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ARTICLE

Positive regulation of raphe serotonin neurons by serotonin 2B receptors

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Serotonin is a neurotransmitter involved in many psychiatric diseases. In humans, a lack of 5-HT_{2B} receptors is associated with serotonin-dependent phenotypes, including impulsivity and suicidality. A lack of 5-HT_{2B} receptors in mice eliminates the effects of molecules that directly target serotonergic neurons including amphetamine derivative serotonin releasers, and selective serotonin reuptake inhibitor antidepressants. In this work, we tested the hypothesis that 5-HT_{2B} receptors directly and positively regulate raphe serotonin neuron activity. By ex vivo electrophysiological recordings, we report that stimulation by the 5-HT_{2B} receptor agonist, BW723C86, increased the firing frequency of serotonin Pet1-positive neurons. Viral overexpression of 5-HT_{2B} receptors in these neurons increased their excitability. Furthermore, in vivo 5-HT_{2B}-receptor stimulation by BW723C86 counteracted 5-HT_{1A} autoreceptor-dependent reduction in firing rate and hypothermic response in wild-type mice. By a conditional genetic ablation that eliminates 5-HT_{2B} receptor expression specifically and exclusively from Pet1-positive serotonin neurons (*Htr2b*$^{5\text{-}HTKO}$ mice), we demonstrated that behavioral and sensitizing effects of MDMA (3,4-methylenedioxy-methamphetamine), as well as acute behavioral and chronic neurogenic effects of the antidepressant fluoxetine, require 5-HT_{2B} receptor expression in serotonergic neurons. In *Htr2b*$^{5\text{-}HTKO}$ mice, dorsal raphe serotonin neurons displayed a lower firing frequency compared to control *Htr2b*$^{lox/lox}$ mice as assessed by in vivo extracellular recordings and a stronger hypothermic effect of 5-HT_{1A}-autoreceptor stimulation was observed. The increase in head-twitch response to DOI (2,5-dimethoxy-4-iodoamphetamine) further confirmed the lower serotonergic tone resulting from the absence of 5-HT_{2B} receptors in serotonin neurons. Together, these observations indicate that the 5-HT_{2B} receptor acts as a direct positive modulator of serotonin Pet1-positive neurons in an opposite way as the known 5-HT_{1A}-negative autoreceptor.

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is involved in many psychiatric diseases including depression, addiction, impulsivity, or psychosis. The 5-HT neurons that innervate forebrain originate predominantly from the rostral cell group of neurons in the dorsal raphe nucleus (DRN) [1, 2]. These neurons express the serotonergic markers tryptophan hydroxylase (TPH2) and 5-HT transporter (SERT), and also the negative autoreceptors, 5-HT_{1A} and 5-HT_{1B} receptors, whose expression is restricted to somatodendritic compartments of 5-HT neurons, and to axonal terminals, respectively [3]. The 5-HT_{1A} autoreceptor activation elicits an outward current carried through G protein-coupled inwardly rectifying potassium channels (GIRK) of the Kir3 family leading to membrane hyperpolarization and inhibition of 5-HT neuron firing [4]. The presence of synaptic vesicles in dendrites of 5-HT neurons led to the suggestion that autoinhibition is mediated via dendritic release of 5-HT, for a review see Andrade et al. [5]. However, activity of 5-HT DRN neurons can also be positively modulated by 5-$HT_{2A/2B/2C}$ receptors triggering directly or indirectly inward currents [6–10]. Upon electrical stimulation of leech 5-HT neurons, transmembrane Ca^{2+} entry through L-type channels first evokes an early dendritic exocytosis; subsequently, the released 5-HT activates dendritic 5-HT_2 autoreceptors coupled to Gq and phospholipase C, resulting in a positive feedforward loop that maintains sustained exocytosis [11]. It has thus been proposed that DRN neurons can display responses ranging from inhibition to excitation depending on a balance of functional 5-HT_{1A} and 5-HT_2 receptors [12]. However, a direct action of 5-HT_2 receptor subtypes at 5-HT neurons has not yet been clearly established.

In humans, a loss-of-function polymorphism of 5-HT_{2B} receptors is associated with 5-HT-dependent phenotypes, including impulsivity and suicidality [13]. Pharmacological experiments indicated

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that the 5-HT_{2B} receptor preferential agonist BW723C86 [14] mimicked serotonin-specific reuptake inhibitor (SSRI) action in the forced swimming test (FST), which was abolished by injection of RS127445, a highly selective 5-HT_{2B} receptor antagonist, or in mice totally knocked out (KO) for 5-HT_{2B} receptor gene (*Htr2b*$^{-/-}$) [15]. Long-term behavioral and neurogenic SSRI effects were also abolished after either genetic ablation of 5-HT_{2B} receptors or chronic exposure to RS127445 [16]. Genetic (KO) or pharmacologic (antagonist) manipulation of 5-HT_{2B} receptors interferes similarly with effects of other molecules that target 5-HT neurons including amphetamine derivatives, 5-HT releasers MDMA (3,4-methylene-dioxy-methamphetamine) and dexfenfluramine [14, 17, 18]. For example, the locomotor response to the psychostimulant MDMA was abolished in *Htr2b*$^{-/-}$ or RS127445-treated wild-type mice [18]. Furthermore, local infusion of BW723C86 in DRN by microdialysis increased extracellular 5-HT that was blocked by RS127445, supporting a functional role of this receptor within the raphe and the selectivity of BW723C86 for this effect [18].

Together, these studies suggested that 5-HT_{2B} receptors could be implicated in 5-HT-dependent behavior by acting directly and positively onto the 5-HT neurons. Nevertheless, the precise localization and way of action of 5-HT_{2B} receptors are still poorly investigated. Main difficulties include a lack of specific antibody as well as low level of expression of 5-HT_{2B} receptors in mouse [19] or human brains [20, 21]. Nevertheless, expression of 5-HT_{2B} receptor mRNA was confirmed in several brain nuclei including DRN [13, 22]. Besides, previous study using single-cell reverse transcription PCR [16] permitted to establish a 5-HT_{2B} receptor expression not only in raphe but more specifically in 5-HT neurons. Nevertheless, precise role of 5-HT_{2B} receptors in 5-HT neurons remains elusive since pharmacologic manipulations or full gene KO mice do not distinguish direct effects on 5-HT neuron from circuit effects.

In this work, we tested the hypothesis of a direct positive regulation of 5-HT neurons activity by 5-HT_{2B} receptors. First, we established that stimulation of 5-HT_{2B} receptors can increase the firing frequency of 5-HT neurons, and counteract 5-HT_{1A} autoreceptor-dependent reduction in firing rate and hypothermic response in wild-type mice. Then, a conditional genetic ablation that eliminates 5-HT_{2B}-receptor expression specifically and exclusively from 5-HT neurons (*Htr2b*$^{5\text{-}HTKO}$ mice) demonstrated a lack of effects of drugs, including SSRIs and MDMA, targeting 5-HT neurons, which present a lower firing rate and a stronger 8-OHDPAT-induced hypothermic response revealing its importance to maintain serotonergic tone. These observations established the direct modulation of 5-HT neurons by 5-HT_{2B} receptors acting in an opposite way as to 5-HT_{1A}-negative autoreceptors.

