

5-HT_{2B} receptors are required for serotonin-selective antidepressant actions

Running title: 5-HT_{2B} receptors in antidepressant responses

Silvina Laura Diaz, Stéphane Doly, Nicolas Narboux-Nême, Sebastián Fernández, Pierre Mazot, Sophie Marie Banas, Katia Boutourlinsky, Imane Moutkine, Arnauld Belmer, Anne Roumier, and Luc Maroteaux

INSERM UMR-S 839, F75005, Paris, France; Université Pierre et Marie Curie, F75005, Paris; Institut du Fer à Moulin, F75005, Paris.

*Corresponding author :

Luc Maroteaux,

Institut du Fer à Moulin

UMR-S839 INSERM/UPMC

17 rue du Fer à Moulin

75005 Paris

Tel : (33) 01 45 87 61 23 Fax : (33) 01 45 87 61 32

Email : luc.maroteaux@upmc.fr

Abstract

The therapeutic effects induced by serotonin-selective reuptake inhibitor (SSRI) antidepressants are initially triggered by blocking the serotonin transporter and rely on long-term adaptations of pre- and post-synaptic receptors. We show here that long-term behavioral and neurogenic SSRI effects are abolished after either genetic or pharmacologic inactivation of 5-HT_{2B} receptors. Conversely, direct agonist stimulation of 5-HT_{2B} receptors induces an SSRI-like response in behavioral and neurogenic assays. Moreover, the observation that (i) this receptor is expressed by raphe serotonergic neurons, (ii) the SSRI-induced increase in hippocampal extracellular serotonin concentration is strongly reduced in the absence of functional 5-HT_{2B} receptors, and (iii) a selective 5-HT_{2B} agonist mimics SSRI responses, supports a positive regulation of serotonergic neurons by 5-HT_{2B} receptors. The 5-HT_{2B} receptor appears, therefore, to positively modulate serotonergic activity and to be required for the therapeutic actions of SSRIs. Consequently, the 5-HT_{2B} receptor should be considered as a new tractable target in the combat against depression.

Keywords: serotonin levels; 5-HT_{2B} receptors; antidepressants; neurogenesis;

Introduction

The most widely used antidepressants, the selective serotonin reuptake inhibitors (SSRIs), induce an increase in extracellular serotonin (5-hydroxytryptamine, 5-HT) concentrations by acutely blocking the specific serotonin transporter (SERT) (1, 2). The SERT regulates the extracellular serotonin concentration by removing 5-HT from the synaptic cleft (3), and diverse molecules modulate this activity, including the A₃ adenosine receptor (4) and kinases such as PKG/p38 MAPK (5). Previous studies have suggested a functional interaction between SERT and the 5-HT_{2B} receptor – a receptor implicated in 5-HT-dependent phenotypes, including impulsivity, aggressivity and suicidality (6). *Ex vivo* studies have indicated that 5-HT_{2B} receptors might participate in the control of SERT in raphe neurons (7), while *in vivo* studies further confirmed that 5-HT_{2B} receptors contribute to the behavioral and physiological effects of the SERT-targeting 5-HT releasers, MDMA (the club-drug ecstasy) and dexfenfluramine (8-10). Furthermore, we recently reported that the acute response to SSRIs in the forced swimming test (FST), a classical test for antidepressant activity, is absent in mice lacking 5-HT_{2B} receptors (11). Conversely, a 5-HT_{2B} receptor agonist induced an antidepressant-like action in the FST, suggesting that this receptor is required for acute SSRI effects and might modulate serotonergic tone.

Whereas blockage of SERT and increase of 5-HT levels are immediately attained after SSRI administration, therapeutic effects are only observed after weeks of treatment. The delay before the onset of clinical effects in depressive individuals appears to rely on the time required for stabilization of monoamine levels and other neuroadaptations, including neurogenesis (12). As well, regulation of 5-HT receptors appears to be required for either the acute or chronic effects of SSRIs, as it was largely demonstrated for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT₄ receptors (12-16). Importantly, fewer than 50% of all patients with depression show remission with optimized available antidepressant treatments (1). Despite extensive research, the neurobiological mechanisms underlying antidepressant effects or the resistance

to antidepressants are not yet well understood (17, 18). Therefore, we studied the chronic actions of SSRIs in behavioral and neurogenic assays and SSRI-induced alterations in synaptic 5-HT levels in mice with genetic or pharmacological ablation of 5-HT_{2B} receptors and demonstrate herein that 5-HT_{2B} receptors are required for SSRI antidepressant acute and long-term effects, possibly by presynaptic modulation of extracellular 5-HT levels.

Materials and Methods

Animals- The 5-HT_{2B}^{-/-} mice used in all experiments are in a 129S2/SvPas (129S2) background (19), while SERT^{-/-} and Pet-1^{-/-} mice are on C57Bl/6NCrI background (20, 21). Adult (7- 9 week-old) male 5-HT_{2B}^{-/-}, SERT^{-/-} and Pet-1^{-/-} mice, and their respective wild type (WT) control mice (originally obtained from Charles River Laboratories, L'Arbresle, France), were derived from heterozygous crosses and were bred at our animal facility. All mice were maintained according to the EC directive 86/609/CEE. Forced Swimming Test (FST) was conducted as already described (22). The NSF test was basically conducted as already described (17), with some modifications (See Suppl. information).

Chronic treatments, Proliferation and Survival assays and Tissue Preparation- For all the drugs employed in this study, the lowest acute effective dose was selected based on our previous data from WT mice (11). Fluoxetine (3 mg/kg/day in 129S2 or 10 mg/kg/day i.p. in C57Bl6/6NCrI mice) or vehicle (0.9% NaCl) was injected once daily for 4 weeks. WT mice chronically treated with RS127445 continuously received the 5-HT_{2B} antagonist at 1 mg/kg/day or vehicle (50% DMSO) via subcutaneous osmotic pumps (Alzet® model 2004) for 4 weeks in addition to the daily i.p. injections of Flx or Veh. The last day of this 4-week experimental protocol, mice received 2 injections of 150 mg/kg BrdU (2-h interval between injections). Then, 24 hours after BrdU administration, a pool of mice was perfused and brains were recovered for cell proliferation studies. Another pool of mice continued receiving the SSRI or vehicle for 4 more weeks after BrdU administration and was finally perfused and the brains recovered for cell survival studies. Similar experimental protocols were conducted for cell proliferation induced by paroxetine (2 mg/kg) or BW723C86 (3 mg/kg/day in 129S2 or 10 mg/kg/day i.p. in C57Bl6/6NCrI mice). All these drugs were injected once daily, and at the end of the chronic treatments (24 h and 4 weeks after BrdU, respectively for proliferation and survival assays), mice were anesthetized and their brains were removed, fixed, and processed

for immunohistochemistry. See supplemental information.

