusions took place after a delay of 120 minutes after the end of the swim session to allow for the expression and accumulation of Fos protein.

Locomotor activity—In a different set of SERT-KO mice locomotor activity was automatically measured in clear plexiglass cages (10" $\times$ 19" $\times$ 8"h) (Photobeam Activity System, San Diego Instruments, San Diego, CA) starting 5 minutes after administration of either saline or WAY-100635. Accumulated beam breaks including ambulatory, fine and rearing movements were registered over twelve bins of 5 minutes for 60 minutes.

Tissue Preparation and Immunohistochemistry—Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and then perfused through the ascending aorta with 10 ml saline (wash), followed by 40 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (4°C, pH 7.6). Brains were removed and stored in the same fixative solution overnight (4°C), and then equilibrated in a solution of 30% sucrose in 0.1 M phosphate buffer. Brains were frozen and sectioned 40 μ m thick. Sections were processed while floating.

Sections were incubated overnight at room temperature with primary antisera diluted in 0.1 M phosphate-buffered saline (PBS) with 0.3% Triton X-100, 0.1% bovine serum albumin and 0.01% sodium azide. The 5-HT neurons were detected using an antiserum raised against tryptophan hydroxylase (TPH), the synthetic enzyme for 5-HT, raised in sheep (Millipore, Billerica, MA, #AB1541; 1:2000). This antiserum yields a labeling pattern consistent with the distribution of 5-HT cells (VanderHorst and Ulfhake, 2006). Fos immunoreactivity was detected by incubating sections in rabbit anti-Fos antisera (EMD Chemicals, Gibbstown, NJ, #PC38; diluted 1:10000). Sections were then incubated for 90 minutes with secondary antisera raised in donkey with minimal cross reactivity to other species and conjugated to CY3 (Jackson Immunoresearch, West Grove, PA) or Alexa 488 (Invitrogen, Carlsbad, CA; diluted 1:100). Sections were rinsed, mounted, dried, and cover-slipped with a glycerol-based mounting medium.

Fos Analysis—Tissue was systematically sampled by analyzing every third section through the entire DRN and forebrain. Sections were imaged and digitally photographed using an Olympus fluorescence microscope with a 10X objective, a Hamamatsu Orca ER camera and Slidebook software (3i). Fos-containing cells were counted in the medial region of the prefrontal cortex including prelimbic and infralimbic regions (from 1.98 to 1.70 mm relative to Bregma; Paxinos and Franklin, 2001) using a sampling mask of 450 $\mu\text{m} \times 450 \mu\text{m}$. Nuclei counting in the medial prefrontal cortex was done with FIJI software using “threshold” and “analyze particles” functions. The threshold was set manually to include all nuclei that appeared filled with immunolabeling with a minimum of noise. In the DRN, cells dually immunolabeled with Fos and TPH were counted in 7 separate subregions (Sperling and Commons, 2011). Dually labeled cells in the DRN were manually enumerated by visualization of the individual and/or merged images of each channel. For the latter analysis, three rostrocaudal divisions of the DRN were made: rostral was -4.16 to -4.24 mm, middle was -4.36 to -4.84 mm, and caudal was -4.96 to -5.20 mm relative to Bregma (Paxinos and Franklin, 2001). In each animal, analysis of every third section yielded at least two or three sections per region. Results from these were averaged together to yield a region mean per animal. After that, region means were averaged into group means.

Statistics—Data were analyzed by multifactorial ANOVA using the IBM SPSS Statistics 19.0 (Chicago, IL). Genotype and Treatment were analyzed as between-subject factors for neuroanatomical and swimming data (two levels each). For locomotor measurements, Treatment and genotype were analyzed as between-subject factor (two levels each) while Bin represents the repeated measures factor (twelve levels). Significant interactions were further analyzed using simple-effect slice ANOVAs at each level. When necessary, multiple comparisons were made using the post-hoc Tukey-Kramer's test. Significance was established at $p < 0.05$. Data are shown as mean values $\pm$ standard error of the mean.

Electrophysiology

Brain slice preparation—The Faculty of Medicine Animal Care and Use Committee at the University of Toronto approved the animal procedures. Homozygous WT or SERT-KO male mice were used, originating from heterozygous SERT mice obtained from Columbia University (Dr. Jay Gingrich). Male adult brains from WT ($n = 4$) or SERT-KO group-

housed mice ($n = 5$), bred from heterozygous SERT parents and group-housed, were rapidly cooled with 4 °C oxygenated (95% oxygen and 5% carbon dioxide) sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose was substituted for NaCl) and 400 μ m thick coronal mPFC slices were cut on a Dosaka Linear Slicer (SciMedia). Brain slices were immediately transferred into a pre-chamber with 30 °C oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 24 mM NaHCO₃, 2 mM CaCl₂, 2 mM MgSO₄, 3 mM KCl, 1.25 mM NaH₂PO₄; pH 7.4) where they were allowed to recover for at least 1 hour prior to recording.

