Neurobiology of Disease

Serotonin 2B Receptors in Mesoaccumbens Dopamine Pathway Regulate Cocaine Responses

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Addiction is a maladaptive pattern of behavior following repeated use of reinforcing drugs in predisposed individuals, leading to lifelong changes. Common among these changes are alterations of neurons releasing dopamine in the ventral and dorsal territories of the striatum. The serotonin 5-HT_{2B} receptor has been involved in various behaviors, including impulsivity, response to antidepressants, and response to psychostimulants, pointing toward putative interactions with the dopamine system. Despite these findings, it remains unknown whether 5-HT_{2B} receptors directly modulate dopaminergic activity and the possible mechanisms involved. To answer these questions, we investigated the contribution of 5-HT_{2B} receptors to cocaine-dependent behavioral responses. Male mice permanently lacking 5-HT_{2B} receptors, even restricted to dopamine neurons, developed heightened cocaine-induced locomotor responses. Retrograde tracing combined with single-cell mRNA amplification indicated that 5-HT_{2B} receptors are expressed by mesolimbic dopamine neurons. *In vivo* and *ex vivo* electrophysiological recordings showed that 5-HT_{2B}-receptor inactivation in dopamine neurons affects their neuronal activity and increases AMPA-mediated over NMDA-mediated excitatory synaptic currents. These changes are associated with lower ventral striatum dopamine activity and blunted cocaine self-administration. These data identify the 5-HT_{2B} receptor as a pharmacological intermediate and provide mechanistic insight into attenuated dopamine tone following exposure to drugs of abuse.

Key words: **cocaine self-administration; electrophysiological recordings; mesolimbic dopamine neurons; mouse knock-out; retrograde tracing; serotonin receptors**

Significance Statement

Here we report that mice lacking 5-HT_{2B} receptors totally or exclusively in dopamine neurons exhibit heightened cocaine-induced locomotor responses. Despite the sensitized state of these mice, we found that associated changes include lower ventral striatum dopamine activity and lower cocaine operant self-administration. We described the selective expression of 5-HT_{2B} receptors in a subpopulation of dopamine neurons sending axons to the ventral striatum. Increased bursting *in vivo* properties of these dopamine neurons and a concomitant increase in AMPA synaptic transmission to *ex vivo* dopamine neurons were found in mice lacking 5-HT_{2B} receptors. These data support the idea that the chronic 5-HT_{2B}-receptor inhibition makes mice behave like animals already exposed to cocaine with higher cocaine-induced locomotion associated with changes in dopamine neuron reactivity.

Introduction

The serotonin [5-hydroxytryptamine (5-HT)] system is implicated in the establishment of drug use-associated behaviors via various receptors (for review, see Müller and Homberg, 2015). Addictive behaviors commonly involve neurons that release dopamine (DA) in the mesocorticolimbic system, as demonstrated via elegant and diverse investigations in both animals and humans (Hyman et al., 2006). This system comprises midbrain projections from the ventral tegmental area (VTA) and substantia nigra to cortical territories and subcortical limbic areas, including the dorsal striatum and nucleus accumbens (NAcc). DA neurons can be regulated through 5-HT receptors expressed in the VTA and NAcc (Hayes and Greenshaw, 2011). Both of these regions receive 5-HT projections from the dorsal raphe nucleus, which thereby can regulate DA neurotransmission (Di Giovanni et al., 2010). Cocaine binds with high affinity to DA, norepinephrine, and 5-HT transporters; blocks reuptake of these monoamines; and increases their extracellular concentrations in the brain. The 5-HT has also been shown to modulate cocaine action in both clinical and preclinical studies (Filip et al., 2010).