Immunohistochemistry & Immunofluorescence- Newborn cells were detected by classical peroxidase immunostaining of 5-bromo-2'deoxyuridine (BrdU), an exogenous marker of cell division, or Ki67, an endogenous cell cycle marker. Identification of newly formed cells was performed by double-labeling cells with neuronal or astroglial markers and BrdU or Ki67, followed by confocal microscopy (see supplemental information).

Microdialysis in freely moving mice- Microdialysis and high-pressure liquid chromatography (HPLC) were performed as already published by our laboratory (8-10). All measurements were obtained from freely moving mice following injections of RS127445 (0.5 mg/kg, i.p.) and/or paroxetine (2 mg/kg, i.p.) (see supplemental information).

Single-cell RT-PCR- Pet-1-EGFP mice, generated from a cross of Pet-1-CRE (23) with RCE:loxP (24) mice, were used for single-cell RT-PCR experiments. Mice were anesthetized and decapitated, and the brain was rapidly dissected out. Coronal slices (250- μ m thick) were prepared using a vibratome, in artificial cerebral spinal fluid aCSF supplemented with sucrose. The methodology involving harvesting of cytoplasmic content and subsequent single cell PCR amplification has been described elsewhere (25).

5-HT_{1A} receptor quantitative PCR- Raphe nucleus and hippocampus were dissected and homogenized in 1 ml of RNAsolv. Genomic DNA was removed by digestion with Amplification Grade DNase I (Sigma-Aldrich). First-strand cDNA was synthesized by reverse transcription of 3 μ g of total RNA with Superscript-II reverse transcriptase (Invitrogen) according to standard protocols. Reverse transcriptase was omitted in some samples as negative control. Relative expression levels of 5-HT_{1A} mRNA were determined by

real time RT-PCR using Absolute SYBR Green Mix (ABgene) and a set of primers specific for 5-HT_{1A} receptor. 5-HT_{1A} expression was normalized to mouse CyclophilinB mRNA expression. Data were analyzed with the 2⁻DCt method, and values are expressed as the mean of four separate experiments performed in duplicate.

In Situ Hybridization- Coronal sections from the same experimental subjects used in immunohistochemistry studies were employed for in situ hybridization, which was performed as previously described (26) on a pool of two coronal free-floating sections per mouse from each experimental group (WT-Veh; WT-Flx; 5-HT_{2B}^{-/-}-Veh and 5-HT_{2B}^{-/-}-Flx). 5-HT_{1A} (IMAGE:8861702) dig-UTP-labeled probe was used and detected using an antibody coupled to alkaline phosphatase (1/2000, Roche). AP activity was revealed using NBT/BCIP.

8-OH-DPAT-induced-hypothermia- Body temperature was measured intrarectally using a lubricated probe inserted ~2 cm and a digital thermometer (CEM advanced thermometer; DT-610B) (27). Mice were moved to the behavioral room and two baseline temperature measurements were taken. After 10 min, animals received 8-OH-DPAT 0.5mg/kg, i.p., and body temperature was recorded every 15 min for a total of 45 min.

Statistical analysis- To determine differences between the experimental groups, responses were analyzed by either an unpaired Student's t test or a two-way analysis of variance (ANOVA) with genotypes and treatments as main factors, depending on the experimental design. Previously, normal distributions and homoscedasticity were verified by Shapiro-Wilk's test and Levene's test, respectively. Bonferroni's or Dunnett's test was used for *post hoc* comparisons. The 5-HT extracellular levels and hypothermic responses in both genotypes were compared by two-way repeated measures ANOVA. In all cases, $p < 0.05$ was considered statistically significant. A summary of tests employed and statistics are presented in Table S1.

Results

Statistical analyses of the data are summarized in Table S1.

5-HT_{2B} receptors in the chronic behavioral effects of SSRIs

Although the FST remains a key screen for acute antidepressant activity with good reliability and predictive validity, this test cannot reveal the neurobiological mechanisms underlying antidepressant therapeutic effects that are only observed after weeks of treatment. We thus studied the putative participation of 5-HT_{2B} receptors in chronic behavioral effects triggered by SSRIs using the novelty-suppressed feeding (NSF) test, one of the few behavioral tests sensitive to chronic antidepressant treatments. Consistent with the impulsive phenotype shown by 5-HT_{2B}^{-/-} mice (6), their basal latency to feed in the NSF test was decreased compared to WT mice (**Fig. 1a-b**). To ensure that 5-HT_{2B}^{-/-} mice are able to respond in this test, we injected the anxiolytic drug diazepam and observed a further decrease in the latency to feed (**Fig. 1a**). Then, we chronically treated WT and 5-HT_{2B}^{-/-} mice with either Flx or Prx for 4 weeks and conducted an NSF test. Interestingly, even though Flx and Prx chronic treatments significantly reduced the latency to feed in WT mice, no differences were observed between control and SSRI-treated 5-HT_{2B}^{-/-} mice (**Fig. 1b**). These mutant mice also failed to respond in the NSF after injections of 1 or 20 mg/kg Flx (**Fig. S1a**). To eliminate putative bias in the NSF test, food intake in the home cage was measured during the 15 minutes immediately following the NSF test. No significant changes were registered according to either genotype or treatment (**Fig. S1b**).

To verify that the results obtained in 5-HT_{2B}^{-/-} mice were not due to developmental alterations, we also tested a group of WT mice after simultaneous chronic treatment with RS127445 and Flx or vehicle. In the NSF test, chronic pharmacological blockade of 5-HT_{2B} receptors mimicked the effects of 5-HT_{2B} ablation (**Fig. 1c**), including the reduced basal latency and the lack of SSRI-antidepressant action.