MATERIALS AND METHODS

Generation and use of mice

KO mice, *129S2.Cg-Htr2b*^{tm1Lum}*/Htr2b*^{tm1Lum} (*Htr2b*$^{-/-}$), were generated on the 129S2/SvPas strain (see extended methods in Supplementary materials). Floxed mice, *Htr2b*^{tm2Lum}*/Htr2b*^{tm2Lum}, (*Htr2b*$^{lox/lox}$), were generated on a mixed B6;129S2 background and backcrossed >10 times onto the 129S2 strain. *Htr2b*$^{lox/lox}$ mice were inactivated for *Htr2b* in 5-HT neurons by crossing with *129S2.Cg-Tg(Fev-cre)*^{1Esd}*/0* (e*Pet1-Cre* BAC transgenic mice or *Pet1-Cre*$^{+}$*/0*) [23] generating *129S2.Cg-Pet1-Cre*$^{+}$*/0; Htr2b*^{lox}*/Htr2b*^{lox} conditional KO mice (*Htr2b*$^{5\text{-}HTKO}$) and littermate controls (*Htr2b*$^{lox/lox}$). Then, *129S2.Cg-Tg(ROSA)26Sor*$^{tm1(CAG-EGFP)Fsh}$ (Rosa26;CAG-loxP-STOP-loxP-EGFP or RCE) were crossed with *Pet1-Cre*$^{+}$*/0* generating (*Pet1-GFP*) that express GFP in Pet1-positive 5-HT neurons only after Cre recombination. All experiments involving mice were approved by the local ethical committee (No. 1170.02).

Ex vivo electrophysiology: cell attached

Electrophysiological recordings and molecular characterization of 5-HT neurons was conducted in acute brain slices from male *Pet1-GFP* mice aged 3–4 weeks old according to published procedures [24, 25].

Viral constructs and stereotaxic injection

To express HA-tagged 5-HT_{2B} receptor specifically in 5-HT neurons, we use a double floxed inverse orientation (DIO) adeno-associated virus (AAV) construct that allows Cre-mediated expression of the transgene (pAAV-EF1A-DIO-WPRE-pA vector; Addgene). The viruses packaged into AAV2.9 serotype with titers of 10^{12}–10^{13} viral particles/ml were obtained (UNC Vector Core, Chapel Hill, NC, USA). AAV2.9 DIO-TdTomato was used as a control. AAVs were injected in the B7 raphe nuclei of *Pet1-GFP* following described procedure [26].

Ex vivo electrophysiology: current clamp

Mice of either sex aged ~9 weeks were deeply anesthetized with ketamine and xylazine (150 and 10 mg/kg, respectively; intraperitoneally; Sigma-Aldrich, France) and immediately sacrificed. Coronal brain slices containing the raphe nucleus were prepared as previously described [27].

8-OHDPAT-induced hypothermia

Body temperature was measured intrarectally in mice every 10 min after the agonist injection during 40 additional minutes [28].

In vivo electrophysiology of DRN 5-HT neurons

The extracellular recordings of 5-HT neurons in DRN were performed in anesthetized mice (chloral hydrate, 400 mg/kg; intraperitoneally) according to Rainer et al. [29]. The DRN 5-HT neurons were identified according to the criteria of Aghajanian and Vandermaelen [30].

Locomotor response to novelty and MDMA-induced hyperlocomotion and sensitization

Locomotor activity was measured as previously described [18], in a circular corridor with four infrared beams placed at every 90° (Imetronic, Passac, France). MDMA-induced locomotor sensitization was performed in a two-injection protocol as previously described [17].

Prepulse inhibition of acoustic startle

Sensorimotor gating was assessed as previously described [31].

Forced swimming test

Mice FST was conducted as described [15].

Fluoxetine treatments, proliferation assay

Fluoxetine dose selection and FST were performed as described [15]. Neurogenesis study was performed as described [16].

Synaptosome study

Crude synaptosomes were prepared as previously described [32]. Synaptosomes 5-HT uptake and citalopram competition experiments were performed as described [18].

DOI-induced head-twitch

DOI-induced head-twitch experiment was performed in clear plastic cages lined with bedding. Head-twitches (lateral movements of the head from side to side) were induced by ±DOI (1, 3, and 5 mg/kg, intraperitoneally), and after 10 min, were scored for 10 min, by an experimenter unaware of mice genotype.

Statistical analysis

To determine differences between experimental groups, responses were analyzed by two-tailed unpaired Student's *t* test or Mann–Whitney test when distribution was not normal. Also, a

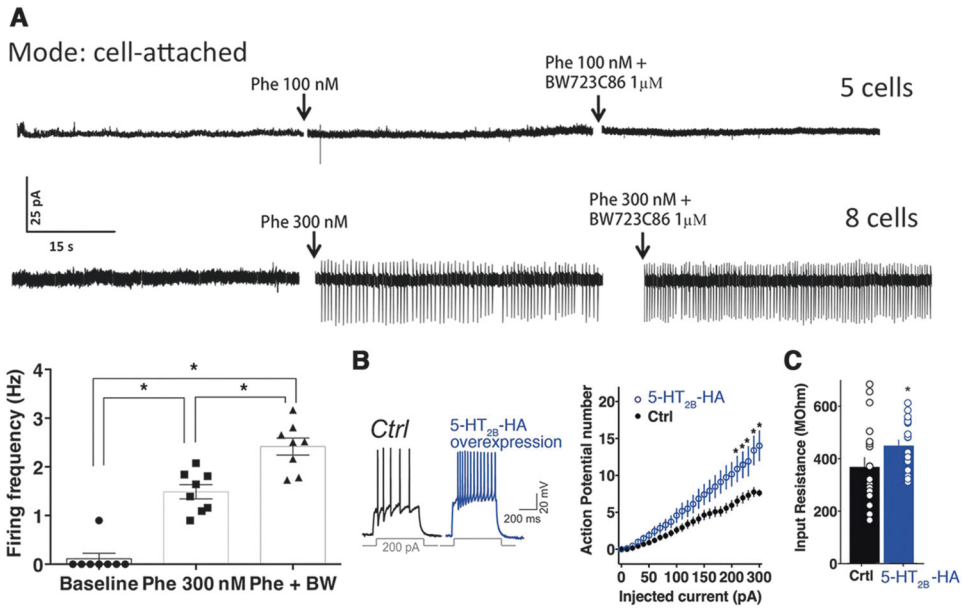

Fig. 1 Ex vivo electrophysiological recordings of wild-type *Pet1-GFP* mice. **a** Recordings of 5-HT neuron in ex vivo wild-type slices. Cell-attached recordings made in identified *Pet1-GFP* neuron did not detect firing in the presence of 100 nM phenylephrine (Phe) or of 1 µM of the 5-HT_{2B}-receptor agonist BW723C86 (top line—5 cells). In the presence of higher concentration of phenylephrine (300 nM), Pet1-positive neuron firing was observed, and BW723C86 (1 µM) was able to increase this firing rate (8 cells). Representative traces (top) and quantification (bottom left) reveal a significant BW723C86-induced increase in firing frequency (one-way repeated measures (RM) ANOVA; bar graph and scatter plots and mean ± SEM; Bonferroni post test, $^*P < 0.05$). **b** Current-clamp recordings of ex vivo raphe slices from AAVs injected *Pet1-GFP* mice, overexpressing 5-HT_{2B}-HA receptor in Pet1-positive 5-HT neuron. (Left) Sample traces of action potential for a +200 pA current step in two experimental groups. (Right) Quantification of the number of action potentials obtained in function of injected currents showed a significant increase in action potential number in Pet1-positive 5-HT neurons of mice overexpressing 5-HT_{2B}-HA receptor, compared to controls ($n = 20$ cells from three control mice and 21 cells from four 5-HT_{2B}-HA mice; two-way ANOVA RM, Bonferroni post test, $^*P < 0.05$; data are mean ± SEM). **c** Input resistance values. Bar graph and scatter plot for input resistance values showed that input resistance was also increased in mice overexpressing HA-5-HT_{2B} receptor in Pet1-positive 5-HT neuron (unpaired *t* test, $^*P < 0.05$; data are mean ± SEM)

one- or two-way analysis of variance (ANOVA) repeated measure (RM) when appropriate, with Bonferroni's test was used for post hoc multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Ex vivo electrophysiological responses to 5-HT_{2B}-receptor stimulation or overexpression