The 5-HT_2 receptors have been implicated as likely candidates for mediating the influence of 5-HT in cocaine abuse (De Deurwaerdère and Spampinato, 1999) as well as for limiting traits (e.g., impulsivity) that contribute to the development of cocaine-use disorder and relapse in humans (for review, see Howell and Cunningham, 2015). The role of 5-HT_{2B} receptors in cocaine addiction remains poorly understood. Recent pharmacological studies have shown that the injection of 5-HT_{2B}-receptor antagonists in rats had no effect on cocaine-induced DA outflow in the NAcc shell or core, or in the dorsal striatum (Devroye et al., 2015). In addition, the same group showed that 5-HT_{2B}-receptor antagonist treatment significantly reduced basal DA levels in the NAcc shell (Devroye et al., 2015), had no effect on the dorsal striatum, and increased DA levels in the prefrontal cortex (PFC; Devroye et al., 2016). They also showed that the preferential 5-HT_{1A}-receptor antagonist WAY 100635 blocks the changes in PFC and NAcc basal DA levels induced by 5-HT_{2B}-receptor antagonists, supporting interplay between PFC 5-HT_{1A} and 5-HT_{2B} receptors (Devroye et al., 2017). Despite these findings, it has remained unclear whether the 5-HT_{2B} receptor serves as a long-term modulator of the the DA/reward system and the mechanisms involved.

Here, we report that mice with permanent genetic ablation even restricted to DA neurons, or with long-term pharmacologic blockade (chronic antagonist treatment) of 5-HT_{2B} receptors, exhibit heightened cocaine-induced locomotor responses. Associated changes include lower ventral striatum DA activity and lower cocaine operant self-administration. Furthermore, a lack of this receptor increases the cocaine reactivity of VTA DA neurons projecting to the NAcc shell and the ratio of AMPA-mediated over NMDA-mediated EPSCs in these neurons. These data support the idea that the 5-HT_{2B} receptor expressed by DA neurons is an intermediate in drug-evoked plasticity.

Materials and Methods

Reagents

Cocaine, SKF 81297 [(±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide; Sigma-Aldrich], SB206553 [3,5-dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b*']dipyrrole-1(2*H*)-carboxamide],RS127445[2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine], and quinpirole hydrochloride (Tocris Bioscience) were slowly dissolved in 0.9% (w/v) NaCl solution (saline). All drugs were administered intraperitoneally (0.1 ml/10 g body weight). RS127445 was found to have subnanomolar affinity for the 5-HT_{2B} receptor ($pK_i = 9.5 \pm 0.1$) and ≥1000-fold selectivity for this receptor compared with numerous other receptors and monoamine uptake sites (Diaz et al., 2012). Based on initial studies showing that RS127445 completely (at 1, 0.5, and 0.1 mg/kg, i.p.) or partially (at 0.05 mg/kg) blocked 3,4-methylenedoxymethamphetamine (MDMA)-induced locomotion in mice and had no effect on basal locomotor activity (Doly et al., 2008, 2009; Banas et al., 2011; Diaz et al., 2012), we used 1 (data not shown) or 0.5 mg/kg dose, intraperitoneally, for the acute injection. Injections were administered 30 min before the test session in acute studies. *Htr2b*$^{+/+}$ mice chronically treated with RS127445 continuously received the 5-HT_{2B} antagonist at 1 mg/kg/d or vehicle (10 mM DMSO) via subcutaneous osmotic pumps (Alzet, model 2004) for 4 weeks.

Animal studies

Htr2b$^{-/-}$ mice (MGI:1888735) used in these experiments were maintained in a 129S2/SvPas (129S2) background. Wild-type 129S2 mice (8–10-week-old) used as a control group were derived from heterozygote crosses and were bred at our animal facility. Swiss-Webster mice carrying *drd2*-EGFP bacterial artificial chromosome transgenes were generated by the Gene Expression Nervous System Atlas program at Rockefeller University (MGI:3843608; Gong et al., 2003). Initial experiments evaluating the cocaine responses were performed independently on small groups of males and females. Since no statistical difference was found between genders, later groups were composed of males for other experiments. All mice were maintained on a 12 h light/dark schedule (lights on at 8:00 A.M.) and housed after weaning in groups of 3–5 of the same genetic background and sex. Mice were moved to the experimental room in their home cage ≥5 d before testing to allow for habituation to the environment and stayed there until the end of experiments. Behavioral studies were performed in the afternoon (2:00–8:00 P.M.). The observer was blind to experimental conditions being measured. Behavioral tests and animal care were conducted in accordance with standard ethical guidelines (National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and European Directive 2010/63/UE) and were approved by the local Ethics Committee for Animal Experiments (No. 01170.02).