Since acute injection of BW723C86 mimicked the effect of SSRIs in WT mice in the FST (11), we tested the effect of chronic direct activation of 5-HT_{2B} receptors. A 4-week treatment with BW723C86 induced a significant decrease in the latency to feed in WT mice, with no effect on 5-HT_{2B}^{-/-} mice (**Fig. 1d**). Thus, direct chronic activation of 5-HT_{2B} receptors appears sufficient to induce chronic SSRI-like effects in the NSF test.

Pro-neurogenic and plastic effects induced by SSRIs

The well-established neurogenic effect of chronic antidepressant treatment (28) has been proposed to be necessary for behavioral responses to SSRIs (17). Therefore, cell proliferation and survival were studied in mice in which 5-HT_{2B} receptors were genetically or pharmacologically abolished. Chronic administration of either Flx or Prx failed to induce a significant increase in the incorporation of the exogenous DNA tracer, BrdU, in the hippocampal subgranular zone (SGZ) in 5-HT_{2B}^{-/-} mice, whereas an SSRI neurogenic effect was clearly observed in WT mice (**Fig. 2a**). These results were confirmed by the endogenous cell cycle progression marker Ki-67 (**Fig. S2a**). Also, no alterations in cell proliferation in either WT or mutant mice were observed in the subventricular zone, confirming that the neurogenic effect of SSRIs is specific to the SGZ of the dentate gyrus (**Fig. S2b**). Moreover, chronic Flx did not increase cell proliferation in WT mice following chronic pharmacologic inactivation of 5-HT_{2B} receptors (**Fig. 2b**). Altogether, these results confirm that a lack of functional 5-HT_{2B} receptors is sufficient to abolish the chronic actions of SSRIs at the cellular level.

Again, as in behavioral tests, chronic treatment with the 5-HT_{2B} receptor agonist BW723C86 induced an antidepressant-like action only in WT mice, evidenced by a significant increase in BrdU incorporation (**Fig. 2c**). We verified that the chronic cellular effects of SSRIs are mediated by SERT and not by direct action at 5-HT_{2B} receptors, as suggested by a previous study (29) in spite of the low affinity of Flx for mouse 5-HT_{2B}

receptors ($K_i=5.65\pm 0.52$) (10). After a 4-week treatment with Flx, SERT^{-/-} mice did not show any increase in cell proliferation (**Fig. S2c**), confirming that the observed Flx effects in WT mice depend on SERT.

Cell survival was also analyzed after administration of Flx for 8 weeks (BrdU was injected after the first 4 weeks), and a significant increase, dependent on the maintenance of Flx, was only observed in WT but not in 5-HT_{2B}^{-/-} mice (**Fig. 2d**). Regarding the fate of these newly generated cells, colabeling of BrdU with neuronal and astroglial markers was conducted in proliferation (24 h after BrdU) and survival (4 weeks after BrdU) conditions, and no difference in cell differentiation was found between genotypes (**Fig. S3**).

5-HT_{2B} receptors are required for SSRI-induced extracellular 5-HT accumulation

Although the neurobiological mechanisms mediating the behavioral and neurogenic effects of SSRIs are not completely understood, it is clear that responses to SSRIs do not occur after 5-HT depletion (30). We therefore evaluated extracellular hippocampal 5-HT levels, by means of microdialysis assays, after acute administration of SSRIs to freely moving mice. In WT mice, a single administration of Prx induced a maximal increase in 5-HT concentrations 60 min after injection, and levels remained elevated for at least 2 h (**Fig. 3a**). In 5-HT_{2B}^{-/-} mice, as in RS127445-pretreated WT mice, the maximal increase in 5-HT concentrations following a single Prx injection was significantly lower (around a quarter of the increase in WT mice). This particular response in mice with non-functional 5-HT_{2B} receptors could not be attributed to decreased basal levels of 5-HT, given that 5-HT concentrations in both genotypes are similar in the hippocampus, and even slightly higher in the raphe nuclei, of 5-HT_{2B}^{-/-} mice (**Fig. S4a**), consistent with the lack of major alterations in tissue 5-HT levels in 5-HT_{2B}^{-/-} or chronically RS127445-treated WT mice after Flx treatment (**Fig. S4b**). Moreover, SERT binding parameters as well as raphe and hippocampus SERT protein expression were similar in either genotype (**Fig. S4c-d**).

To reinforce the hypothesis that 5-HT_{2B} receptors directly participate in the control of 5-HT levels, we then evaluated 5-HT_{2B} receptor expression in raphe serotonergic neurons. We performed single-cell RT-PCR experiments on selected GFP-positive dorsal and median raphe neurons from Pet-1-EGFP mice (Pet-1 is a transcription factor specific for 5-HT neurons, and Pet-1-EGFP mice express EGFP only in 5-HT-positive neurons) (23). Among the Pet-1-positive neurons, a large majority of the Tph2 (the limiting enzyme in 5-HT synthesis) -expressing cells also expressed 5-HT_{2B} receptor mRNA (n=10 out of 12 neurons) (**Fig. 3b**), establishing for the first time the expression of this receptor in raphe serotonergic neurons.

Nevertheless, these experiments cannot exclude a contribution to antidepressant effects from potential postsynaptic 5-HT_{2B} receptors expressed on non-serotonergic neurons. We thus re-evaluated the acute Flx and BW723C86 effects in 2 different animal models in which presynaptic serotonergic components are altered: Pet-1^{-/-} mice, in which 5-HT neurons do not differentiate and 5-HT levels are reduced by 80% (21), and SERT^{-/-} mice, in which 5-HT neurons do not uptake 5-HT (20) and thus, 5-HT storage is decreased (31). Neither of these groups of mice responded to acute Flx or BW723C86 stimulation in the FST (**Fig. 3c-d**). After a 4-week treatment with the 5-HT_{2B} receptor agonist, SERT^{-/-} mice did not show any increase in cell proliferation (**Fig. S2d**). Thus, as Flx, the efficacy of BW723C86 requires both SERT and serotonergic neurons – results that are consistent with a presynaptic role for the 5-HT_{2B} receptor.