To examine the ability of 5-HT_{2B} receptors to regulate 5-HT neuron activity, we first performed cell-attached electrophysiological recordings of identified DRN 5-HT neurons in coronal slices of wild-type mice expressing GFP in Pet1-positive neurons (*Pet1-GFP* mice). In vivo, DRN neurons fire with a regular pattern varying from 0.5 to 5 Hz. 5-HT neurons in ex vivo coronal brain slices are electrically quiescent, but an excitatory noradrenergic tone facilitates firing [33]. In order to reproduce noradrenergic input, subsaturating concentrations of phenylephrine (Phe, 100–300 nM) were added to the bath [25]. Low concentration (100 nM) of Phe was unable to initiate regular firing nor was the application of a preferential 5-HT_{2B} receptor agonist BW723C86 (1 µM). Higher concentration of Phe (300 nM) was able to initiate and maintain a regular firing. Subsequent bath application of BW723C86 (1 µM) induced a significant increase in firing frequency (1.73 ± 0.23-fold) compared to Phe alone (Fig. 1a) (one-way ANOVA RM, effect of treatment, $F_{1.6,\ 11.0} = 98$; $P < 0.0001$, $n = 8$ cells). Bonferroni's post hoc analysis showed a significant increase in firing frequency between each group (Fig. 1a). These data suggested that 5-HT_{2B}-receptor stimulation could increase Pet1-positive raphe 5-HT neuron firing activity.

To further establish that 5-HT_{2B} receptors can affect 5-HT neuron activity, we overexpressed a HA-tagged 5-HT_{2B} receptor specifically in Pet1-positive neurons, using a DIO AAV construct that allows Cre-mediated expression of the tagged receptor. Viruses packaged into AAV2.9 serotype, AAV-DIO-5-HT_{2B}-HA or control AAV-DIO-TdTomato, were unilaterally injected into B7 raphe nucleus of *Pet1-GFP* mice (Fig. S1A). We confirmed the proper injection site by colocalization of either TdTomato expression or HA immunofluorescence with *Pet1-GFP*-positive neurons (Fig. S1B). Coronal raphe-containing brain slices were used to record *Pet1-GFP*-positive neuron excitability performed in current clamp mode. Recordings at various current steps showed that the number of action potentials obtained in function of the injected current was significantly increased in Pet1-positive neurons overexpressing 5-HT_{2B} receptors (two-way ANOVA RM, effect of overexpression, $F_{1,\ 39} = 4.94$, $P = 0.032$, $n = 20$ and 21 cells from 3 and 4 mice) (Fig. 1b). Input resistance value from recorded neurons was also significantly increased in 5-HT_{2B}-HA-overexpressing neurons (unpaired *t* test, $t_{39} = 2.05$, $P = 0.047$) (Fig. 1c). Ex vivo recordings indicated that the 5-HT_{2B}-receptor overexpression increased Pet1-positive neuron excitability.

In vivo responses to 5-HT_{1A} and 5-HT_{2B} receptor agonists

Since our initial ex vivo results indicated that 5-HT_{2B} receptors could increase 5-HT neuron activity, we hypothesized that it could work in an opposite way as to 5-HT_{1A}-negative autoreceptor. We thus tested the effect of BW723C86 on 5-HT_{1A} receptor agonist 8-OHDPAT-induced inhibition of neuronal firing frequency in vivo. Interestingly, 8-OHDPAT was significantly less potent in suppressing putative 5-HT neuron firing activity after BW723C86 injection as shown by two-way ANOVA RM (main effect of BW723C86 treatment, $F_{1,\ 9} = 7.34$; $P = 0.024$, $n = 6$–5 mice). Bonferroni's post hoc analysis revealed a reduced effect of 8-OHDPAT at 50 and 100 µg/kg on putative 5-HT neuron firing activity (Fig. 2a). 8-OHDPAT

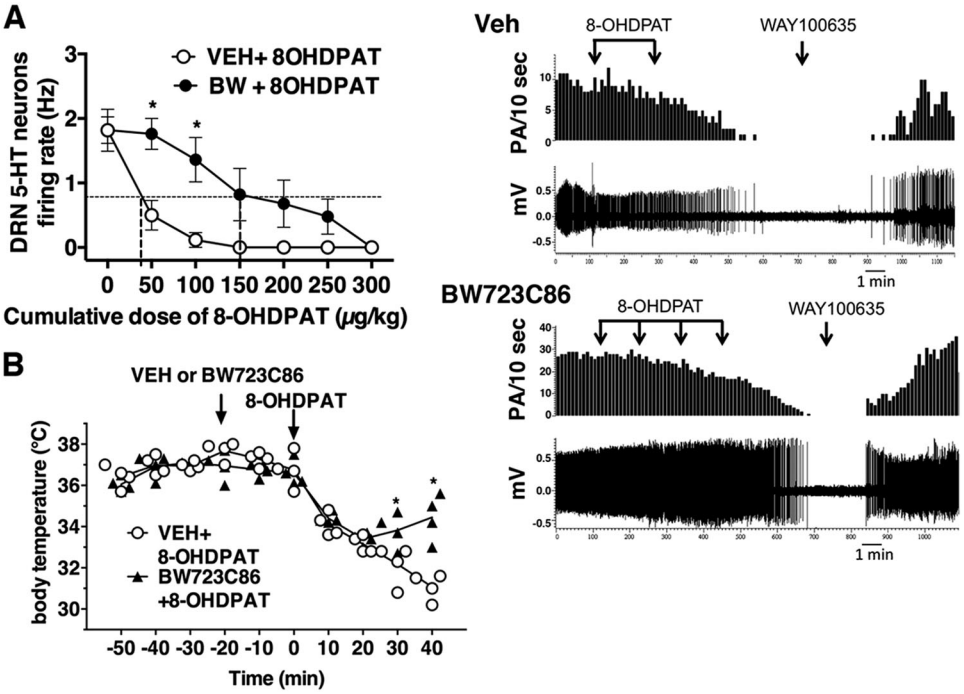

Fig. 2 In vivo response of wild-type mice to combined 5-HT_{2B} and 5-HT_{1A} receptor agonists. **a** In vivo extracellular electrophysiological recordings of putative raphe neurons in anesthetized wild-type mice. (Left) BW723C86 (5 mg/kg subcutaneously (s.c.)) injected 20 min before test counteracted the 5-HT_{1A} agonist 8-OHDPAT (0.05 mg/kg s.c.) inhibitory cumulative effects to putative 5-HT neuron firing ($n = 6$–5 mice per group; two-way ANOVA RM, followed by Bonferroni's multiple comparisons test, $^*P < 0.05$; data are mean ± SEM). (Right) Examples of typical recordings of putative DRN 5-HT neurons obtained in each experimental group. Each arrow represents an injection of 8-OHDPAT (0.05 mg/kg s.c.). The injection of the 5-HT_{1A} receptor antagonist WAY100635 (0.3 mg/kg s.c.) completely reversed the inhibitory effect of 8-OHDPAT. **b** In vivo hypothermic effects. The 5-HT_{2B}-receptor agonist BW723C86 (5 mg/kg s.c.) injected 20 min before the test was able to counteract 5-HT_{1A} agonist 8-OHDPAT (0.3 mg/kg s.c.) hypothermic effects on wild-type mice (scattered plot, $n = 4$–4 mice two-way ANOVA RM, followed by Bonferroni's multiple comparisons test, $^*P < 0.05$)

ED50 was shifted about 3.3-fold from 45 to 148 µg/kg; BW723C86 alone did not modify firing (not illustrated).

