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Chronic SSRI Treatment Exacerbates Serotonin Deficiency in Humanized *Tph2* Mutant Mice

William B. Siesser,[†] Benjamin D. Sachs,[†] Amy J. Ramsey,[‡] Tatyana D. Sotnikova,[§] Jean-Martin Beaulieu,[∥] Xiaodong Zhang,[⊥,#,▽] Marc G. Caron,[†,○] and Raul R. Gainetdinov*[,†,§]

[†]Department of Cell Biology, Duke University, Durham, North Carolina 27710, United States

[‡]Department of Pharmacology and Toxicology, University of Toronto, ON, Canada M5S 1A8

[§]Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia (IIT), Via Morego 30, Genova, 16163 Italy

[∥]Department of Psychiatry and Neuroscience, Université Laval/IUSMQ, Québec, Canada

[⊥]Neuroscience & Behavioral Disorders Program, Duke−NUS Graduate Medical School Singapore, Singapore

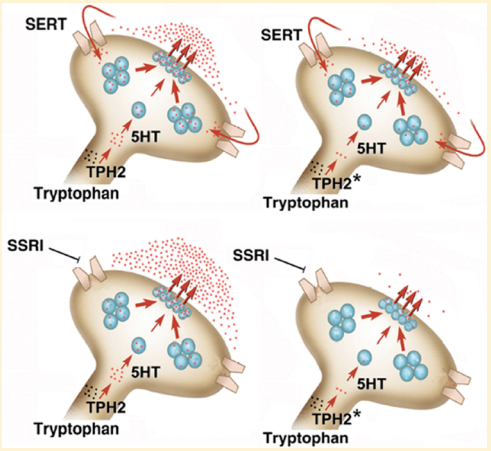
[#]Department of Physiology, National University of Singapore, Singapore

[▽]Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, North Carolina 27710, United States

[○]Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, United States

Supporting Information

ABSTRACT: Selective serotonin reuptake inhibitors (SSRIs) are a major class of antidepressants that act by blocking inward transport of serotonin (5-HT) into presynaptic neurons mediated by the serotonin transporter (SERT). Both reuptake and ongoing synthesis are essential in supporting intraneuronal serotonin concentrations in serotonergic neurons. A rare mutation in tryptophan hydroxylase 2 (*Tph2*), the rate limiting enzyme for 5-HT synthesis, was identified in several patients with major depression, and knock-in mice expressing the analogous mutation (R439H *Tph2* KI) show 80% reduction in 5-HT synthesis and tissue levels. Chronic treatment with SSRIs (fluoxetine and paroxetine) resulted in a dramatic further depletion of 5-HT tissue levels in R439H *Tph2* KI mice (down to 1−3% of wild type levels) while having little effects in wild-type controls. Treatment with the 5-HT precursor 5-hydroxytryptophan (5-HTP) restored 5-HT tissue content in mutant mice, and cotreatment with 5-HTP and fluoxetine essentially prevented the depleting effect of a chronic SSRI. These data demonstrate that chronic SSRI treatment could further exacerbate the 5-HT deficiency in *Tph2* mutation carriers, and this can be prevented by 5-HTP supplementation.



KEYWORDS: *Tryptophan hydroxylase, serotonin, antidepressant, SSRI, depression, SNP*

Selective serotonin reuptake inhibitors (SSRIs) are the most widely prescribed pharmacological treatment for depression and anxiety disorders.[1] SSRIs exert their therapeutic effect by blocking the serotonin transporter (SERT), thereby increasing extracellular levels of serotonin (5-HT). Patients require several weeks or more of treatment before showing an improvement in mood, and many patients show only partial remission or fail to respond entirely.[1,2] There are multiple reports indicating that chronic SSRI treatment can result in specific alterations in 5-HT system homeostasis including alterations in 5-HT synthesis and release, SERT activity, and 5-HT autoreceptor functions.[3] Intriguingly, although treatment with SSRIs generally increases extracellular 5-HT levels, it has been shown that chronic SSRIs can reduce the 5-HT tissue levels reflecting decreased intraneuronal 5-HT concentrations.[4−6] While these effects can be explained in part by the contribution of 5-HT autoreceptor-mediated negative feed-back control,[3,7−10] a less recognized possibility suggests a contribution of reduced SERT-mediated recycling of extracellular 5-HT following sustained blockade of SERT by chronic SSRIs.