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Experimental design and statistical analysis

The total number of animals per group was defined according to the SD and the difference score observed for each small group in a pilot experiment. Mice were randomly assigned to different experimental groups of 4–6 animals and independent experiments were performed ≥3 times. Normal distributions and homoscedasticity were verified by Shapiro–Wilk's test and Levene's test, respectively. Putative outliers were determined by the ROUT method. The statistical analysis was developed with the software Infostat and GraphPad Prism 6. Microdialysis and locomotor activity data were analyzed by two-way repeated-measures (RM) ANOVA with gene or drug treatment and time as factors. Food and cocaine self-administration was analyzed by three-way ANOVA with hole, day, and genotype as factors. Behavioral and biochemical assays were analyzed by two-way ANOVA with treatment and genotype as main factors. One-way ANOVA or *t* test and Bonferroni's test were used for *post hoc* comparisons depending on the experiment. $p < 0.05$ was predetermined as the threshold for statistical significance.

Generation of Htr2b floxed mice. Genomic contigs of *Htr2b* encompassing exon 1 and 2 and flanking sequence were obtained by screening of a 129S2 mouse genomic library. For the gene-targeting construct, a 10 kb BamHI-XhoI fragment containing the two first exons was selected, while a 4 kb SacI-SacI fragment containing exon 2, which includes the ATG start codon and 5′ UTR, was used to induce the targeted deletion. A LoxP site was inserted in the 5′-UTR and a neomycine-resistance cassette flanked by two LoxP sites in the ClaI site of the second intron. Then, the SacI-SacI fragment comprising the floxed construct was excised and electroporated into 129S2 embryonic stem (ES) cells, which were subjected to G418 selection. Targeted homologous recombination was confirmed by PCR and Southern blot analysis. A positive ES-cell clone was injected into C57BL/6NCrl blastocysts and implanted into pseudopregnant mice. A chimeric male displaying germ-line transmission was then used to propagate the floxed *Htr2b* (*Htr2b*$^{fl/fl}$) allele on a C57BL/6NCrl background for the first two generations. More than 10 backcrosses of *Htr2b*$^{fl/fl}$ mice with 129S2 (*Htr2b*$^{+/+}$) mice were performed. *Htr2b*$^{fl/fl}$ alleles were detected by PCR using the oligonucleotide F1: CTAACATTTTTCATCCA CATCTA as forward primer located in the 5′ UTR (position of the primers is indicated in Fig. 6*a*). Paired to this primer, the reverse primer R1: TCCCTCGAAGCTTATCGGCGCG, located in the 5′ end of the second intron, led to the amplification of a 1 kbp product in the presence of the *Htr2b*$^{fl/fl}$ allele, while the reverse primer R2: ACTTTAATTGGGACTC GCTGAT, located in the 3′ side of the ClaI site, permits amplification of a 309 bp amplicon indicative of the *Htr2b*-null allele.

DA neurons selective ablation of Htr2b. Adult *Htr2b*$^{fl/fl}$ mice were exclusively inactivated in DA neurons for *Htr2b* by crossing with DAT-Cre$^{+/0}$ (BAC–Slc6-icre) mice (Turiault et al., 2007). *Htr2b*$^{fl/fl}$;*DAT-Cre*$^{+/0}$ (*Htr2b*^{DAKO}) were generated on a mixed 129S2.B6 background used as F3 on 129S2 strain with littermate *Htr2b*$^{fl/fl}$;*DAT-Cre*$^{0/0}$ (*Htr2b*$^{fl/fl}$) as control mice. Identification of DA neurons was performed in *DAT-Cre*$^{+/0}$;*Gt(ROSA)26Sor*$^{tm1.1(CAG-EGFP)Fsh}$(*DAT-Cre*$^{+/0}$;*RCE*or*Dat-GFP*) mice, which expressed EGFP only after recombination [Jackson Laboratory; also named RCE:loxP mice, which harbor the R26R CAG-boosted EGFP (RCE) reporter allele with a loxP-flanked STOP cassette upstream of the enhanced green fluorescent protein (EGFP) gene: MGI: 4412373.