Whole-cell recordings and pharmacology—Brain slices were placed in a modified superfusion chamber (Warner Instruments) mounted on the stage of an Olympus BX50WI microscope (Olympus) with 32 °C oxygenated ACSF flowing at a rate of 3–4 ml/minute. Using infrared differential interference contrast (IR-DIC) microscopy, pyramidal neurons in layer V of the medial prefrontal cortex (cingulate and prelimbic regions) were patched under visual control. Patch pipettes (2–3 M Ω) contained 120 mM potassium gluconate, 5 mM KCl, 2 mM MgCl₂, 4 mM K₂-ATP, 0.4 mM Na₂-GTP, 10 mM Na₂-phosphocreatine, and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH). Whole-cell recordings were conducted using a Multiclamp 700b (Molecular Devices).

Intrinsic neuronal properties—Intrinsic neuronal properties were assessed in current clamp. Depolarizing current steps were used to elicit action potentials in order to calculate spike amplitude, measured as the voltage difference between the threshold and the peak.

Hyperpolarizing current steps were used to obtain the steady state change in membrane potential needed to calculate input resistance. We observed no difference between the intrinsic properties of neurons from WT and SERT-KO mice (unpaired t test, resting membrane potential, $p = 0.2$; spike amplitude, $p = 0.2$; input resistance, $p = 0.5$). Combined, the neurons had the following intrinsic properties: resting membrane potential -83.3 ± 3.6 mV; spike amplitude, 83.4 ± 0.8 mV; and input resistance, 207.3 ± 13.2 Ω M.

Outward currents elicited by 5-HT—The currents elicited by 5-HT were recorded using continuous single electrode voltage-clamp mode at a holding potential of -75 mV, near the equilibrium potential for chloride ions. The holding currents did not differ significantly between genotypes (WT: 54.2 ± 5.9 pA; KO: 62.5 ± 7.9 pA; $p = 0.4$). Data were acquired and low-pass filtered at 3 kHz using pClamp10.2/ Digidata1440 software (Molecular Devices). We recorded for a baseline period before adding 5-HT (10 μ M; Sigma) to the bath perfusion for 30 s and then allowed a washout period of at least 5 minutes. This protocol gave reproducible 5-HT-elicited currents with repeated applications (**Supplementary Figure 2 A,B**). The selective 5-HT_{1A} antagonist WAY-100635 (30 nM; 10 minutes; Tocris) was used to examine the contribution of this receptor to the 5-HT-elicited outward current. In prefrontal pyramidal neurons of both genotypes, WAY-100635 had negligible effects on the holding current (change in holding current: WT, -0.03 ± 2.5 pA; SERT-KO, -1.3 ± 3 pA). All compounds were stored in stock solutions at -20 °C before being diluted and applied to the slice in oxygenated ACSF.

Analysis of electrophysiological data—The peak amplitude of the 5-HT current was measured using Clampfit software (Molecular Devices) by subtracting the mean current at

the peak of the 5-HT response from the mean holding current at baseline. All data are expressed as the mean $\pm$ SEM. All electrophysiological data was tested for Gaussian distribution using D'Agostino & Pearson omnibus normality test. Comparisons within-subjects (before and after WAY-100635 experiments) and between-subjects (genotype) were made using paired *t* test and either Mann-Whitney test or unpaired *t* test, respectively, at a significant level of 0.05. For illustration purposes, electrophysiological recordings were averaged using Axograph software.

RESULTS

Stress-coping behavior and locomotor activity

We focused on a behavioral manipulation, acute swim stress, to probe adaptations in 5-HT_{1A} receptor function in the SERT-KO because this stimulus produces a well-characterized activation of the 5-HT system (Roche et al., 2003; Commons, 2008). In addition, this acute stress was selected because many pharmaceutical agents, particularly serotonergic, have been characterized in this test (reviewed by (Cryan et al., 2005)). Here, we analyzed behavior during a single 15 minutes swim session by scoring climbing, swimming and immobility events in SERT-KO or WT mice with and without pretreatment with the 5-HT_{1A} antagonist, WAY-100635. ANOVA results of behavior showed a significant interaction between genotype and treatment factors ($F_{1,35}= 26.95$, $p<0.001$). In WT mice, there was no difference in swimming behavior between saline and WAY-100635 pretreated mice (**Figure 1 A**). In contrast, saline treated SERT-KO mice swam significantly less than WT mice ($F_{1,19}= 34.07$, $p<0.001$). Acute administration of WAY-100635 in SERT-KO mice increased swimming events ($F_{1,20}=69.08$, $p<0.001$), to even higher levels than in WT mice treated with WAY-100635 ($F_{1,16}= 7.35$, $p<0.02$) (**Figure 1 A**). Reciprocal changes were seen in immobility score (**Figure 1 A**), while climbing events were rare and similar across groups.