In general, both synthesis and transporter-mediated reuptake mechanisms seem to be critical for proper maintenance of intracellular monoamine stores.[11] In fact, genetic inactivation of the members of the plasma membrane monoamine transporter family—the dopamine transporter (DAT), norepinephrine transporter (NET), and SERT—produces drastic depletions (60−95%) of tissue levels of cognate monoamines.[12−14] Furthermore, mice lacking the DAT or NET show remarkable

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depletions (virtually, disappearance) of dopamine or norepinephrine, respectively, following treatments affecting their synthesis.[15,16] Thus, it is reasonable to expect that the ability of chronic reuptake inhibitors to induce monoamine depletions could be exacerbated under conditions of deficient monoamine synthesis machinery.

The rate-limiting step in the synthesis of brain 5-HT is catalyzed by the enzyme tryptophan hydroxylase 2 (TPH2).[17,18] Multiple polymorphisms in *Tph2* have been associated with depression, bipolar disorder, suicide attempts, and response to antidepressant treatment.[19−22] Among them, several functional mutations that affect the activity of the enzyme and thus 5-HT synthesis have been recently identified.[23] For example, a functional *Tph2* mutation (P206S) has been found in a cohort with bipolar disorder;[24] other functional mutations have been found to segregate with Attention-Deficit/Hyperactivity Disorder.[25,26] Likewise, a rare mutation in *Tph2* (G1463A) that decreases 5-HT synthesis by ∼80% was discovered in several elderly patients with protracted major depression.[27] While this single nucleotide polymorphism was not found in other cohorts of patients with depression,[28] a recent study demonstrated G1463A mutation in RNA edited form in human post mortem amygdala samples obtained from individuals with various psychiatric disorders.[29] Mice expressing a mutation analogous to G1463A (R439H *Tph2* KI mice) have been developed and show an 80% reduction in 5-HT synthesis and tissue levels as well as aberrant responses in tests of anxiety, aggression and behavioral despair.[30] Furthermore, these mice display several abnormalities in putative 5-HT biomarkers similar to those reported in patients with depression.[31] To test if sustained blockade of SERT leads to the exacerbation of 5-HT deficiency in subjects with deficient 5-HT synthesis we evaluated effects of chronic SSRIs on brain 5-HT tissue levels of R439H *Tph2* KI mice. In addition, we evaluated the ability of 5-hydroxytryptophan (5-HTP) to restore 5-HT levels and prevent the depleting effects of SSRI treatment in R439H *Tph2* KI mice.

RESULTS AND DISCUSSION

In the first set of experiments, mice were treated with fluoxetine in the drinking water for 6 weeks and then assessed for changes in 5-HT (Figure 1A,B) and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Table S1, Supporting Information) tissue content with HPLC by electrochemical detection. As previously reported,[30] the vehicle-treated homozygous (HO) R439H *Tph2* mice had ∼20% and ∼10% wild-type (WT) 5-HT and 5-HIAA levels, respectively (Figures 1−3, Tables S1−S5, Supporting Information). Chronic fluoxetine in the drinking water markedly reduced 5-HT levels in the HO R439H *Tph2* mice (down to 3% of wild type levels) and only minimally affected wild-type levels in the frontal cortex (Figure 1A) and striatum (Figure 1B) [Frontal cortex: Genotype, Drug, and Genotype × Drug: all p's < 0.001; WT vs HO, p < .001; Striatum: Genotype, Drug, both p's < 0.001; Genotype × Drug, p = 0.006; WT vs HO, p < 0.001]. Importantly, dramatic depletion of brain 5-HT in mutant versus control mice was observed while the dosage of fluoxetine received did not differ between genotypes as indicated by similar plasma levels of the drug (Figure S1, Supporting Information). To assess the effects of another SSRI and route of administration, we tested daily injections (i.p.) for 3 weeks of paroxetine. Chronic paroxetine treatment (5 and 10 mg/kg) in HO R439H *Tph2* caused further depletion of 5-HT levels in the frontal cortex to ∼2% of wild-type 5-HT levels [Genotype, Drug, and Genotype × Drug: all p's < 0.001; WT vs HO p < 0.001], whereas the same treatment had little effect on 5-HT levels in WT mice (Figure 1C). In the striatum, the HO R439H *Tph2* mice also showed a much greater depletion of 5-HT tissue content than WT mice with a 99% loss of 5-HT observed (Figure 1D) [Genotype, Drug, and Genotype × Drug: all p's < 0.001; WT vs HO p < 0.001]. In summary, two SSRIs delivered in the drinking water or by daily injection showed minor or no effects on wild-type 5-HT tissue content in different brain regions but exerted dramatic further depletion of the 5-HT levels in HO R439H *Tph2* mice sometimes down to 1% of wild-type levels. Corresponding alterations in 5-HIAA levels following chronic fluoxetine and paroxetine were also observed (Tables S1 and S2, Supporting Information).