5-HT_{1A} receptors are not altered by the absence of 5-HT_{2B} receptors

The 5-HT_{1A} autoreceptor is involved in chronic SSRI effects (17, 32). The proposed mechanism for SSRI action is a temporary increase in serotonergic tone that downregulates presynaptic 5-HT_{1A} expression (12, 33). We wondered if the absence of 5-HT_{2B} receptors could modify expression and/or functionality of 5-HT_{1A} receptors. Raphe nuclei or

hippocampal basal expression of 5-HT_{1A} receptors in 5-HT_{2B}^{-/-} mice – as evaluated by qPCR, binding assays and *in situ* hybridization – was not different from WT mice (**Fig. 4a-c**). Moreover, we evaluated the functionality of these receptors by two different tests. Acute administration of the 5-HT_{1A} agonist 8-OH-DPAT induces a decrease in body temperature which is mediated by presynaptic 5-HT_{1A} receptors (27). Mice from both genotypes showed a similar hypothermic response after an acute administration of 8-OH-DPAT at 0.5 mg/kg, i.p. (**Fig. 4d**). In addition, the response in the FST to 8-OH-DAT is mediated by post-synaptic receptors, and at higher doses of this 5-HT_{1A} agonist, mice develop a serotonin syndrome. Again, for this test, mice from both genotypes had similar responses (**Fig. 4e**), indicating no differences in functionality for this receptor. Therefore, the lack of extracellular 5-HT accumulation, behavioral or neurogenic responses after SSRI treatment in 5-HT_{2B}^{-/-} mice cannot be explained by alterations in 5-HT_{1A} receptor expression or functionality.

Discussion

Based on the results presented herein, the activation of 5-HT_{2B} receptors is necessary for acute and chronic SSRI actions, and chronic stimulation with a 5-HT_{2B} receptor agonist is sufficient to mimic SSRI effects in WT mice. Furthermore, the moderate increase in extracellular 5-HT levels induced by SSRIs in the absence of functional 5-HT_{2B} receptors might be insufficient to trigger behavioral and neurogenic actions. These data, together with the single-cell RT-PCR results showing expression of 5-HT_{2B} receptors in raphe serotonergic neurons, are consistent with a presynaptic action of 5-HT_{2B} receptors mediating the acute and chronic SSRI effects.

Although many data have been published on the topic, the exact role of hippocampal neurogenesis in the clinical response to antidepressants remains equivocal (18, 34). Neuroplastic adaptations induced by chronic antidepressants appear to result from slow and fine regulations allowing a re-establishment of monoamine levels (35) and 5-HT receptor adaptation (12). In this context, considering the strong reduction in the SSRI-induced increase in extracellular 5-HT both in 5-HT_{2B}^{-/-} mice and RS127445-treated WT mice, the lack of SSRI-induced proliferative and survival effects in these mutant animals is not unexpected. The neurogenic actions of antidepressants have been proposed to be responsible for the behavioral effects induced by chronic treatment (17). Accordingly, our results show that the absence of neurogenic effects induced by chronic SSRIs in mice with pharmacologically or genetically abolished 5-HT_{2B} receptors correlates with a lack of a response in the NSF test. Conversely, chronic agonist stimulation of the 5-HT_{2B} receptor is associated with neurogenic effects and a positive response in the NSF test, supporting a link between neurogenesis and behavior.

Previous studies from our laboratory demonstrate that the absence of 5-HT_{2B} receptors impairs the response to drugs targeting the 5-HT system. In particular, 5-HT_{2B} receptors are necessary for the 5-HT releasing effect of SERT-targeting drugs, like MDMA (the club-drug

ecstasy) and dexfenfluramine (8-10). In line with these results, we shown here that acute SSRI administration to 5-HT_{2B}^{-/-} mice induces also a reduced (1/4) increase in hippocampal 5-HT levels compared to WT mice. Furthermore, we demonstrate for the first time that 5-HT_{2B} receptors are expressed by serotonergic neurons of the raphe nuclei, which is consistent with a positive regulatory role for these receptors in synaptic 5-HT homeostasis. This inhibition of SSRI-induced extracellular 5-HT accumulation in mice with no functional 5-HT_{2B} receptors likely reflects a lack of positive control exerted by 5-HT signaling in raphe neurons. Indeed, *In vivo* microdialysis experiments performed with the agonist BW723C86 confirm that local 5-HT_{2B} receptor activation in the raphe nuclei is sufficient to induce extracellular 5-HT accumulation (9). Moreover, stimulation of 5-HT_{2B} receptors appears to be presynaptic since BW723C86-induced acute behavioral effects are abolished in Pet-1^{-/-} mice (a genetic model of 5-HT depletion) or in SERT^{-/-} mice.

Together, these data identify, for the first time, 5-HT_{2B} receptors as possible positive 5-HT autoreceptors, acting in an opposing manner to the 5-HT_{1A} and 5-HT_{1B} receptors, which are well established as negative autoreceptors (36). This concept is consistent with previous electrophysiology studies showing reduced or abolished 5-HT-dependent depolarization of serotonergic neurons by 5-HT₂ receptor antagonists (ketanserin or mesulergine) in the presence of the 5-HT_{1A} receptor antagonist (WAY100635) (37), but extensive electrophysiological studies are required to fully test this hypothesis. Moreover, given that no specific anti-mouse 5-HT_{2B} receptor antibody is currently available (each giving different results as well as residual labeling in 5-HT_{2B}^{-/-} mice), we are not currently able to evaluate whether these autoreceptors are localized presynaptically or in the soma of the raphe serotonergic neurons. This last point could have important physiological consequences in the mode of action of the receptor.