We tested whether the effects of acute treatment with WAY-100635 on swimming behavior in SERT-KO mice resulted from a non-specific effect on locomotion. For this, we measured the locomotor activity of SERT-KO mice in a novel cage after acute injections of either saline or WAY-100635 following the same time course used in swimming experiments. These results showed a non-significant interaction between treatment and time. Moreover, WAY-100635 treatment did not affect locomotor activity in the cage when compared to saline treated animals ($F_{1,12}= 0.74$, $p=0.41$) (**Figure 1 B**) consistent with previous observations (Holmes et al., 2003).

Fos expression in the mPFC after swim

Since the mPFC has a key role in orchestrating coping behavior as well as in controlling the activity of DRN 5-HT neurons (Celada et al., 2001; Warden et al., 2012), we next asked whether there are genotype and treatment differences in neuronal activation in this cortical region in response to swim stress. We found that SERT-KO mice showed less Fos activation within mPFC after swim stress than WT mice, but that this genotype difference occurred only in the absence of 5-HT_{1A} receptors blockade (**Figure 2**). Saline-treated SERT-KO mice showed significantly lower levels of Fos-positive cells when compared to WT mice

($F_{1,18} = 5.72$, $p < 0.03$). While Fos activation in the mPFC of WT mice was not affected by WAY-100365 (**Figure 2**), it was significantly increased by WAY-100635 application preceding swim stress in SERT-KO mice ($F_{1,20} = 9.76$, $p < 0.01$), restoring Fos levels to WT values (**Figure 2**).

In order to determine if these differences between genotypes were dependent on the stress condition, we also analyzed Fos expression in the mPFC of WT and SERT-KOs receiving saline or WAY-100635 injections but did not swim. In the absence of swim there were similar trends for an enhanced effect of WAY-100635 in the SERT-KO, although differences did not reach the threshold for significance (Supplementary Figure 1).

Modulation of mPFC neurons by 5-HT

We hypothesized that altered 5-HT_{1A} heteroreceptor activity may occur in SERT-KO mice, with consequences for neuronal activity in the mPFC. Here, we examined mPFC 5-HT_{1A} receptor currents in brain slices from an independent cohort of SERT-KO and WT mice. Visually-identified layer V pyramidal neurons were selected for whole-cell recording (**Figure 3 A**), since this population of neurons has been implicated both directly and indirectly in long-range feedback control of the DRN (Hajós et al., 1999; Celada et al., 2001; Gabbott et al., 2005; Warden et al., 2012). While brief mention has been made of elevated prefrontal 5-HT responses in SERT-KO mice (Goodfellow et al., 2014), the properties and underlying receptor mechanisms of these abnormal responses were examined only in mice heterozygous for SERT and not in SERT-KO animals. Here, bath application of 5-HT (10 μ M) again elicited significantly larger outward currents in SERT-KO mice as compared to WT mice (Mann Whitney, $p < 0.01$). The averaged 5-HT responses for a large group of neurons from each genotype are shown in **Figure 3 B** and the reproducibility of such responses within individual neurons is demonstrated in **Supplementary Figure 2 A**. Kinetic analysis reveals that the total duration of the currents was significantly longer in SERT-KO mice (WT 78 ± 2.8 s; SERT-KO 105.5 ± 9.3 s; Mann Whitney, $p < 0.05$). These larger and prolonged 5-HT inhibitory currents occurred in the absence of significant genotype differences in intrinsic properties of the neurons (see Methods). In cell-paired experiments from SERT-KO mice, we observed that the 5-HT_{1A} antagonist WAY-100635 abolished these larger 5-HT outward currents ($n=5$; paired t test, $p < 0.01$; **Figure 3 C**). An additional replication shows that WAY-100635 strongly suppresses the 5-HT outward currents in prefrontal neurons of both WT and SERT-KO mice (**Supplementary Figure 2 C,D**), thus eliminating the genotype difference. Concentration-response experiments demonstrate that prefrontal pyramidal neurons in SERT-KO mice are sensitive to inhibition across a wide range of 5-HT concentrations, as illustrated in **Supplementary Figure 2 E,F**. Prefrontal neurons of SERT-KO mice appear to be more sensitive to low levels of 5-HT than WT. In both genotypes, however, we find that prefrontal neurons respond similarly to strong 5-HT stimulation, which contrasts to the autoreceptor desensitization observed with strong stimulation in the DRN of SERT-KO mice (Araragi et al., 2013). Taken together, we find that SERT-KO mice have enhanced 5-HT_{1A} receptor effects in prefrontal cortex, which may be relevant to long-range feedback control of the DRN.