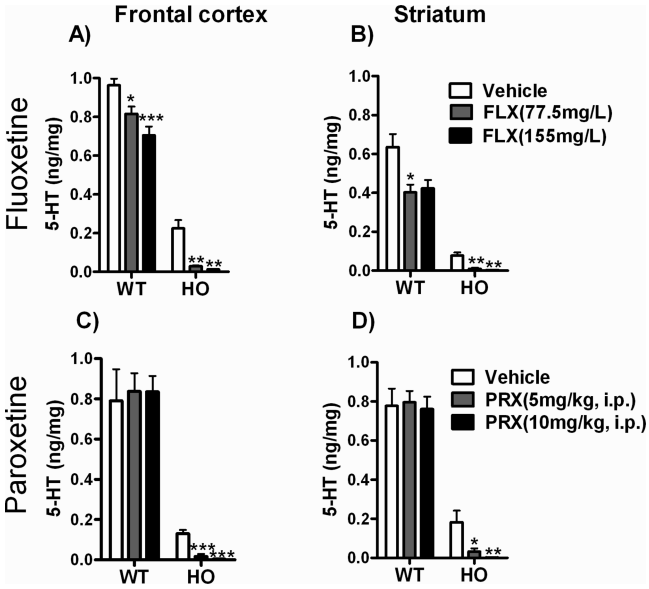

Figure 1. Effects of chronic fluoxetine and paroxetine treatment on 5-HT tissue levels in TPH2 mutant and wild-type mice. Levels of 5-HT in HO R439H *Tph2* mice, which are normally 20% of wild-type baseline levels, are depleted further and to a greater extent than in wild-type mice by chronic fluoxetine treatment in the drinking water for 6 weeks. (A) 5-HT levels in the frontal cortex. (B) 5-HT levels in the striatum. As observed with fluoxetine in the drinking water, chronic paroxetine treatment (i.p.) also depleted 5-HT levels to a greater extent in HO R439H *Tph2* mice in the (C) frontal cortex and (D) striatum. Data are expressed as ng/mg wet tissue weight and presented as mean ± SEM *, **, ***, p < 0.05, 0.01, 0.001 vs vehicle. FLX, fluoxetine. PRX, paroxetine.

TPH2 synthesizes 5-HTP from tryptophan, the rate limiting step in the synthesis of 5-HT. In an attempt to rescue 5-HT levels in the R439H *Tph2* KI mice, mice were treated with the 5-HT precursor, 5-HTP, acutely or chronically, and then assessed for changes in 5-HT tissue content. The HO R439H *Tph2* mice mice were significantly more sensitive to 5-HTP treatment than wild-type controls (Figure 2). Acute 5-HTP treatment (50 mg/kg) restored 5-HT tissue content to wild-type levels in the HO R439H *Tph2* mice in the frontal cortex (Figure 2A) and the striatum (Figure 2B). In the frontal cortex, 5-HTP treatment had a greater effect on 5-HT tissue content in HO R439H *Tph2* mice than wild-type mice [Genotype, Drug, all p's < 0.001; Genotype × Drug, p = 0.001]. In the striatum, low doses of 5-HTP (10 and 20 mg/kg) had no effect on wild-type 5-HT levels but increased 5-HT in HO R439H *Tph2* mice