Although the mechanism of 5-HT_{1A} receptor agonist-induced hypothermia is incompletely understood [34], the 8-OHDPAT-induced hypothermia in mice is known to be mediated by 5-HT_{1A} autoreceptor [35]. We thus used the 8-OHDPAT-induced hypothermia as another in vivo readout of the functional status of 5-HT_{1A} autoreceptor and tested putative effects of stimulating 5-HT_{2B} receptors. In agreement with the above-mentioned effect on firing, a pretreatment of wild-type mice with BW723C86 (5 mg/kg s.c,) was able to significantly reduce the ability of 8-OHDPAT (0.3 mg/kg s.c.), to induce hypothermia in wild-type mice (Fig. 2b), as shown by two-way ANOVA RM analysis (interaction between the time and treatment, $F_{9,\ 54} = 10.34$; $P < 0.0001$, $n = 4$ mice per group). Bonferroni's post hoc analysis showed a significant reduction by BW723C86 at 30 and 40 min post-injection of the 8-OHDPAT-induced hypothermia (Fig. 2b). These data indicated that 5-HT_{1A} autoreceptor inhibitory activities can be attenuated by concomitant activation of 5-HT_{2B} receptors.

Absence of response to ecstasy, MDMA, in *Htr2b*$^{5\text{-}HTKO}$ mice

To establish putative direct actions of 5-HT_{2B} receptors on 5-HT neurons, we developed mice with conditional ablation of this receptor gene specifically in Pet1-positive neurons. We inserted recombination sites (loxP) flanking the second exon of *Htr2b* gene (*Htr2b*$^{lox/lox}$) and crossed these mice with mice expressing the Cre recombinase under *Pet1* gene promoter (BAC transgenic). *Pet1* gene expression in the brain is restricted to most of differentiated 5-HT neurons and their postmitotic precursors [23]. We thus generated *Pet1-Cre*$^{+}$*/0; Htr2b*$^{lox/lox}$ mice (*Htr2b*$^{5\text{-}HTKO}$) (Fig. S2A). Restricted recombination in raphe 5-HT neurons was revealed by analysis of raphe DNA and by colocalization of a Cre-dependent GFP reporter with 5-HT staining (Fig. S2B–C) [23]. We quantified the efficiency of recombination and found that 83.6 ± 6.1% of the Pet1-Cre-dependent GFP was colocalized with 5-HT staining, 11.6 ± 3.9% was only 5-HT positive and <5% (4.8 ± 2.9%) was only GFP positive ($n = 12$ from 3 different mice), supporting an efficient and specific recombination in 5-HT neurons (Fig. S2C).

MDMA, the active compound of ecstasy, is a substrate of SERT leading to massive 5-HT release from synaptic vesicle stores, which is partially calcium-dependent [36]. The *Htr2b*$^{5\text{-}HTKO}$ mice injected with MDMA (20 mg/kg) did not display increased locomotor responses in contrast to control *Htr2b*$^{lox/lox}$ littermate mice (Fig. 3a, b). Two-way ANOVA RM analysis showed a main effect of genotype ($F_{1,\ 30} = 4.22$; $P = 0.049$, $n = 16$ mice per group). Bonferroni's post hoc analysis showed a significant difference between genotypes from $t = 25$ to $t = 45$ min post-injection (Fig. 3a). The analysis of the total locomotor activity over the first 60 min after MDMA injection confirmed the significant effect of genotypes (two-way ANOVA RM, $F_{1,\ 60} = 4.61$; $P = 0.036$, $n = 16$ mice per group). Bonferroni's post hoc analysis showed a significant increase in locomotion in control *Htr2b*$^{lox/lox}$ mice, but not in *Htr2b*$^{5\text{-}HTKO}$ mice (Fig. 3b). We further assessed the contribution in Pet1-positive neurons of 5-HT_{2B} receptors to MDMA-induced locomotor sensitization using a two-injection protocol [37]. A significant increase in locomotor activity was observed in *Htr2b*$^{lox/lox}$ control mice as shown by two-way ANOVA RM analysis (main effect of genotype, $F_{1,\ 14} = 5.12$; $P = 0.04$). Bonferroni's post hoc analysis showed a significant increase in locomotion in control *Htr2b*$^{lox/lox}$ mice at the second injection of MDMA (20 mg/kg) (first injection 639 ± 222 vs. second injection 1,291 ± 295 1/4 of turns, $n = 8$), but not in *Htr2b*$^{5\text{-}HTKO}$ (first injection 254 ± 132 vs. second injection 353 ± 174, $n = 8$) (Fig. 3c). These results indicated a lack of behavioral and sensitizing effects