5-HT_{1A} receptor-dependent feedback inhibition of DRN 5-HT cells

DRN autoreceptor 5-HT_{1A} function is thought to be severely dampened in SERT-KO mice (Li et al., 1999, 2000; Fabre et al., 2000; Mannoury la Cour et al., 2001), yet 5-HT_{1A} heteroreceptor function appears to be enhanced in mPFC neurons. To investigate the net effect of this differential plasticity on feedback inhibition to the DRN, we examined Fos activation within 5-HT neurons in SERT-KO and WT mice, with and without WAY-100635 treatment (**Figure 4 A**). If 5-HT_{1A} receptors are actively inhibiting 5-HT neurons, then removing that inhibition with WAY-100635 should disinhibit Fos expression. Since our question focused on feedback regulation of DRN 5-HT neurons, Fos was not quantified in non-5-HT neurons. Non-5-HT neurons with Fos were visible in every group often laterally flanking 5-HT neurons, but their location was variable and no qualitative differences were noted.

With saline treatment, swim produced the comparable levels of Fos expression within 5-HT neurons in WT and SERT-KO mice. However, administration of the 5-HT_{1A} antagonist WAY-100635 preceding swim stress elicited in average a 4.3-fold increase in the activation of DRN 5-HT neurons in SERT-KO mice, compared to the 1.3-fold increase in WT mice (**Figure 4 A-F**). There were significant interactions between genotype and treatment factors in ANOVA analyses for all DRN subregions examined (RD: $F_{1,35} = 24.61$, $p < 0.001$; RV: $F_{1,35} = 57.32$, $p < 0.001$; MD: $F_{1,35} = 55.37$, $p < 0.001$; MV: $F_{1,35} = 52.29$, $p < 0.001$; LW: $F_{1,35} = 36.13$, $p < 0.001$; CD: $F_{1,35} = 37.39$, $p < 0.001$; CV: $F_{1,35} = 14.36$, $p < 0.001$) (**Figure 4 G**). In WT mice, the increase in Fos after WAY-100635 treatment occurred in only a subset of regions within the DRN. In contrast, WAY-100635 treatment increased Fos expression in every area of the DRN in SERTKO mouse (**Figure 4 G**), and the areas with the lowest absolute number of Fos/5-HT neurons corresponded to regions that contain fewer total 5-HT neurons (LW, CV, CD), such that the activation of Fos by WAY-100635 in the SERT-KO was both profound and pervasive.

To determine if these enhanced effects of WAY-100635 depended on the stress, we also analyzed WT and SERT-KO mice that did not swim. This analysis revealed that even in the absence of swim, WAY-100635 had a more profound effect on Fos in SERT-KO than in WT animals. WAY-100635 increased Fos expression by 4-7 fold in SERT-Kos but only about 1.3 fold in WTs, regardless of stress condition (Supplementary Figure 3).

DISCUSSION

In response to an acute swim stress, SERT-KO mice show increased passive coping behavior coupled with attenuated Fos activation in the mPFC, and these effects are completely reversed by blocking 5-HT_{1A} receptors. Consistent with these data, SERT-KO mice have increased inhibitory 5-HT_{1A} receptor-mediated currents in the major output neurons of mPFC. Furthermore, systemic blockade of 5-HT_{1A} receptors results in a substantially greater disinhibition of DRN 5-HT neurons in SERT-KO mice, as compared to that observed in WT mice. Thus overall it appears that the net feedback 5-HT_{1A} inhibition on 5-HT neurons is maintained or enhanced in the face of prolonged increases in extracellular 5-HT observed in the SERT-KO (Shen et al., 2004).

Augmented 5-HT_{1A} heteroreceptor function could contribute to the behavioral phenotype of the SERT-KO mouse. In the current study, acute treatment with the selective 5-HT_{1A} receptor antagonist WAY-100635 preceding acute swim stress did not modify stress-coping strategies in WT mice. However in the SERT-KO mouse, WAY-100635 pretreatment markedly increased use of an active coping strategy. These observations echo previous work showing that blockade of 5-HT_{1A} receptors normalizes anxiety-like behavior in the SERT-KO (Holmes et al., 2003). In both cases the effect of WAY-100635 in SERT KO mice could not be attributed to a general increase in locomotor activity. While the location of 5-HT_{1A} receptors that underlie these effects is unknown, these observations support the behavioral importance of adaptations in 5-HT_{1A} receptor function for the phenotype of the SERT-KO mouse.

Neuronal activity in the mPFC, as measured by Fos activation, predicted active coping in response to the swim stress. The low appearance of Fos from our *in vivo* work in SERT-KO mice fits well with the increased inhibitory, 5-HT_{1A} receptor currents we measured electrophysiologically in mPFC neurons *ex vivo*. However, it should be noted that Fos may be activated as a direct consequence of 5-HT_{1A} receptor inhibition and/or indirectly as mPFC networks change state. Since there is abundant 5-HT to stimulate 5-HT_{1A} receptors in mPFC of SERT-KO mice (Shen et al., 2004), it is consistent that blocking these receptors would change the balance of activity in mPFC networks and thus influence coping strategy in the swim. These observations are consistent with studies that have implicated this structure in the selection and implementation of stress-coping strategies (Amat et al., 2005; Warden et al., 2012).