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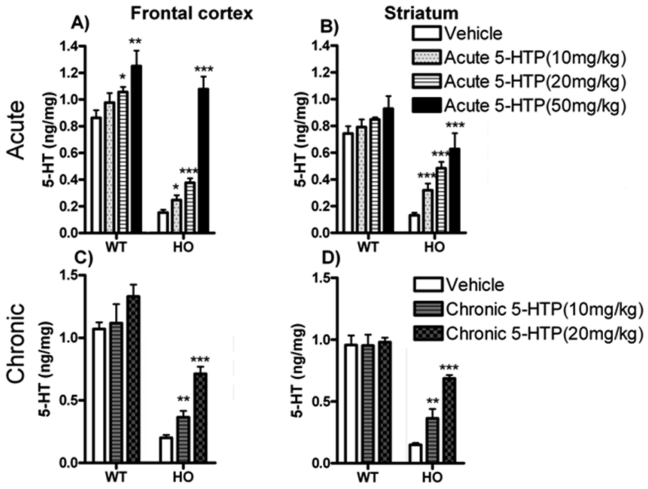

Figure 2. Effects of acute and chronic 5-hydroxytryptophan (5-HTP) treatment on 5-HT levels in TPH2 mutant and wild-type mice. Treatment with the 5-HT precursor 5-HTP can restore 5-HT to wild-type levels in the HO R439H *Tph2* mice. (A) Acute 5-HTP treatment (i.p.) increases 5-HT levels in the frontal cortex 2 h after injection. At the highest dose used (50 mg/kg), 5-HT levels were restored to wild-type levels in the HO R439H *Tph2* mice. (B) In the striatum, HO R439H *Tph2* mice were more sensitive to acute 5-HTP treatment than wild-type controls: low doses (20 mg/kg) significantly increased 5-HT levels in HO R439H *Tph2* mice but had no effect on 5-HT levels in wild-type controls. (C) Chronic treatment with 5-HTP (i.p., twice per day for 2 weeks) significantly increased 5-HT in the frontal cortex of HO R439H *Tph2* mice without affecting 5-HT levels in wild-type controls. (D) In the striatum, chronic 5-HTP treatment increased 5-HT levels only in HO R439H *Tph2* mice. Data are expressed as ng/mg wet tissue weight and presented as mean ± SEM *, **, ***, $p <$ .05, 0.01, 0.001 vs vehicle.

[HO: vehicle vs 5-HTP 20 mg/kg, $p = 0.013$]. Chronic treatment with 5-HTP twice per day for 2 weeks had no effect on wild-type 5-HT tissue content but significantly increased HO R439H *Tph2* 5-HT levels in the frontal cortex (Figure 2C) and striatum (Figure 2D) [Frontal Cortex: Genotype, Drug, Genotype × Drug, all p's < 0.001; HO: vehicle vs 5-HTP 10 mg/kg, p=.044; HO: vehicle vs 5-HTP 20 and 50 mg/kg, $p <$ 0.001; Striatum: Genotype, Drug, Genotype × Drug, all p's < 0.001; HO: vehicle vs 5-HTP 10 mg/kg, $p = 0.003$; HO: vehicle vs 5-HTP 20 and 50 mg/kg, $p <$ 0.001]. Taken together, these data indicate that the low 5-HT levels in HO R439H *Tph2* mice mice can be effectively restored to control levels by 5-HTP treatment. Similarly, both acute and chronic 5-HTP treatment significantly increased 5-HIAA levels in HO R439H *Tph2* mice, while only acute 5-HTP induced significant effect in WT mice (Tables S3 and S4, Supporting Information)

To assess if cotreatment of chronic 5-HTP will prevent the 5-HT depleting effects of chronic fluoxetine in the HO R439H *Tph2* mice we treated mice with a combination of chronic fluoxetine (20 mg/kg, i.p.) and chronic 5-HTP (20 mg/kg, i.p., twice daily) for 3 weeks (Figure 3). As presented in Figure 2C,D, 5-HTP alone (20 mg/kg, i.p., twice daily) significantly increased 5-HT concentrations in HO R439H *Tph2* mice with little effect observed in wild-type mice. Similar to the effects observed with chronic i.p. administration of paroxetine as well as chronic fluoxetine added in the drinking water, chronic i.p. administration of fluoxetine alone significantly depleted 5-HT levels in the frontal cortex (Figure 3A) and striatum (Figure 3B). However, cotreatment with 5-HTP reversed the 5-HT