A similar lack of acute and chronic effects were obtained in mice in which the 5-HT_{2B} receptor was pharmacologically blocked or genetically ablated, confirming that effects seen in

mutant mice are not a consequence of developmental compensation. Notably, the baseline response in the NSF test is altered in both groups of mice and might result from specific settings established as a consequence of chronically inactive 5-HT_{2B} receptors. The SSRI-reduced latency to feed in the NSF is associated with anxiolytic-like effects. The ability of 5-HT_{2B}^{-/-} mice to show anxiolysis clearly persists, as evidenced by their response to diazepam. The fact that both 5-HT_{2B} receptor agonist and antagonist induced a similar decrease in the latency to feed appears paradoxical. Nevertheless, all the results obtained herein for BW723C86 are consistent with antidepressant-like actions, whereas the response induced in the NSF test by RS127445 may be a consequence of the impulsive phenotype already described for these animals (6) rather than an anxiolytic effect. Moreover, the fact that basal neurogenesis is unaltered in either WT mice chronically treated with RS127445 or in 5-HT_{2B}^{-/-} mice is more consistent with an effect of impulsivity in the NSF in mice with non functional 5-HT_{2B} receptors than an antidepressant-like action.

The recent report that a single-nucleotide polymorphism (SNP) introducing a stop codon at the beginning of the human 5-HT_{2B} receptor (Q20*) is associated with psychiatric diseases marked by high impulsivity and suicidal behavior (6) supports a possible action of 5-HT_{2B} receptors on serotonergic tone. Herein, we show that the presence of a functional 5-HT_{2B} receptor is required for behavioral and neurogenic SSRI actions. One of the unanswered questions about antidepressant effects remains the lack of explanation for the limited percentage of patients responding to chronic treatment (1). Interindividual variation in antidepressant efficacy is thought to be at least partly genetic. A number of studies have tested the association between allelic variations in candidate genes of the serotonergic pathway involved in the synthesis, transport, recognition or degradation of 5-HT and the response to various SSRIs. However, even in positive studies (i.e., long/short alleles in the SLC6A4/SERT promoter) (38), these polymorphisms do not explain all of the variance seen in clinical responses, suggesting the involvement of variants within other genes. To date, none

of these studies investigated the potential role of the 5-HT_{2B} receptor. We believe that our data pinpoint the need to investigate the association between treatment response to SSRIs and SNPs in the 5-HT_{2B} receptor gene. Interestingly, 70% of the Q*20 male cases displayed impulsive suicidal behavior, and 66% had at least one life-threatening suicide attempt by age 33.5 (6). Thus, it would be interesting to collect and analyze clinical data related to SSRI responses from these cases. Moreover, 5-HT_{2B} receptor mutant mice appear to be a useful animal model for studying the clinical resistance to SSRI antidepressant effects.

The contribution of 5-HT_{2B} receptors to the effects of SSRI antidepressants is undoubtedly demonstrated by the results presented above, and agonist stimulation of this receptor induces SSRI-like effects. However, it is clear that 5-HT_{2B} receptor agonists cannot be used as antidepressant molecules, given established harmful cardiopulmonary peripheral effects of such compounds (39, 40). Despite notable advances, many patients with anxiety/depressive disorders fail to adequately respond to existing pharmacologic treatments. Augmenting antidepressant efficacy with other drugs has generated considerable clinical interest. Our study demonstrating that behavioral and neurogenic SSRI effects are abolished after pharmacologic inactivation of 5-HT_{2B} receptors should be taken into account when designing polypharmacology treatment strategies, given that actions mediated by this receptor might hinder the desired effects. As novel antidepressants are being developed to both ameliorate efficacy of treatments and increase the number of responders, the positive contribution of the 5-HT_{2B} receptor to serotonergic homeostasis should be considered.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. The 5-HT_{2B} receptor is necessary for the chronic behavioral effects of SSRIs

Chronic behavioral responses to antidepressants were evaluated by the Novelty-Suppressed Feeding test (NSF). Given the decreased basal (Veh) latency shown by 5-HT_{2B}^{-/-} mice in the NSF test (**a**, **b**), their ability to respond in this paradigm was evaluated by acute administration of an anxiolytic (**a**): diazepam (0.5 mg/kg) significantly decreased latency to feed in both WT and 5-HT_{2B}^{-/-} mice (**b**). Chronic treatment (4 weeks) with Flx (3 mg/kg/day) or Prx (2 mg/kg/day) significantly decreased latency to feed in WT mice, but no effects were detected in 5-HT_{2B}^{-/-} mice. (**c**) Similarly, Flx did not induce any change in the latency to feed in WT mice implanted with s.c. pumps chronically delivering the selective 5-HT_{2B} antagonist RS127445 (1 mg/kg/day for 4 wks). (**d**) In addition, chronic administration of the 5-HT_{2B} receptor agonist BW723C86 (3 mg/kg/day for 4 weeks) significantly decreased the latency to feed in WT mice, but no effects were detected in 5-HT_{2B}^{-/-} mice. *** p<0.0001; ** p <0.01; * p <0.05 compared to WT-Veh group; + p <0.05 compared to 5-HT_{2B}^{-/-}-Veh group. Data are expressed as mean ± SEM; n = 5-6 (**a**); 11-14 (**b**); 8-10 (**c**); 8-9 (**d**) mice per group. See also Figure S2.

Figure 2. Hippocampal neurogenic effects of SSRIs are impaired in the absence of 5-

HT_{2B} receptors. (**a**) Photographs correspond to representative BrdU immunolabeling in the SGZ 24 h after BrdU administration to the 4 experimental groups (**a**; scale bar, 50µm). The SSRIs Flx (3 mg/kg/day) or Prx (2 mg/kg/day), administered daily for 4 weeks, induced a significant increase in cell proliferation in the SGZ of WT mice, but no effect was observed in 5-HT_{2B}^{-/-} mice. (**b**) Similarly, Flx did not modify BrdU labeling in WT mice implanted with s.c. pumps delivering the selective 5-HT_{2B} antagonist RS127445 (1mg/kg/day) for 4 weeks. (**c**) Chronic treatment with BW723C86 (3 mg/kg/day) for 4 weeks significantly increased cell proliferation in the SGZ of WT mice, but no effects were detected in 5-HT_{2B}^{-/-} mice. (**d**) Cell

survival in the cell granular layer was also analyzed 4 weeks after BrdU administration. Animals received Flx or Veh for 4 weeks before BrdU treatment followed by 4 more weeks of Flx or Veh. Again, only WT mice showed a significant increase in cell survival after the Flx+Flx treatment, with no significant change in 5-HT_{2B}^{-/-} mice. *** p <0.001; ** p <0.01; * p <0.05 comparing to WT-Veh group. Data are expressed as mean ± SEM; n = 4-6 (a); 5-8 (b); 5-9 (c); 5-7 (d) mice per group. See also Figure S3.