Blocking 5-HT_{1A} receptors systemically also resulted in a massive disinhibition of Fos within DRN 5-HT neurons in the SERT-KO. This finding indicates that 5-HT_{1A} receptor function in the SERT-KO includes the capacity to exert feedback inhibition of 5-HT neurons *in vivo* under physiological conditions. That is, blocking 5-HT_{1A} receptors disinhibited Fos expression, implicating the prior presence of functional feedback inhibition. Consistent with this observation, it has previously been noted that systemic administration of WAY-100635 can increase firing rates of DRN neurons in anesthetized SERT-KO mice (Gobbi et al., 2001). Yet, the locus of the relevant 5-HT_{1A} receptors has not been identified. While the 5-HT_{1A} autoreceptors within the DRN are thought to be downregulated and desensitized (Li et al., 1999, 2000; Mannoury la Cour et al., 2001), they could contribute to this effect. Indeed, a recent study using a raphe slice preparation argued that residual 5-HT_{1A} autoreceptors retain the capacity to provide feedback inhibition of 5-HT neurons in the SERT-KO and therefore desensitization does not predict their capacity to exert autoinhibition (Araragi et al., 2013). The current data also suggest that 5-HT_{1A} controlled long-feedback loops could be enhanced in SERT-KO mice since mPFC neurons have increased 5-HT_{1A} receptor currents. Thus there could be convergent mechanisms contributing to enhanced feedback control of 5-HT neurons in the SERT-KO.

The DRN and mPFC are reciprocally interconnected and can influence each other bi-directionally (Celada *et al*, 2001; Celada et al., 2013). Therefore we could speculate that in the SERT-KO, WAY-100635 disinhibits cortical pyramidal neurons, and this results in a shift in descending control of the DRN from feedback inhibition to direct excitation.

However, there are many potential circuit diagrams that could explain the current results. Participation of additional brain regions, other adaptations in the SERTKO and the ultimate drivers of activation within the DRN and mPFC all remain unknown. Therefore in evaluating these scenarios, it is worthwhile to keep in mind that while Fos experiments give unique insight into adaptations in the SERT-KO, they are hypothesis generating in terms of mechanism.

Our results show that elevated 5-HT levels observed in SERT-KO mice lead to different types of plasticity for the heteroreceptor and autoreceptor subpopulations of 5-HT_{1A} receptors and the nature of 5-HT_{1A}-receptor dependent feedback inhibition of the DRN is fundamentally different in the SERT-KO mouse. Future work is necessary to understand the mechanisms responsible for these changes. However, these findings are consistent with the idea that altered 5-HT_{1A} receptor function could be an etiological factor in mood disorders (Miller et al., 2009). Not only may 5-HT_{1A} receptors be influenced by genetic factors, but they may also be stress-sensitive (Rozeske et al., 2011). Therefore, overall a greater focus on interactions between genes and treatment may be essential to understand the neurobiology of stress-related psychopathology.

Conclusions

In the current study, SERT-KO mice showed altered behavioral performance in the swim and parallel changes in mPFC Fos expression, which were both reversed by blocking 5-HT_{1A} receptors. Enhanced response of mPFC neurons to 5-HT_{1A}-heteroreceptor activation raised the possibility of altered long-range feedback to the DRN. Here, we found that net 5-HT_{1A}-receptor inhibition of DRN 5-HT neurons is greatly increased in the SERT-KO mouse. Taken together, these findings suggest that there is greater diversity in the plasticity of 5-HT_{1A}-mediated feedback inhibition in this mouse model of stress sensitivity than previously realized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the help of Katherine E. Price, Dr. Guo Yueping and Alayna Silberstein at Boston Children's Hospital and Dr. Derya Sargin of the University of Toronto for invaluable assistance in completing the experiments.

Funding: This work was supported by NIH grant DA021801 (KGC), as well as funding from the Natural Science and Engineering Research Council (EKL) and an Early Researcher Award from the Province of Ontario (EKL). EKL holds the Canada Research Chair in Developmental Cortical Physiology. NMG was supported by a Banting and Best Canada Graduate Scholarship from the Canadian Institutes of Health Research.

REFERENCES

- Amat J, Baratta MV, Paul E, Bland ST, Watkins LR, Maier SF. Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nat. Neurosci.* 2005; 8:365–371. doi:10.1038/nn1399. [PubMed: 15696163]