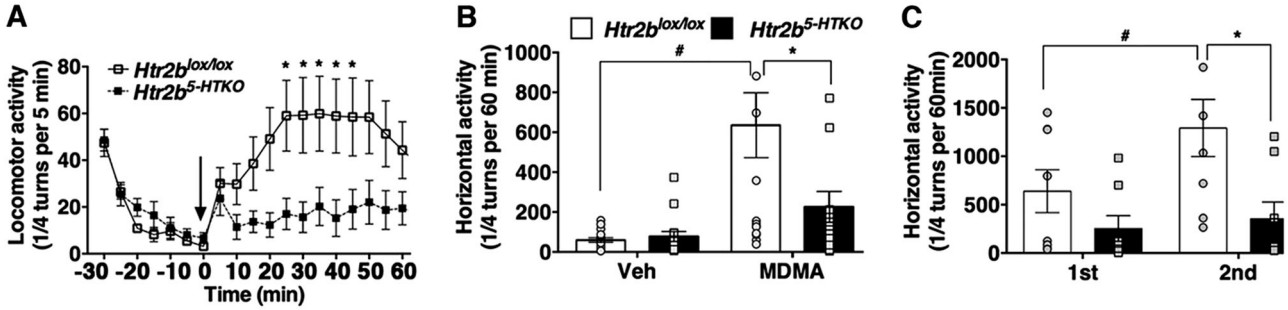

Fig. 3 Behavioral response to the 5-HT releaser MDMA in *Htr2b*$^{5\text{-}HTKO}$ mice. **a** MDMA-induced locomotion. Mice were injected with MDMA (20 mg/kg i.p.) (arrow) after 30 min habituation. A lack of MDMA-induced locomotion was observed in *Htr2b*$^{5\text{-}HTKO}$ mice, while control *Htr2b*$^{lox/lox}$ mice showed a clear increase in locomotion. Data between −30 to +60 min were analyzed using two-way ANOVA RM (means ± SEM, $n = 16$ mice per group) and a Bonferroni post test was applied on each graph, $^{*}P < 0.05$. **b** Cumulative MDMA-induced locomotion. Cumulative locomotion during the first hour following MDMA injection showed a significant difference between the two genotypes. Data were analyzed using two-way ANOVA ($n = 16$–16 mice, scattered plot, mean ± SEM). A Bonferroni post test was also applied on each graph ($^{*}P < 0.05$ *Htr2b*$^{5\text{-}HTKO}$ vs. *Htr2b*$^{lox/lox}$; $^{\#}P < 0.05$ MDMA vs. Veh). **c** Locomotor sensitization by two MDMA injection protocol. The stimulant effect of a challenge dose of MDMA (20 mg/kg i.p.) 7 days after the first (2nd) was significantly enhanced compared to the first injection in control *Htr2b*$^{lox/lox}$ mice, while it had no effect in *Htr2b*$^{5\text{-}HTKO}$ mice. Data were analyzed using two-way ANOVA RM ($n = 8$–8 mice, scattered plot, mean ± SEM). Bonferroni post test was also applied on each graph ($^{*}P < 0.05$ *Htr2b*$^{5\text{-}HTKO}$ vs. *Htr2b*$^{lox/lox}$; $^{\#}P < 0.05$ 1st vs. 2nd injection)

of MDMA in *Htr2b*$^{5\text{-}HTKO}$ mice as found in full KO, *Htr2b*$^{-/-}$ mice, confirming the need for this receptor in Pet1-positive 5-HT neurons.

Absence of response to the SSRI fluoxetine in *Htr2b*$^{5\text{-}HTKO}$ mice

SSRIs are known to block 5-HT reuptake by SERT, leading to extracellular 5-HT accumulation following vesicular 5-HT release. A classical outcome for acute response to SSRIs is the reduced immobility time observed in FST. We tested the effect of acute fluoxetine injection (3 mg/kg intraperitoneally (i.p.)), the optimal dose determined for 129S2 strain in FST (Diaz et al., 2011). Fluoxetine injection did not affect immobility time in *Htr2b*$^{5\text{-}HTKO}$ mice (Fig. 4a) as shown by two-way ANOVA analysis (time and genotype interactions, $F_{1,\ 24} = 10.67$; $P < 0.003$, $n = 7$–7). However, a significant reduction of immobility time was observed in control *Htr2b*$^{lox/lox}$ littermates as Bonferroni's post hoc analysis showed the only significant difference between vehicle and fluoxetine-treated *Htr2b*$^{lox/lox}$ control mice (Fig. 4a).

Long-term effects of SSRI are known to be associated with hippocampus subgranular zone (SGZ) neuronal proliferation. We performed a daily i.p. injection for 4 weeks of fluoxetine (3 mg/kg), bromodeoxyuridine (BrdU) injections the 27th day, and quantified BrdU incorporation in SGZ neurons of the hippocampus at the 28th day. Chronic injection of fluoxetine did not produce changes in BrdU incorporation in *Htr2b*$^{5\text{-}HTKO}$ mice (Fig. 4b) (two-way ANOVA analysis showed a trend for treatment and genotype interactions, $F_{1,\ 26} = 3.88$; $P = 0.059$, $n = 7$–8). An increase was detected in control *Htr2b*$^{lox/lox}$ littermates as Bonferroni's post hoc analysis showed the only significant difference between vehicle and fluoxetine-treated *Htr2b*$^{lox/lox}$ controls (Fig. 4b). These results indicated that the lack of both acute behavioral and chronic neurogenic effects of SSRIs found in *Htr2b*$^{-/-}$ mice was due to 5-HT_{2B}-receptor elimination from Pet1-positive neurons.

We recently reported that *Htr2b*$^{-/-}$ mice displayed novelty-induced hyperlocomotion and a global deficit in sensorimotor gating [31]. However, locomotor activity in a new environment was not different between *Htr2b*$^{5\text{-}HTKO}$ and *Htr2b*$^{lox/lox}$ littermate control mice over the first 60 min (Fig. S3A). Similarly, prepulse inhibition (PPI) of startle reflex and startle amplitude were not different between *Htr2b*$^{5\text{-}HTKO}$ and *Htr2b*$^{lox/lox}$ littermate control mice (Fig. S3B). Although present in *Htr2b*$^{-/-}$ mice, these deficits were not found in mice lacking the 5-HT_{2B} receptor selectively in 5-HT neurons, supporting the specificity of these conditional mice.

Since SERT-targeting drug (MDMA and fluoxetine) action is affected by the lack of 5-HT_{2B} receptors in Pet1-positive neurons, we next determined possible alterations of SERT expression or function in brain synaptosome preparations from *Htr2b*$^{5\text{-}HTKO}$ mice. Heterologous competition binding experiments showed no difference in the density of citalopram binding sites (B_{max} 305 ± 18 vs. 389 ± 26 fmol/mg of protein) or affinity (K_i 0.4 ± 0.1 vs. 0.17 ± 0.06 nM) between *Htr2b*$^{5\text{-}HTKO}$ mice and their *Htr2b*$^{lox/lox}$ littermate control mice (Fig. 4c). Additionally, 5-HT uptake experiments on brain synaptosomes showed no difference in 5-HT transport maximum velocity (V_{max}; 5.57 ± 0.32 vs. 6.49 ± 0.38 fmol/sample/min) or apparent affinity (K_m 40.7 ± 2.6 vs. 27.7 ± 2.5 nM) between *Htr2b*$^{5\text{-}HTKO}$ mice and their *Htr2b*$^{lox/lox}$ littermate controls (Fig. 4c). Together, these results demonstrated that selective ablation of 5-HT_{2B} receptors in raphe Pet1-positive neurons eliminates MDMA and fluoxetine actions but does not affect SERT expression and activity.

Htr2b$^{5\text{-}HTKO}$ mice display a hyposerotonergic phenotype

To determine whether the 5-HT_{2B} receptor had an overall effect on 5-HT neurons, we measured firing rates of putative 5-HT DRN neurons in vivo in *Htr2b*$^{5\text{-}HTKO}$ mice. Neurons were included in this analysis based on characteristics and averaged traces of their action potentials [25]. *Htr2b*$^{5\text{-}HTKO}$ mice display a higher percentage of putative 5-HT neurons discharging with a low firing mode (16.1% vs. 9.3% < 1 Hz) and a lower percentage of neurons with a high firing mode (5.9% vs. 13.9% > 4 Hz) relative to *Htr2b*$^{lox/lox}$ mice. This observation was reflected by a significant shift in cumulative distribution of neurons with lower firing rate in *Htr2b*$^{5-HTKO}$ mice compared to control *Htr2b*$^{lox/lox}$ mice ($n = 108$ and 118 neurons, respectively; Kolmogorov–Smirnov test; $P = 0.0008$) (Fig. 5a). These results revealed that ablation of 5-HT_{2B} receptors in raphe Pet1-positive neurons is sufficient to modify 5-HT neuron firing rate.