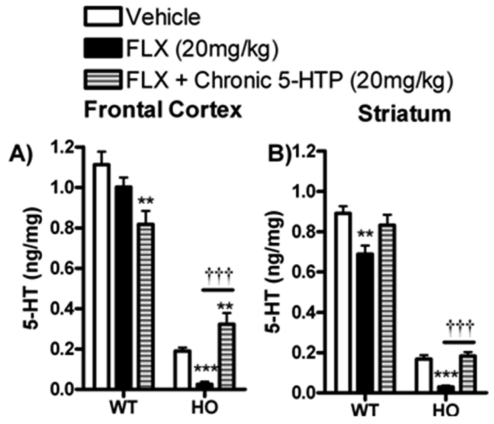

Figure 3. Chronic 5-hydroxytryptophan (5-HTP) prevents the depleting effects of chronic SSRI treatment in TPH2 mutant mice. Mice were treated chronically with vehicle, fluoxetine (i.p.) or fluoxetine and 5-HTP (i.p.) for 3 weeks. Chronic fluoxetine treatment further depleted 5-HT levels in HO R439H *Tph2* mice, but cotreatment of fluoxetine with 5-HTP blocked this depletion. (A) 5-HT levels in the frontal cortex. (B) 5-HT levels in the striatum. Data are expressed as ng/mg wet tissue weight and presented as mean ± SEM **, ***, $p <$ 0.01, 0.001 vs vehicle. †††, $p <$ 0.001 vs fluoxetine treatment. FLX, fluoxetine.

depletion effects of the SSRI and maintained 5-HT tissue content at baseline HO R439H levels or higher [Drug, Genotype × Drug, all p's < 0.001; HO: Fluoxetine alone vs Fluoxetine plus 5-HTP, $p <$ 0.001]. Accordingly, low 5-HIAA levels in HO R439H mice following chronic fluoxetine were also restored by 5-HTP treatment (Table S5, Supporting Information).

The present study demonstrates that the chronic treatment with SSRIs further depletes the 5-HT levels of *Tph2* deficient mice. As previously reported, the HO R439H *Tph2* mice have a decreased capacity to synthesize 5-HT and have only 20% of wild-type 5-HT tissue levels.[30] However, when treated with SSRI antidepressants either by daily i.p. injections or in the drinking water, 5-HT was further depleted in these mice down to 1% of wild type levels. Treatment with the 5-HT precursor 5-HTP, which bypasses the rate limiting step of synthesis mediated by TPH2, restored 5-HT levels in HO R439H *Tph2* mice and prevented the depletion of 5-HT tissue content by chronic SSRI treatment.

The primary mechanism of action for SSRIs is the inhibition of SERT, thereby increasing extracellular 5-HT levels but also disrupting inward 5-HT transport. Several studies have shown that chronic SSRI treatment can induce modest decreases in 5-HT levels in normal animals.[4−6] While 5-HT autoreceptor-mediated inhibition of 5-HT synthesis, observed following SSRIs,[7−10] could in part contribute to the effects observed, profound depletions in 5-HT levels observed in knockout mice lacking the SERT[12] indicate also a direct role of disrupted inward transport of 5-HT caused by SSRIs. The dramatically increased sensitivity of the HO R439H *Tph2* mice to the depleting effects of SSRIs demonstrates that *Tph2* is essential for appropriate 5-HT homeostasis during chronic reuptake blockade. With an impaired ability to synthesize 5-HT, the HO R439H *Tph2* mice lose almost all of the 5-HT tissue content in the brain. Strikingly, in knockout mice lacking plasma membrane monoamine transporters DAT or NET, an inhibition of DA and NE synthesis causes immediate disappearance of DA or NE content, respectively.[15,16] Taken

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together, these data demonstrate cooperative roles that transporters and synthesizing machinery play in maintaining proper monoamine levels in presynaptic neurons.

Previous reports have shown an interaction between single nucleotide polymorphisms in *Tph2* and antidepressant response, and there is anecdotal evidence suggesting resistance to SSRI treatment in patients with a mutation analogous to R439H.[19,27] The data in the present study showing dramatic depletion of brain 5-HT levels after chronic SSRI treatment provide one possible mechanism for *Tph2*'s role in antidepressant response. While it is unknown at present how a subject with an impaired ability to synthesize 5-HT would respond behaviorally to chronic SSRIs, the level of 5-HT deficiency achieved following these treatments in our study (1−3% of normal levels) is unlikely can be considered as a favorable situation.