- Araragi N, Mlinar B, Baccini G, Gutknecht L, Lesch K-P, Corradetti R. Conservation of 5-HT_{1A} receptor-mediated autoinhibition of serotonin (5-HT) neurons in mice with altered 5-HT homeostasis. *Front Pharmacol.* 2013; 4:97. doi:10.3389/fphar.2013.00097. [PubMed: 23935583]
- Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mössner R, Westphal H, Lesch KP. Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine (“Ecstasy”) in serotonin transporter-deficient mice. *Mol. Pharmacol.* 1998; 53:649–655. [PubMed: 9547354]
- Bruchas MR, Schindler AG, Shankar H, Messinger DI, Miyatake M, Land BB, Lemos JC, Hagan CE, Neumaier JF, Quintana A, Palmiter RD, Chavkin C. Selective p38 α MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. *Neuron.* 2011; 71:498–511. doi:10.1016/j.neuron.2011.06.011. [PubMed: 21835346]
- Celada P, Puig MV, Artigas F. Serotonin modulation of cortical neurons and networks. *Front Integr Neurosci.* 2013; 7:25. doi:10.3389/fnint.2013.00025. [PubMed: 23626526]
- Celada P, Puig MV, Casanovas JM, Guillazo G, Artigas F. Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin-1A, GABA(A), and glutamate receptors. *J. Neurosci.* 2001; 21:9917–9929. [PubMed: 11739599]
- Commons KG. Evidence for topographically organized endogenous 5-HT-1A receptor-dependent feedback inhibition of the ascending serotonin system. *Eur. J. Neurosci.* 2008; 27:2611–2618. doi: 10.1111/j.1460-9568.2008.06235.x. [PubMed: 18513318]
- Cryan JF, Valentino RJ, Lucki I. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neurosci Biobehav Rev.* 2005; 29:547–569. doi:10.1016/j.neubiorev.2005.03.008. [PubMed: 15893822]
- Detke MJ, Rickels M, Lucki I. Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl.)*. 1995; 121:66–72. [PubMed: 8539342]
- Fabre V, Beaufour C, Evrard A, Rioux A, Hanoun N, Lesch KP, Murphy DL, Lanfumey L, Hamon M, Martres MP. Altered expression and functions of serotonin 5-HT_{1A} and 5-HT_{1B} receptors in knock-out mice lacking the 5-HT transporter. *Eur. J. Neurosci.* 2000; 12:2299–2310. [PubMed: 10947809]
- Fletcher A, Forster EA, Bill DJ, Brown G, Cliffe IA, Hartley JE, Jones DE, McLenachan A, Stanhope KJ, Critchley DJ, Childs KJ, Middlefell VC, Lanfumey L, Corradetti R, Laporte AM, Gozlan H, Hamon M, Dourish CT. Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT_{1A} receptor antagonist. *Behav. Brain Res.* 1996; 73:337–353. [PubMed: 8788530]
- Fornal CA, Metzler CW, Gallegos RA, Veasey SC, McCreary AC, Jacobs BL. WAY-100635, a potent and selective 5-hydroxytryptamine_{1A} antagonist, increases serotonergic neuronal activity in behaving cats: comparison with (S)-WAY-100135. *J. Pharmacol. Exp. Ther.* 1996; 278:752–762. [PubMed: 8768728]
- Gabbott PLA, Warner TA, Jays PRL, Salway P, Busby SJ. Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. *J. Comp. Neurol.* 2005; 492:145–177. doi: 10.1002/cne.20738. [PubMed: 16196030]
- Gobbi G, Murphy DL, Lesch K, Blier P. Modifications of the serotonergic system in mice lacking serotonin transporters: an in vivo electrophysiological study. *J. Pharmacol. Exp. Ther.* 2001; 296:987–995. [PubMed: 11181933]
- Goodfellow NM, Sargin D, Ansoorge MS, Gingrich JA, Lambe EK. Mice with compromised 5-HTT function lack phosphotyrosine-mediated inhibitory control over prefrontal 5-HT responses. *J. Neurosci.* 2014; 34:6107–6111. doi:10.1523/JNEUROSCI.3762-13.2014. [PubMed: 24760870]
- Haenisch B, Bönisch H. Depression and antidepressants: insights from knockout of dopamine, serotonin or noradrenaline re-uptake transporters. *Pharmacol. Ther.* 2011; 129:352–368. doi: 10.1016/j.pharmthera.2010.12.002. [PubMed: 21147164]
- Hajós M, Hajós-Korcsok E, Sharp T. Role of the medial prefrontal cortex in 5-HT_{1A} receptor-induced inhibition of 5-HT neuronal activity in the rat. *Br. J. Pharmacol.* 1999; 126:1741–1750. doi: 10.1038/sj.bjp.0702510. [PubMed: 10372816]