Locomotor activity. Locomotor activity was measured in an actimeter (circular corridor with four infrared beams placed at 90° intervals; Imetronic), as described previously (Doly et al., 2009). Counts were incremented by consecutive interruption of two adjacent beams (i.e., mice moving through one-quarter of the corridor). Mice were individually placed in the activity box for 30 min followed by an intraperitoneal injection of a saline solution and recorded for another 60 min over 3 consecutive days for habituation before all locomotor experiments. The day of the experiment, mice were injected with a saline solution or 5-HT$_{2B}$ antagonists (RS127445, 0.5 mg/kg, or SB206553, 3 mg/kg) and individually placed in the activity box for 30 min before being intraperitoneally injected with a saline or cocaine (7.5–20 mg/kg) solution and the locomotor activity was recorded for 2 h.

Locomotor sensitization. For locomotor sensitization in this study, we used a two-injection protocol shown to be as efficient as a repeated-injection protocol but involving much less handling of mice, thus minimizing stress and contextual habituation, which can be confounding factors (Valjent et al., 2010). After 30 min in the actimeter, mice received a first injection of cocaine (7.5, 15, or 20 mg/kg) and the locomotor activity was recorded for 2 h. Mice were then challenged 7 d later with a second injection of cocaine at the same concentration as the first injection, same injection protocol, and the locomotor activity was recorded for 2 h.

Microdialysis in freely moving mice. The microdialysis experiment was performed using awake mice as described previously (Doly et al., 2009). Initially, anesthetized animals were placed in a stereotaxic frame (David Kopf Instruments) and a stainless-steel guide cannula (CMA/12, CMA Microdialysis; outer diameter, 0.7 mm) was implanted in the NAcc. The cannula was then secured to the skull with dental cement, and the skin was sutured. Animals were kept in individual cages for a 7 d recovery period. Dialysis probes were equipped with a Cuprophan membrane (membrane length, 1 mm; diameter, 0.24 mm; cutoff, 5000 Da; Microdialysis). Using the Paxinos and Franklin (2001) atlas as our guide, we established stereotaxic coordinates for the NAcc as follows: 1.2 mm anteroposterior and 0.6 mm mediolateral relative to bregma, and 4.2 mm dorsoventral relative to the dura surface. After insertion, probes were perfused at a constant rate of 1 μl/min with artificial CSF containing the following: 154.1 mM Cl^-, 147 mM Na^+, 2.7 mM K^+, 1 mM Mg^{2+}, and 1.2 mM Ca^{2+}, adjusted to pH 7.4 with 2 mM sodium phosphate buffer. The microdialysis experiment was performed using awake mice. Dialysates were collected every 20 min. All measurements were performed 150 min after the beginning of perfusion, by which time a steady state was achieved. Mice were injected with cocaine (20 mg/kg, i.p.) 20 min after the beginning of measurements. At the end of the experiment, all brains were fixed in a 4% formaldehyde solution and serial coronal slices were cut on a microtome. Histological examination of cannula tip placement was subsequently made on 100 μm safranine-stained coronal sections. Dialysate samples were injected without any purification into an HPLC system that consists of a pump linked to an automatic injector (Agilent, 1100), a reverse-phase column (3.5 lm, 150·4.6 mm; Zorbax SB C18, Agilent Technologies), and a coulometric detector (Coulochem III, ESA Biosciences) with a 5011 analytical cell to quantify DA or 5-HT. The first electrode was fixed at −100 mV and the second electrode at +300 mV. The gain of the detector was set at 50 nA. The signal of the second electrode was connected to an HP Chemstation for HPLC. The composition of the mobile phase was 50 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.65 mM octyl sodium sulfate, and 14% (v/v) methanol, pH 3.5. The flow rate was set at 1 ml/min. The quantity of neurotransmitters was calculated based on standard injected in each HPLC run in the same range of concentrations as experimental samples.