Treatment with 5-HTP bypasses the rate-limiting step in the synthesis of 5-HT that requires TPH2. When the HO R439H *Tph2* mice were treated with 5-HTP, it restored their 5-HT levels to wild-type levels. In addition, the HO R439H *Tph2* mice were more sensitive to 5-HTP treatment than wild-type controls, and cotreatment of 5-HTP with fluoxetine prevented the depleting effects of fluoxetine. Further studies involving oral 5-HTP treatment and analysis of effects of increased peripheral serotonin by the combination of SSRIs with 5-HTP are necessary to evaluate the potential applicability of these findings to clinical practice. Nevertheless, with a growing list of TPH2 polymorphisms affecting 5-HT synthesis,[23] treatment decisions in the clinic could potentially be improved by genotyping for functional mutations in TPH2 before initiating treatment with an SSRI. Thus, a patient with a mutation that interferes with the synthesis of 5-HT might benefit from either another class of antidepressant or cotreatment with 5-HTP and SSRIs. Although most *Tph2* mutations identified so far are rare,[26] there are likely multiple alternative splice variants and extensive RNA editing in *Tph2* that may affect TPH2 function.[29] These observations as well as the recent characterization of a dominant negative form of TPH2 significantly affecting 5-HT synthesis that is present in roughly 20% of the general population may justify consideration of such a genotyping strategy.[32,33]

The present study shows that both synthesis and SERT-mediated reuptake are essential for maintaining proper intraneuronal 5-HT levels and persistent blockade of SERT by SSRIs exacerbates 5-HT deficiency in subjects with impaired TPH2 function. Finally, supplemental treatments with 5-HTP could prevent depletions caused by SSRIs in subjects with 5-HT synthesis deficiency.

METHODS

Animals. R439H *Tph2* KI mice are described in Beaulieu et al.[30] Mice were housed 2−5 per cage in an AALAC-approved vivarium maintained at 23 ± 2 °C on a 12 h light−dark cycle with onset of lights at 8 a.m. Animals were 2−3 months old at the start of chronic SSRI treatment. For the analysis of tissue content, male and female mice were used (n = 5−6 per group). All experiments were approved by the Duke University and Medical Center Institutional Animal Care & Use Committee.

Drugs and Pharmacological Treatments. Fluoxetine HCl and paroxetine maleate were generous unrestricted gifts from Lundbeck Research USA. Drugs were dissolved in water and injected i.p. daily (once a day) for 3 weeks in a volume of 10 mL/kg (fluoxetine,20 mg/kg; paroxetine, 5 or 10 mg/kg). For the drinking water studies, fluoxetine HCl (Spectrum Chemicals) was prepared fresh twice per week by dissolving in tap water (77.5 or 155 mg/L) in opaque water bottles. For acute i.p. injections of 5-HTP (Sigma), the drug was dissolved in saline and injected i.p. (10, 20, or 50 mg/kg) 2 h before euthanasia. For chronic treatment with 5-HTP, the drug was dissolved in saline and injected twice per day for 2 weeks (10 or 20 mg/kg). In all chronic drug experiments, the animals were sacrificed 24 h after last drug exposure.

Neurochemical Assays. For analysis of 5-HT tissue content, frontal cortex, hippocampus, and striatum were rapidly dissected and frozen on liquid nitrogen. Levels of 5-HT and 5-HIAA were analyzed using high-performance liquid chromatography (HPLC) by electrochemical detection as described previously.[14,18]

Data Analysis. Samples that were more than 2 standard deviations from the mean were excluded from analysis. Data were analyzed by two-way ANOVA or one-way ANOVA followed by Bonferroni posthoc test, or two-tailed t test where appropriate. Analyses were performed using SPSS for Windows Rel. 11.5.0.

ASSOCIATED CONTENT

Supporting Information

Figure S1 and Tables S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Mailing address: Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, Via Morego 30, Genoa, 16167, Italy. Telephone: +39 010 71781513. E-mail: raul.gainetdinov@iit.it.

Author Contributions

W.B.S., J.M.B., T.D.S., X.Z., M.G.C., and R.R.G. conceived and designed the experiments. W.B.S., B.D.S., A.J.R., T.D.S., and R.R.G. executed the experiments and analyzed the data. All authors wrote and reviewed the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; SSRI, selective serotonin reuptake inhibitor; SERT, serotonin transporter; 5-HTP, 5-hydroxytryptophan; KI, knock-in; DA, dopamine; DAT, dopamine transporter; NE, norepinephrine; NET, norepinephrine transporter

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