Figure 3. SSRI-induced increase in extracellular 5-HT concentration is blunted when 5-HT_{2B} receptors are absent. After 4 h of equilibration, the SSRI Prx (2 mg/kg, i.p.) was administered at time zero (0): hippocampal samples obtained by microdialysis were collected each 20 minutes for 3 h, and 5-HT concentrations were measured by HPLC (a). The peak observed at one hour after Prx injection was significantly higher in WT mice than in 5-HT_{2B}^{-/-} mice or RS127445-pretreated WT mice (15 min before Prx injection). Inset represents the area under the curve (AUC) for each experimental group. Basal extracellular serotonin levels were (mean ± SEM, n=3-4) 0.28 ± 0.09 nM in WT mice, 0.23 ± 0.09 nM in 5-HT_{2B}^{-/-} mice, and 0.3 ± 0.1 nM in RS127445-pretreated WT mice (a). See also Figure S3. (b) Analysis of SC-PCR products obtained from single GFP-positive raphe neurons of Pet-1-GFP mice, illustrating a negative (Neuron1-representative of 2 out of 12) and a positive (Neuron2-representative of 10 out of 12) example of the expression of 5-HT_{2B} receptors; both neurons classes express Tph2 enzyme and 5-HT_{1A} receptors. (c) Acute administration of Flx (10 mg/kg) or BW723C86 (10 mg/kg) induced a significant decrease in immobility times in the FST in WT (C57Bl/6NCrl) littermate mice but this effect disappeared in Pet-1^{-/-} mice (c) and in SERT^{-/-} mice (d). * p <0.05; *** p <0.001. Data are expressed as mean ± SEM; n = 3-5 (a); 6-10 (c); 8-10 (d) mice per group.

Figure 4. Expression, affinity, and functionality of 5-HT_{1A} receptors are similar in WT and 5-HT_{2B}^{-/-} mice. (a) Expression of the 5-HT_{1A} receptor was evaluated by qPCR in raphe nucleus and hippocampus and no significant difference was found between WT and 5-HT_{2B}^{-/-} mice. (b) As well, binding assay with the 5-HT_{1A} agonist 8-OH-DPAT on membranes prepared from the raphe and hippocampus showed no differences between genotypes. (c) *In situ* hybridization of 5-HT_{1A} receptors in the raphe (top: scale bar, 50µm; bottom: scale bar, 5µm) and in the hippocampus (scale bar, 50µm) of WT and 5-HT_{2B}^{-/-} mice show similar patterns and levels of expression in either genotype. (d) 8-OH-DPAT (0.5 mg/kg, i.p.) induced a similar time-dependent hypothermic response in both, WT and 5-HT_{2B}^{-/-} mice. (e) In the FST, different doses of 8-OH-DPAT (0.5, 1, and 5 mg/kg, i.p.) induced similar responses in WT and 5-HT_{2B}^{-/-} mice. * p <0.05; ** p <0.01; data is expressed as mean ± SEM; n = 4 (a-b); 8-9 (d); 7-10 (e) mice per group.

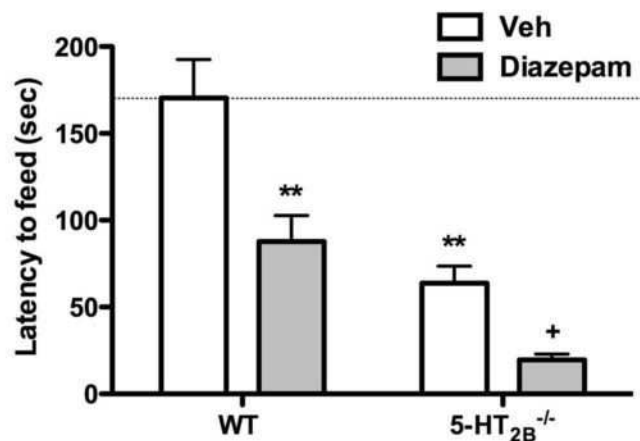
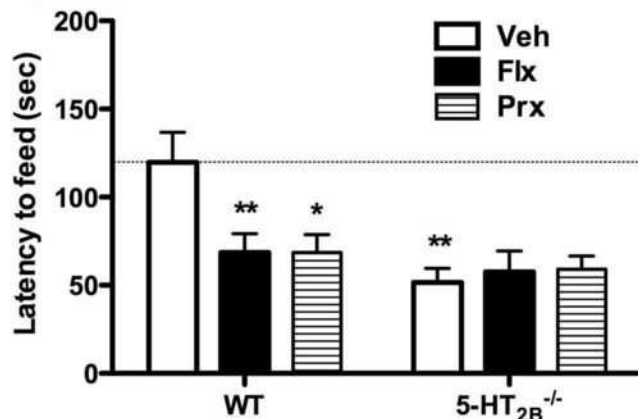
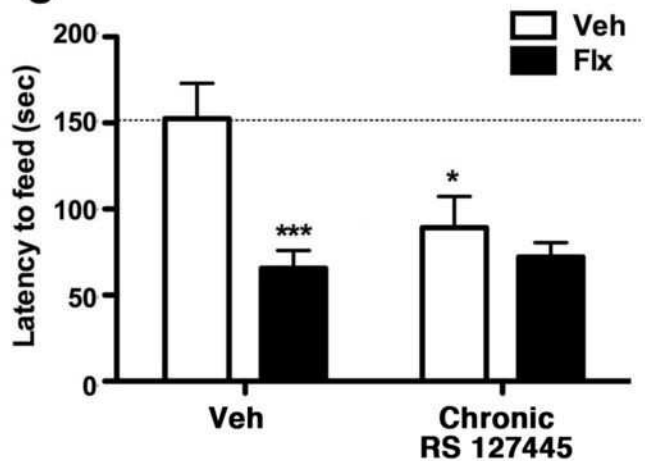
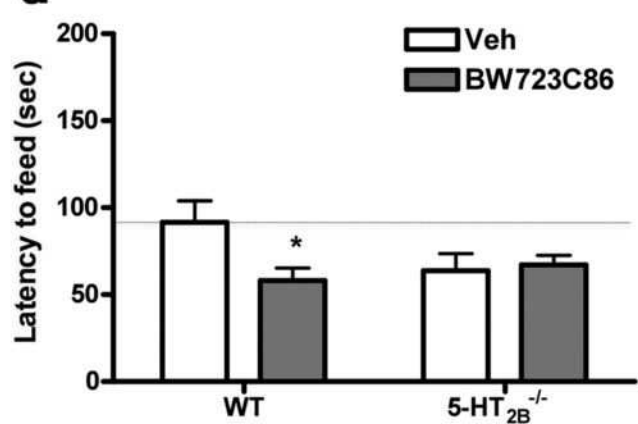
Figure 1**a****b****c****d**