- Holmes A, Yang RJ, Lesch K-P, Crawley JN, Murphy DL. Mice lacking the serotonin transporter exhibit 5-HT(1A) receptor-mediated abnormalities in tests for anxiety-like behavior. *Neuropsychopharmacology*. 2003; 28:2077–2088. doi:10.1038/sj.npp.1300266. [PubMed: 12968128]
- 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. *J. Neurosci*. 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]
- Mannoury la Cour C, Boni C, Hanoun N, Lesch KP, Hamon M, Lanfumey L. Functional consequences of 5-HT transporter gene disruption on 5-HT(1a) receptor-mediated regulation of dorsal raphe and hippocampal cell activity. *J. Neurosci*. 2001; 21:2178–2185. [PubMed: 11245702]
- Miller JM, Brennan KG, Ogden TR, Oquendo MA, Sullivan GM, Mann JJ, Parsey RV. Elevated serotonin 1A binding in remitted major depressive disorder: evidence for a trait biological abnormality. *Neuropsychopharmacology*. 2009; 34:2275–2284. doi:10.1038/npp.2009.54. [PubMed: 19458612]
- Murphy DL, Lesch K-P. Targeting the murine serotonin transporter: insights into human neurobiology. *Nat. Rev. Neurosci*. 2008; 9:85–96. doi:10.1038/nrn2284. [PubMed: 18209729]
- Nakamura K, Matsumoto M, Hikosaka O. Reward-dependent modulation of neuronal activity in the primate dorsal raphe nucleus. *J. Neurosci*. 2008; 28:5331–5343. doi:10.1523/JNEUROSCI.0021-08.2008. [PubMed: 18480289]
- Porsolt RD, Anton G, Blavet N, Jalfre M. Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur. J. Pharmacol*. 1978; 47:379–391. [PubMed: 204499]
- Roche M, Commons KG, Peoples A, Valentino RJ. Circuitry underlying regulation of the serotonergic system by swim stress. *J. Neurosci*. 2003; 23:970–977. [PubMed: 12574426]
- Rozeske RR, Evans AK, Frank MG, Watkins LR, Lowry CA, Maier SF. Uncontrollable, but not controllable, stress desensitizes 5-HT1A receptors in the dorsal raphe nucleus. *J. Neurosci*. 2011; 31:14107–14115. doi:10.1523/JNEUROSCI.3095-11.2011. [PubMed: 21976495]
- Savitz J, Lucki I, Drevets WC. 5-HT(1A) receptor function in major depressive disorder. *Prog. Neurobiol*. 2009; 88:17–31. doi:10.1016/j.pneurobio.2009.01.009. [PubMed: 19428959]
- Sharp T, Boothman L, Raley J, Quéree P. Important messages in the “post”: recent discoveries in 5-HT neurone feedback control. *Trends Pharmacol. Sci*. 2007; 28:629–636. doi:10.1016/j.tips.2007.10.009. [PubMed: 17996955]
- Shen H-W, Hagino Y, Kobayashi H, Shinohara-Tanaka K, Ikeda K, Yamamoto H, Yamamoto T, Lesch K-P, Murphy DL, Hall FS, Uhl GR, Sora I. Regional differences in extracellular dopamine and serotonin assessed by in vivo microdialysis in mice lacking dopamine and/or serotonin transporters. *Neuropsychopharmacology*. 2004; 29:1790–1799. doi:10.1038/sj.npp.1300476. [PubMed: 15226739]
- Sperling R, Commons KG. Shifting topographic activation and 5-HT1A receptor-mediated inhibition of dorsal raphe serotonin neurons produced by nicotine exposure and withdrawal. *Eur. J. Neurosci*. 2011; 33:1866–1875. doi:10.1111/j.1460-9568.2011.07677.x. [PubMed: 21501256]
- Sprouse JS, Aghajanian GK. Electrophysiological responses of serotonergic dorsal raphe neurons to 5-HT1A and 5-HT1B agonists. *Synapse*. 1987; 1:3–9. doi:10.1002/syn.890010103. [PubMed: 3505364]
- Takahashi A, Shimamoto A, Boyson CO, DeBold JF, Miczek KA. GABA(B) receptor modulation of serotonin neurons in the dorsal raphe nucleus and escalation of aggression in mice. *J. Neurosci*. 2010; 30:11771–11780. doi:10.1523/JNEUROSCI.1814-10.2010. [PubMed: 20810897]
- VanderHorst VGJM, Ulfhake B. The organization of the brainstem and spinal cord of the mouse: relationships between monoaminergic, cholinergic, and spinal projection systems. *J. Chem. Neuroanat*. 2006; 31:2–36. doi:10.1016/j.jchemneu.2005.08.003. [PubMed: 16183250]
- Warden MR, Selimbeyoglu A, Mirzabekov JJ, Lo M, Thompson KR, Kim S-Y, Adhikari A, Tye KM, Frank LM, Deisseroth K. A prefrontal cortex-brainstem neuronal projection that controls response

to behavioural challenge. *Nature*. 2012; 492:428–432. doi:10.1038/nature11617. [PubMed: 23160494]

Highlights

- 5-HT_{1A} receptors retain the capacity to exert feedback-inhibition of serotonin neurons in the dorsal raphe in the SERT-KO.
- 5-HT exerts greater inhibition in the mPFC of SERT-KO mice than controls, suggesting long-feedback loops may be involved.
- Blocking 5-HT_{1A} receptors in the SERT-KO normalizes behavioral coping in the swim test.