Head-twitch response is a rhythmic paroxysmal rotational head movement that occurs in mice and rats treated by a variety of serotonergic hallucinogens, including LSD and DOI [38]. This behavior is specifically linked to 5-HT_{2A} receptor activation, since selective 5-HT_{2A} receptor antagonists block head-twitch response induced by DOI and other hallucinogens, and it is absent in *Htr2a*$^{-/-}$ mice [38]. Here, we tested ±DOI at 1, 3, and 5 mg/kg i.p. We found that a dose of 5 mg/kg induced a larger increase in head-twitch in *Htr2b*$^{5\text{-}HTKO}$ mice, as compared to *Htr2b*$^{lox/lox}$ littermate control (+109%, $t_{13} = 2.68$, $n = 3$–5, $P = 0.016$, multiple *t* test) (Fig. 5b). Similarly, a greater head-twitch response was observed in *Htr2b*$^{-/-}$ compared with control mice at the same dose (Fig. S4A). Nevertheless, no significant change in 5-HT_{2A} or 5-

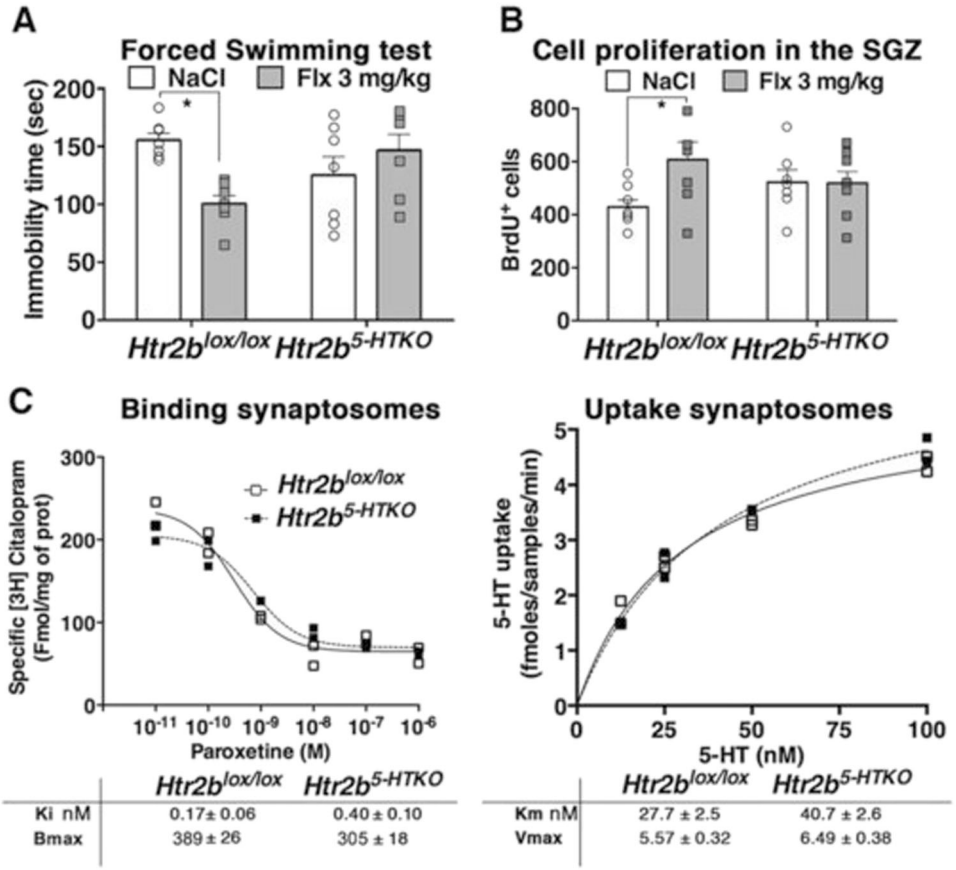

Fig. 4 Antidepressant action in *Htr2b*$^{5\text{-}HTKO}$ mice. **a** Forced swimming test (FST). The time spent immobile in the FST was significantly reduced in control *Htr2b*$^{lox/lox}$ mine but not in *Htr2b*$^{5\text{-}HTKO}$ mice 30 min after SSRI antidepressant fluoxetine (Flx 3 mg/kg i.p.) injection (two-way ANOVA, Bonferroni post tests, $^{*}P < 0.05$; $n = 7–7$ mice, scattered plots with mean ± SEM). **b** Neurogenesis in subgranular zone (SGZ) of the hippocampus. Fluoxetine (3 mg/kg/day i.p.), daily injected for 4 weeks, induced a significant increase in BrdU incorporation in neuron of the SGZ of control *Htr2b*$^{lox/lox}$ mice, but had no effect in *Htr2b*$^{5\text{-}HTKO}$ mice (two-way ANOVA, Bonferroni post test; $^{*}P < 0.05$; $n = 7–8$ mice, scattered plots with mean ± SEM). **c** SERT expression and function. (Left) SERT expression in conditional *Htr2b*$^{5\text{-}HTKO}$ and *Htr2b*$^{lox/lox}$ control mice was evaluated using heterologous competition binding assays of [^{3}H]citalopram on synaptosome membranes prepared from whole brain. No differences in the affinity (K_i) or expression (B_{max}) between *Htr2b*$^{5\text{-}HTKO}$ and *Htr2b*$^{lox/lox}$ genotypes were observed. (Right) Saturation isotherms for [^{3}H]5-HT uptake of this synaptosomal preparation were similar in conditional *Htr2b*$^{5\text{-}HTKO}$ and *Htr2b*$^{lox/lox}$ control mice. Nonlinear regression analysis did not reveal differences in the Km or *V*max. Shown are representative curves of at least two independent experiments performed in duplicates. Individual values are presented

HT_{1A} receptor expression or in 5-HT content and turnover was found in PFC from *Htr2b*$^{-/-}$ compared to control mice (Fig. S4B–D).

Since a drastic increase in the number of DOI-induced head-twitch was observed in the *Tph2-R439H* knock-in mouse [34], a mouse model with 60–80% reduction in TPH2 activity and thus with low serotonergic tone, the increased behavioral response to DOI confirmed a lower serotonergic tone in *Htr2b*$^{5\text{-}HTKO}$ mice.

Finally, we used the 8-OHDPAT-induced hypothermia as an in vivo readout of the functional status of 5-HT_{1A} autoreceptor in *Htr2b*$^{5\text{-}HTKO}$ mice. The hypothermic response to 8-OHDPAT (0.1 mg/kg s.c.) was significantly increased in *Htr2b*$^{5\text{-}HTKO}$ mice compared to control *Htr2b*$^{lox/lox}$ mice as shown by two-way ANOVA RM analysis (interaction between time and treatment, $F_{4,36} = 5.30$; $P = 0.0018$, $n = 3–8$ mice). Bonferroni's *post hoc* analysis showed a significantly stronger hypothermic effect at 20 min post-injection of the 8-OHDPAT (Fig. 5c). These data supported that 5-HT_{1A} autoreceptor inhibitory activity is increased upon inactivation of 5-HT_{2B} receptors in Pet1-positive neurons.

DISCUSSION

Our results show that 5-HT_{2B} receptors can positively modulate 5-HT neuron activity in brain slices of wild-type mice, and counteract 5-HT_{1A}-negative autoreceptor actions. Furthermore, mice lacking 5-HT_{2B} receptors exclusively in Pet1-positive 5-HT neurons, *Htr2b*$^{5\text{-}HTKO}$ mice, display an excess of inhibitory control exerted by 5-HT_{1A} autoreceptors supporting an opposite control of 5-HT_{2B} receptors on 5-HT neurons.