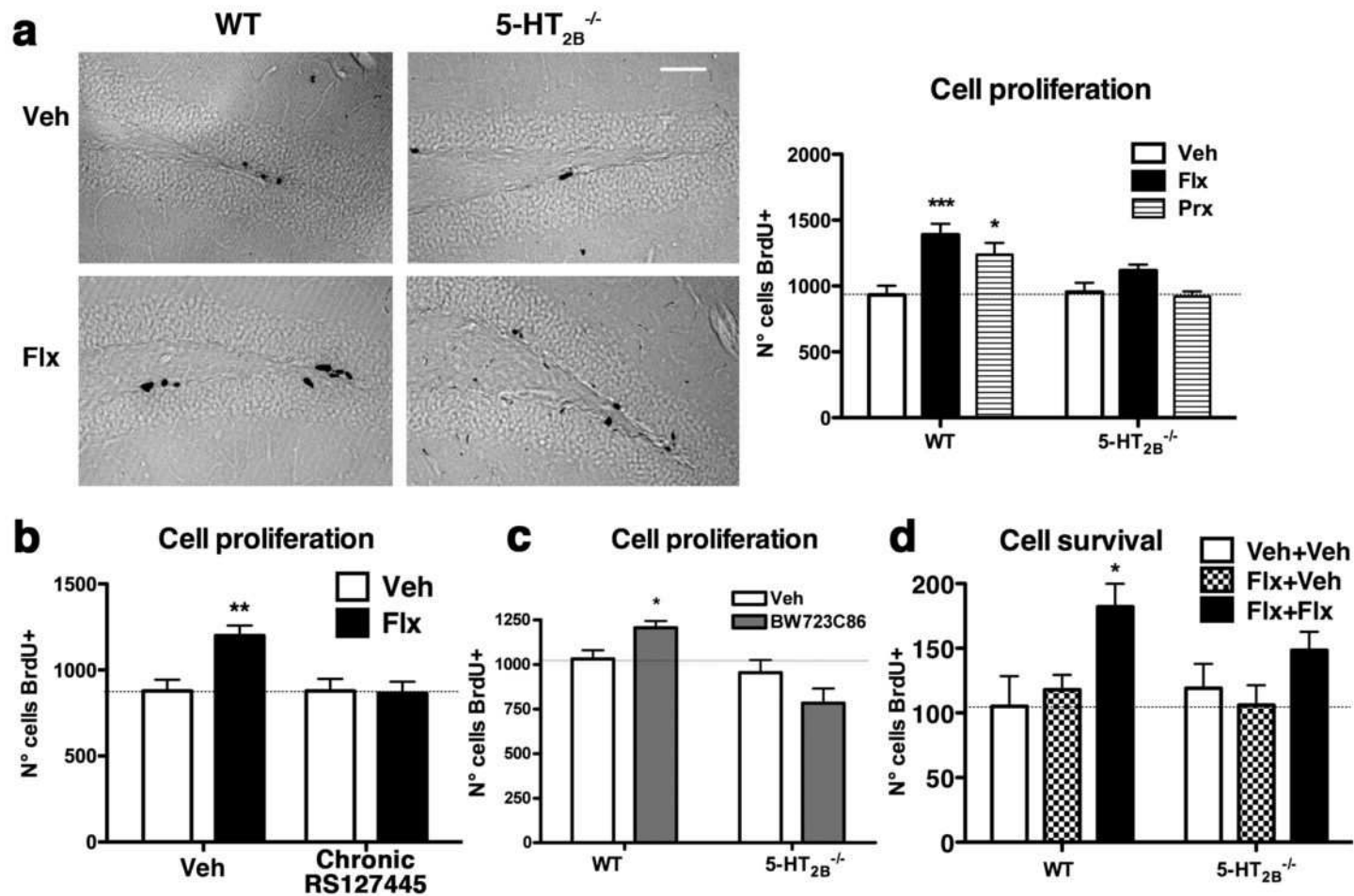
Figure 2

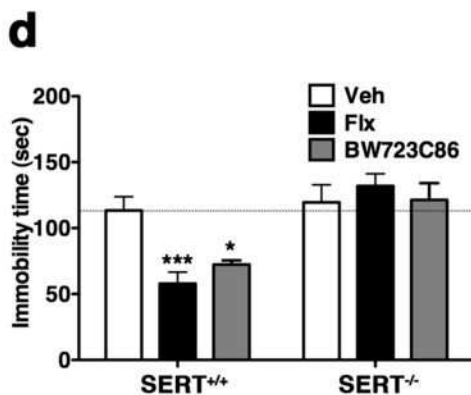
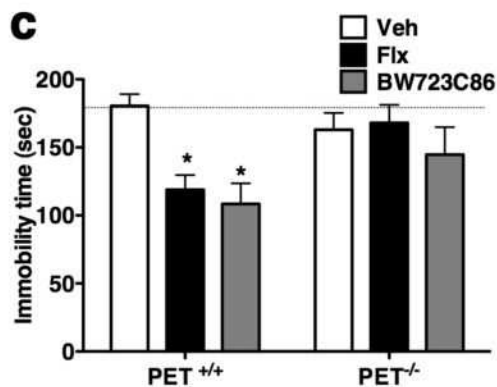
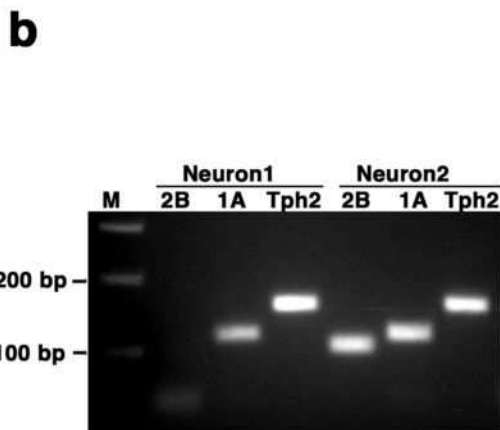
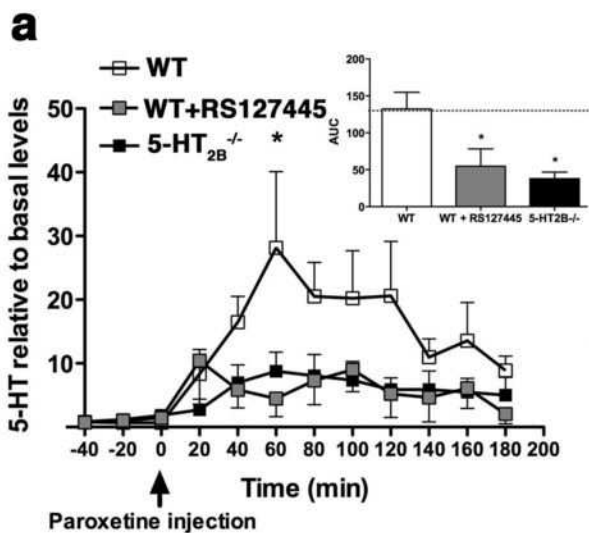