Tissue preparation and immunofluorescence. Ten minutes after cocaine or saline injection and locomotor recording, mice were rapidly anesthetized with pentobarbital (500 mg/kg, i.p.; Sanofi-Aventis) and perfused transcardially with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.5 (Bertran-Gonzalez et al., 2008). Brains were postfixed overnight in the same solution and stored at 4°C. Thirty-micrometer-thick sections were cut with a Vibratome (Leica) and stored at −20°C in a solution containing 30% (v/v) ethylene glycol, 30% (v/v) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.10 M Tris, 0.14 M NaCl), pH 7.4, incubated for 5 min in TBS containing 3% H_2O_2 (v/v) and 10% methanol (v/v), and rinsed three times 10 min in TBS. After 20 min incubation in 0.2% Triton X-100 in TBS (v/v), sections were rinsed three times in TBS, blocked with 30 g/L BSA in TBS, and incubated overnight (or longer as indicated) at 4°C with the primary antibodies. Antibody for tyrosine hydroxylase (TH) is a mouse monoclonal (1:750 with an incubation ≥2 d; Sigma-Aldrich) and for phosphorylated extracellular signal-regulated kinase (pERK) using rabbit polyclonal antibodies against diphospho-Thr-202/Tyr-204-ERK1/2 (1:400; Cell Signaling Technology). After incubation with primary antibody, sections were rinsed three times for 10 min in TBS and incubated for 45 min with chicken Cy3-coupled (1:400; Jackson Laboratory) or goat Alexa Fluor 488-coupled (1:400; Invitrogen) secondary antibodies. Finally, sections were rinsed for 10 min twice in TBS and

twice in Tris buffer (0.25 M Tris, pH 7.4) before mounting in Vectashield (Vector Laboratories). Brain regions were identified using a mouse brain atlas and sections equivalent to 1.54 mm from bregma were taken. Sections were processed as previously described (Doly et al., 2008). Confocal microscopy and image analysis were performed at the Institut du Fer à Moulin Imaging Facility. Labeled images from each region of interest were obtained bilaterally using sequential laser-scanning confocal microscopy (SP2 and SP5, Leica). Neuronal quantification was performed in 375 × 375 μm images by counting nuclear/cytoplasm Cy3 immunofluorescence (for pERK1/2). Cell counts were performed by an observer unaware of the treatment received by the mice.

Food and cocaine self-administration. Before cocaine self-administration testing, mice were individually housed and food-deprived to 85–90% of their *ad libitum*-feeding body weight, and then trained to respond for food pellets in mouse operant chambers (Med Associates) equipped with two nose-poke openings, one randomly selected as the active and the other as the inactive nose-poke opening. A cue light located above the active nose-poke opening was paired contingently with the delivery of the reinforcer. Animals were first trained to respond for food pellets (Testdiet) in 1 h daily self-administration sessions on a fixed ratio 1 (FR1) schedule of reinforcement until responding for food criteria was acquired (≥20 reinforcers, >75% of correct responding, and stabilization for 2–3 d; for time scale of these experiments, see Fig. 8*a*). After this initial training, mice were kept in *ad libitum* conditions to recover their body weight before proceeding with the catheter implantation as previously described (Soria et al., 2005). Mice were anesthetized with a mixture of ketamine/xylazine (5:1; 0.10 ml/10 g, i.p.) and implanted with indwelling intravenous SILASTIC catheters on their right jugular vein, as previously described (Soria et al., 2005). After surgery, animals were allowed to recover for 3 d before initiation of self-administration sessions. To prevent any food-seeking or food-extinction disturbance in succeeding cocaine self-administration, saline solution self-administration sessions were performed until a reliable and stable responding pattern was obtained. Mice were then trained to self-administer cocaine at the dose of 0.250 mg/kg/infusion during 8 d on a FR1 schedule of reinforcement, with stability criteria set at ≥5 cocaine infusions and 75% responding on the active nose-poke opening over 3 d. Once stable patterns of responses were established, all mice underwent dose–response experiments, where cocaine was presented at doses of 0.125 or 0.500 mg/kg/infusion over 4 consecutive days. Subsequently, a progressive ratio procedure was performed to test the motivation of the mice to work for cocaine at the dose of 0.125 mg/kg/infusion, where the response requirements to earn an infusion escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The patency of intravenous catheters was evaluated at the end of the experiments by an infusion of 0.05 ml of thiopental sodium (5 mg/ml; Braun Medical) through the catheter. If prominent signs of anesthesia were not apparent within 3 s of the infusion, the mouse was removed from the experiment.