5-HT_{2B} receptors positively regulate 5-HT neurons

There is a growing consensus that 5-HT neurons are non-homogeneous as supported by anatomical, biochemical, and electrophysiological studies [12, 24, 39, 40]. Sub-populations of 5-HT neurons, either within the DRN or between various raphe nuclei, are interconnected, and form complex circuits [41–43]. The activity of 5-HT neurons can be modulated by both 5-HT_{1A} and 5-$HT_{2A/2B/2C}$ receptors [7, 9]. Identified 5-HT neurons are known to respond to 5-HT_{1A} receptor agonists by a 5-HT-induced outward current [7–9, 44]. A significant proportion of TPH2-positive neurons (about 50%) also respond to 5-HT_2-receptor activation by an inward current [12]. Tonic spiking of 5-HT neurons establishes synaptic 5-HT levels. We show here, by cell-attached recordings of identified wild-type raphe Pet1-positive neurons, that stimulation of 5-HT_{2B} receptors by BW723C86 can increase their firing frequency. The effect on Pet1-positive neuron firing frequency of the 5-HT_{2B} receptor agonist can only be detected in

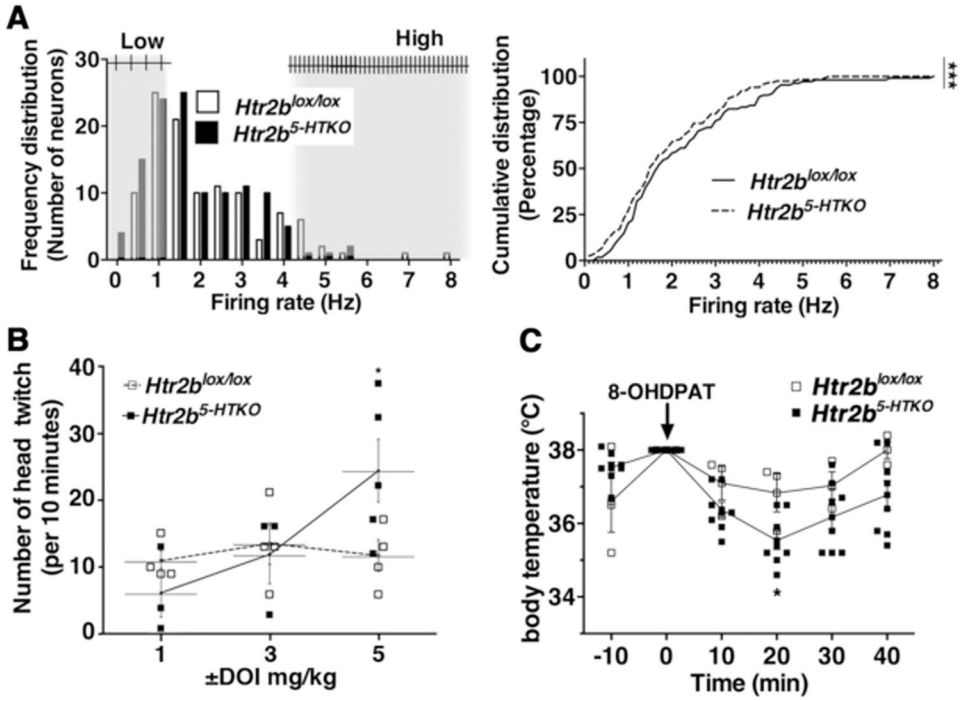

Fig. 5 Hyposerotonergic phenotype of $Htr2b^{5-HTKO}$ mice. **a** In vivo extracellular recordings of putative DRN 5-HT neurons in anesthetized mice. (Left) The firing frequency of individual putative DRN 5-HT neuron was shifted from high to low firing rates in $Htr2b^{5-HTKO}$ mice. (Right) A shift in cumulative distribution was observed with significantly lower firing rate in $Htr2b^{5-HTKO}$ mice than in control $Htr2b^{lox/lox}$ mice ($n = 108$ and 118 neurons, respectively; Kolmogorov–Smirnov test; $^*P < 0.05$). **b** Head-twitch dose–response to DOI. Control $Htr2b^{lox/lox}$ and $Htr2b^{5-HTKO}$ mice were i.p. injected with ±DOI (1, 3, and 5 mg/kg i.p.). Ten minutes later, the head-twitch response was scored for 10 min. DOI-induced head-twitch response was significantly increased in conditional $Htr2b^{5-HTKO}$ ($^*P < 0.05$; multiple *t* test) compared to littermate control mice at 5 mg/kg of DOI (data are presented as scattered plot, $n = 3$–5 mice per group, and means ± SEM). **c** In vivo hypothermic effects of 8-OHDPAT. The hypothermic response to 8-OHDPAT (0.1 mg/kg s.c.) was significantly stronger in $Htr2b^{5-HTKO}$ mice compared to control $Htr2b^{lox/lox}$ mice ($n = 3$–8 mice, scattered plot, two-way ANOVA RM, followed by Bonferroni's multiple comparisons test, $^*P < 0.05$)

the presence of Phe (300 nM), suggesting that although the two receptors share common Gq signal transduction, they are likely acting at different effectors that remains to be identified. Independent electrophysiological current-clamp recordings showed that overexpression of 5-HT_{2B} receptors in Pet1-positive 5-HT neurons was sufficient to increase their excitability. These results indicate that 5-HT_{2B} receptors can positively control the firing of 5-HT neurons. This was confirmed in vivo by extracellular recordings in $Htr2b^{5-HTKO}$ mice of putative 5-HT neurons that showed a significant shift to low firing rate. Together, the present results revealed a need for 5-HT_{2B} receptors in 5-HT neurons to positively regulate their activity.

Serotonergic tone results from an opposite control exerted by 5-HT_{1A} and 5-HT_{2B} receptors

The lack of effects of MDMA and fluoxetine in the absence of 5-HT_{2B} receptors in Pet1-positive 5-HT neurons, previously observed in full KO mice $Htr2b^{-/-}$ associated to reduced extracellular 5-HT accumulation as assessed by microdialysis [16, 18], raised the possibility of an interaction of 5-HT_{2B} receptors with SERT. However, the absence of modification in SERT uptake and expression lowers this possibility. The unique control of dendritic 5-HT release has important implications for DRN physiology and actions of SERT-targeting drugs, SSRIs and MDMA. Packaging by the vesicular monoamine transporter 2 is essential for 5-HT transmission; glutamate receptor activation in dorsal raphe brain slice can evoke somatodendritic release by vesicle exocytosis [45]. SSRI antidepressants, which have a more limited effect on 5-HT release from dendrites than from the soma and terminals, markedly increase extracellular 5-HT in DRN that involves both somatic and dendritic release [45]. The 5-HT released within DRN induces feedback inhibition of 5-HT neuron firing activity by stimulation of somatodendritic 5-HT_{1A}-negative autoreceptors, which results from local release rather than extended diffusion of 5-HT throughout the extracellular space [46]. We found that the hypothermic response to 8-OHDPAT, known to be mediated by 5-HT_{1A} autoreceptors but not heteroreceptors in mice [35], is attenuated by pretreatment with the 5-HT_{2B} receptor agonist BW723C86. However, we never observed an effect of the 5-HT_{2B}-receptor agonist in the absence of 5-HT_{1A}-receptor agonist. These observations support either a dominant effect of 5-HT_{1A} over 5-HT_{2B} receptors or combination of 5-HT_{2B} autoreceptors and heteroreceptors having opposite actions. Richardson-Jones et al. [35] generated a mouse strain differing in 5-HT_{1A} autoreceptor expression by approximately 30–40% below the wild-type level (1A-Low). These 1A-low mice showed reduced 8-OHDPAT-induced hypothermia and their neurons exhibit a shift toward higher firing rates. To the opposite, in the absence of 5-HT_{2B} receptors in Pet1 neurons (in $Htr2b^{5-HTKO}$ mice), we found a significant increase in hypothermic response to 8-OHDPAT and a significant shift toward lower frequency firing neurons. These findings support that the lack of 5-HT_{2B} receptor in Pet1-positive 5-HT neurons is associated with a higher 5-HT_{1A}-autoreceptor reactivity and thus a lower activity of 5-HT neurons. The lower serotonergic tone observed in the absence of 5-HT_{2B} receptors in Pet1-positive neurons would thus result from the opposite control exerted by 5-HT_{1A} and 5-HT_{2B} receptors on DRN neurons. This may explain the lack of actions of SERT-targeting drugs, SSRIs and MDMA, although the detailed mechanism remains to be identified. An interaction between 5-HT_{1A} and 5-HT_{2B} receptors directly or via trans-