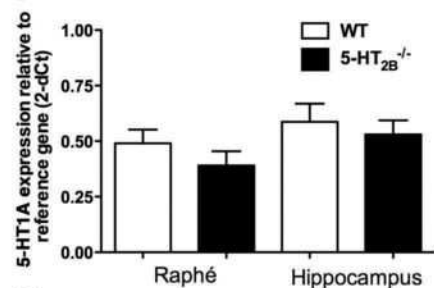
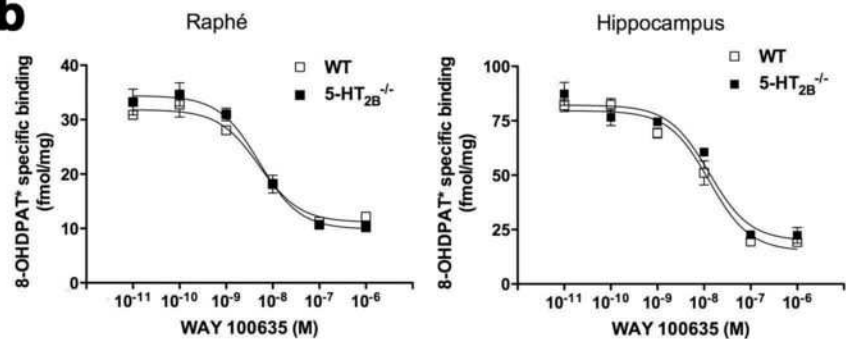
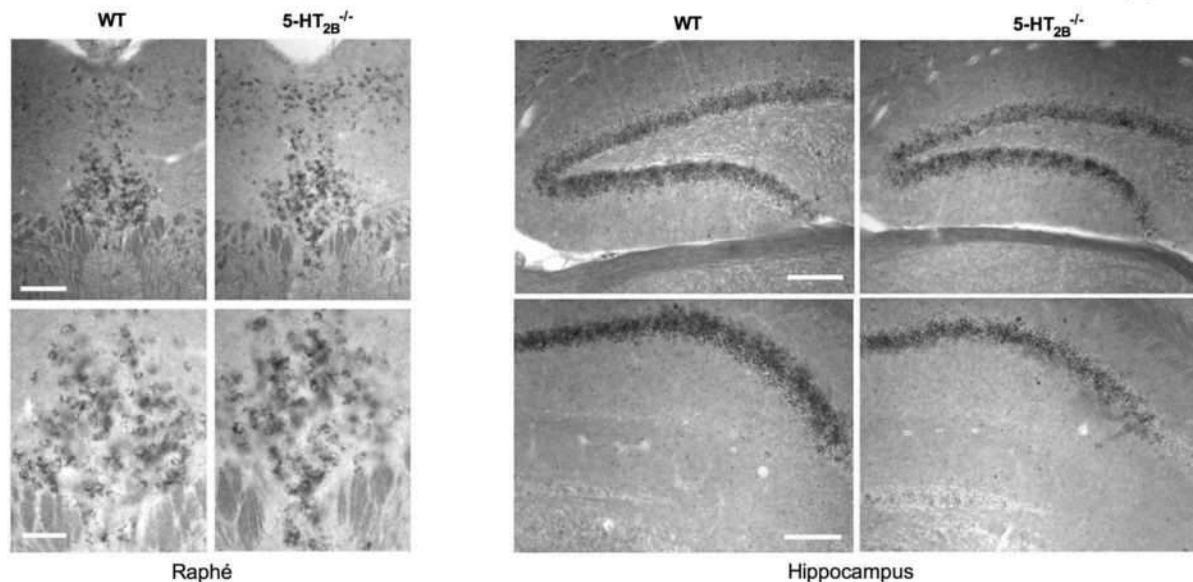
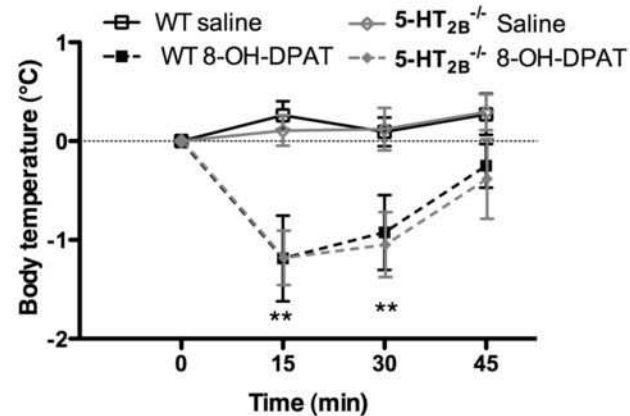
Figure 3

Figure 4

a**b****c****d****e**