A **B**

Figure 1. Stress-coping behavior in WT and SERT-KO mice after acute blockade of 5-HT_{1A} receptors

(A) Swimming and immobility events were scored within the 15 minute session in WT and SERT-KO mice acutely treated with either saline or WAY-100635 5 min before swim (WT-SAL N=8; WT-WAY N=9; SERT-KO-SAL N=12; SERTKO-WAY N=9). (B) Locomotor activity of the SERT-KO mouse after acute treatment with either saline or WAY-100635 (SERT-KO-SAL N=8; SERT-KO-WAY N=6). Values represent average number of beam breaks in the cage over 12 bins of 5 minutes for 60 minutes. Multiple comparisons were made using the Tukey-Kramer's test after ANOVA. $p < 0.05$, * $p < 0.01$.

Figure 2. Fos activation in the medial prefrontal cortex of the WT and SERT-KO mice after acute blockade of 5-HT_{1A} receptors preceding swim
(A) Low magnification montage image showing the region of the medial prefrontal cortex (dashed box) where the number of Fos-expressing cells was analyzed (white dots). This region includes both prelimbic (PrL) as well as infralimbic (IL) prefrontal cortices. **(B-E)** Fos-expressing cells within the medial prefrontal cortex (some indicated by arrows) of WT-SAL **(B)**, WT-WAY **(C)**, SERT-KO-SAL **(D)** and SERT-KO-WAY **(E)** mice. Scale bars = 100 μ m. **(F)** Quantitative analysis of Fos-expressing cells in the medial prefrontal cortex of WT and SERT-KO mice treated with either saline or WAY-100635 preceding acute swim

stress (WT-SAL N=6; WT-WAY N=6; SERT-KO-SAL N=7; SERT-KO-WAY N=6).

* $p < 0.05$, ** $p < 0.01$. All of the objects bright and dim of the size and shape of nuclei (some indicated by arrows) would be included.

Figure 3. Loss of the SERT gene results in enhanced 5-HT_{1A} receptor currents in the medial prefrontal cortex

(A) Schematic of a coronal brain slice indicates the medial prefrontal regions where whole-cell electrophysiological recordings were performed in WT (N=4) and SERT-KO (N=5) mice. **(B₁)** Averages of voltage-clamp recordings of the currents elicited by bath application of 5-HT (10 M; 30 s; horizontal line) from an independent cohort of mice: WT (N=16 neurons) and SERT-KO (N=17 neurons). See Supplementary Figure 2A for examples of recordings from individual neurons to demonstrate the reproducibility of these 5-HT responses. **(B₂)** Scattergrams show that 5-HT elicits a significantly larger outward current in

SERT KO, as compared to WT mice (Mann Whitney, $*p<0.01$). Note, the statistical significance between genotypes remains even when the two largest SERT-KO responses are excluded from the analysis (Mann Whitney, $p=0.01$). (C) Within-cell paired electrophysiological data demonstrate that the larger 5-HT-elicited currents in neurons from SERT-KO mice are completely abolished by the 5-HT_{1A} receptor antagonist WAY-100635 (30 nM; 10 minutes; paired t test, $*p<0.01$). Individual examples from an additional replication show the effects of WAY-100635 in both genotypes are shown in **Supplementary Figure 2C**.

Figure 4. Fos expression in 5-HT neurons in the DRN in WT (A-C) and SERT-KO (D-F) mice after swim, with and without blockade of 5-HT_{1A} receptors

WT (A) and SERT-KO (D) mice pretreated with saline before swim show very low levels of Fos activation (red) within 5-HT neurons identified by TPH immunolabeling (green) within the dorsal part of the mid DRN (arrows). WAY-100635 (WAY) treatment increases Fos immunolabeling within 5-HT cells (some indicated by arrows) in both WT (B) and SERTKO (E) mice in the same DRN subregion. However, this increase is more pronounced in the mutants than in the WT mice (WT-SAL N=8; WT-WAY N=9; SERT-KO-SAL N=12; SERT-KO-WAY N=10). In all cases, many non-5-HT neurons contained Fos. (C and F)

Zoom-in views of the areas indicated in B and E respectively, illustrating the increases in the number of double-labeled cells after acute WAY-100635 treatment preceding swim (some indicated by arrowheads). Scale bars = 100 μ m. (G) Quantitative analysis of swim-induced Fos expression within TPH-labeled cells throughout the seven regions of the DRN in WT and SERT-KO mice: rostral (dorsal, RD; ventral, RV), middle (dorsal, MD; ventral, MV; lateral wings, LW) and caudal (dorsal, CD; ventral, CV). Compared to WT, saline treated SERT-KO mice have significantly fewer 5-HT neurons with Fos in two regions: MV and CV. WAY-100635 treatment increases Fos expression in WT mice in three subregions: MV, LW and CV. WAY-100635 treatment increases Fos expression robustly in every subregion of SERTKO mice. $\circ p < 0.05$ for WT-SAL vs. WT-WAY in MV, LW and CV, and for WT-SAL vs. SERT-KO-SAL in MD and CV. $*p < 0.001$ for SERT-KO-WAY vs. all treatment/genotype combinations, and for WT-SAL vs. SERT-KO-SAL in MV.