regulation could be involved since we previously reported such cross-talks between 5-HT_{1B} and 5-HT_{2B} receptors [47].

5-HT_{2B} receptors contribute to SSRI therapeutic effects

The excess of inhibitory control exerted by 5-HT_{1A} receptors in the absence of 5-HT_{2B} receptors in Pet1-positive 5-HT neurons may also explain the lack of response to chronic SERT blockers (fluoxetine) in *Htr2b*$^{5\text{-}HTKO}$ mice. Chronic SSRI antidepressant responses are at least partially ascribed to desensitization of somatodendritic 5-HT_{1A} receptors [35]. Recent works using chemogenetic approaches (i.e., Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)) showed that CNO activation of SERT-positive or Pet1-positive neurons expressing the Gq-coupled M3Gq DREADD induced an increase in 5-HT neuron firing rate and a reduction in immobility in FST [48, 49]. We showed previously that activation of 5-HT_{2B} Gq-coupled receptors with BW723C86 mimicked both acute and chronic behavioral and neurogenic effects of SSRI antidepressants and led to extracellular 5-HT accumulation, which were eliminated in *Htr2b*$^{-/-}$ mice or by RS127445 [16, 18]. We found here that knocking out the 5-HT_{2B} receptors exclusively from Pet1-positive neurons (*Htr2b*$^{5\text{-}HTKO}$) mice is sufficient to eliminate behavioral effects in FST and neurogenic effects of fluoxetine and that 5-HT_{2B}-receptor overexpression increases Pet1-positive neuron excitability. It appears thus that 5-HT_{2B} receptors contribute to SSRI therapeutic effects by their positive Gq-dependent signaling on adult raphe 5-HT neurons, which may be revealed upon somatodendritic 5-HT_{1A}-receptor desensitization.

The 5-HT neuron firing relies on a balance of functional 5-HT_{1A} and 5-HT_{2B} receptors

The reason why positive 5-HT_{2B} receptors acting in an opposite manner to negative 5-HT_{1A} autoreceptors has not been previously identified could have several explanations. Recently, the role for 5-HT_{1A} receptor-mediated autoinhibition of the DRN in homeostatic control of firing rate has been questioned. As discussed by Andrade et al. [5], 5-HT_{1A} autoinhibition may participate in regulating glutamate signaling to 5-HT neurons [50] or in mediating inputs from distal serotonergic cell groups [43]. De Kock et al. [51] first showed that following calcium influx through *N*-methyl-D-aspartate receptor (NMDA) receptors, 5-HT could be released from DRN neuron dendrites in the absence of postsynaptic firing. Colgan et al. [50] reported that 5-HT release from dendrites is secondary to calcium influx through L-type calcium channels that open in response to the local dendritic depolarization elicited by synaptically released glutamate. A contribution of dendritic 5-HT release to 5-HT_{1A}-autoreceptor activation would thus result from excitatory glutamatergic inputs to DRN via locally triggered calcium influx rather than by neuronal firing. Independently, it has been reported that upon electrical stimulation of leech 5-HT neurons, transmembrane Ca^{2+} entry through L-type channels can first evoke an early dendritic exocytosis; subsequently, the released 5-HT activates 5-HT_2 autoreceptors coupled to Gq and phospholipase C, resulting in a positive feedforward loop that maintains sustained exocytosis [11]. In frog motoneurons, a potentiation of NMDA-induced depolarization has been shown to depend on the activation of 5-HT_{2B} receptors causing an influx of extracellular Ca^{2+} through L-type Ca^{2+} channels and a reduction of the open-channel block of NMDA receptors [52]. Since serotonergic DRN neurons can respond to 5-HT with responses ranging from inhibition to excitation with the net effect of 5-HT relying on a balance of functional 5-HT_{1A} and 5-$HT_{2A/2B/2C}$ receptors [12], combined expression levels of these receptors in various 5-HT subpopulations may set-up DRN firing levels. Since DRN receives serotonergic inputs from caudal raphe nuclei [43], 5-HT released in DRN may also originate from extrinsic serotonergic afferents. Our results show that 5-HT_{2B} receptors can positively modulate 5-HT neuron activity, and counteract 5-HT_{1A}-negative autoreceptor actions. Furthermore, our recent unpublished data revealed a somatodendritic expression of 5-HT_{2B} receptors as 5-HT_{1A} receptors. Whether 5-HT_{2B} receptors act at different 5-HT neurons subtypes, from different raphe nuclei, or by direct interaction with 5-HT_{1A} receptors, remain to be determined.

Concluding remarks

A remaining question is why both positive and negative autoreceptors are needed to regulate 5-HT neuron activity. In locus coeruleus (LC), the major noradrenergic nucleus of the brain, resting membrane potential, and pattern of spontaneous firing neurons in newborns are affected by both α1-adrenergic and α2-adrenergic receptors activation [53]. Application of α2-adrenergic receptor agonists activates GIRK channels, resulting in membrane hyperpolarization and inhibition of spontaneous firing of action potentials [54]. By contrast, stimulating α1-adrenergic receptors, known to couple to a Gq protein, induces membrane depolarization and accelerates spontaneous firing rates in neonatal rat LC neurons [55], whose alterations may trigger pathological conditions [53]. Mixtures of positive and negative feedback has been modeled and appears to be necessary to create oscillatory signal outputs [56], which are important for neuronal network functions such as DRN or LC. Our findings established that Gq-coupled 5-HT_{2B} receptors expressed by Pet1-positive 5-HT neurons act in an opposite manner as to 5-HT_{1A} autoreceptors. The 5-HT_{2B} receptor can thus be considered as a positive modulator of serotonergic tone that acts at 5-HT neuron excitability. This positive modulation has to be taken into account in the studies of the regulatory mechanisms of 5-HT neurons including those of antidepressants.

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AUTHOR CONTRIBUTIONS

AB, EQ, SLD, SPF, SD, SMB, PMP, IM, AM, and AT conducted the experiments; AR conducted and designed the experiments; BPG conducted, designed the experiments, and wrote the paper; MM supervised, wrote the paper, and provided funding; LM supervised, wrote the paper, and provided funding.

ADDITIONAL INFORMATION

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