Sucrose preference. Sucrose preference was evaluated in the two-bottle choice test in individually caged *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice. In the first phase, two bottles of water were available over 2 consecutive days. Then, the liquid from one of the bottles was replaced with a 2% sucrose solution, and made available over 3 d. Total liquid intake, as well as sucrose preference, was determined by weighing the bottles once every morning.

Single-cell reverse-transcription PCR. Swiss-Webster mice carrying *Drd2-EGFP* bacterial artificial chromosome transgenes were generated by the Gene Expression Nervous System Atlas program at Rockefeller University. For single-cell reverse-transcription PCR (RT-PCR) experiments, 1–2-week-old *Drd2-EGFP* mice were used to direct EGFP protein expression to D2-receptor-expressing neurons. Mice were anesthetized and decapitated, and the brain was rapidly dissected out. Horizontal slices (250 μm thick) were prepared using a vibratome, in ACSF supplemented with sucrose. After a 1 h recovery period, individual slices were placed in an electrophysiology chamber continuously perfused with ACSF bubbled with carbogen and maintain at 22°C. Neurons were visualized using an Olympus BX51WI upright microscope holding ×5 and ×40 objectives, a fluorescent lamp, and infrared, red, and green fluorescence filters. The methodology involving harvesting of cytoplasmic content and subsequent single-cell PCR amplification has been described previously (Cauli et al., 1997). Briefly, borosilicate glass pipettes (3–5 MΩ) were made in a HEKA PIP5 puller and filled with 8 μl of autoclaved RT-PCR internal solution containing (in mM) the following: 144 K-gluconate, 3 $MgCl_2$, 0.5 EGTA, 10 HEPES, pH 7.2 (285/295 mOsm). Single DA neurons in the VTA and substantia nigra compacta (SNc) were approached with a pipette and, after a whole-cell connection was established, cytoplasmic content of the cell was harvested by applying gentle negative pressure to the pipette. Cell content was expelled into a tube where an RT reaction was performed in a final volume of 10 μl. Target cDNA sequences were thereafter amplified by conducting a multiplex nested PCR, designed to simultaneously detect the enzyme TH, the D2 receptor, and 5-the HT_{2B} receptors. The primers used were as follows: TH, external sense CTGGCCTTCCGTGTGTTTCAGTG, external antisense CCGGCTGGTAGGTTTGATCTTGG, internal sense AGTGCA CACAGTACATCCGTCAT and internal antisense GCTGGTAGGTTT GATCTTGGTA; D2 receptor external sense GCAGCCGAGCTTTCA GAGCC, external antisense CCTGCGGCTCATCGTCTTAAG, internal sense AGAGCCAACCTGAAGACACCAC and internal antisense CT TAAGGGAGGTCCGGGTTTTG; 5-HT_{2B} external sense CACTGGAG AAAAGGCTGCAGTA, external antisense TTGCACTGATTGGCCTG AATTG, internal sense GGCTATATGGCCCCTCCCAC and internal antisense GGTCCAGGGAAATGGCACAG.

Initially, all three genes were simultaneously amplified in a single tube using 10 μl of cDNA, 200 nM of each primer, and 2.5 U of Taq polymerase in a final volume of 100 μl. The PCR was performed using a 6 min hot start at 94°C, followed by a 21-cycle program (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s). Subsequently, 2 μl of the amplified cDNA was used as the template for the second amplification step, in which each gene was individually amplified in a separate tube, with a 35-cycle program using the same cycling conditions as mentioned above, in a final volume of 100 μl. The products of the second PCR were analyzed by electrophoresis in 2.5% agarose gels using ethidium bromide. The sizes of the PCR-generated fragments were as predicted by the mRNA sequences and were further verified by direct sequencing of the final products.

In vivo *electrophysiology: extracellular single-cell recordings.* Mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) supplemented as required to maintain optimal anesthesia throughout the experiment, and were positioned in a stereotaxic frame (David Kopf Instruments). Body temperature was kept at 37°C by means of a thermostatically controlled heating blanket. Procedures for DA cell electrophysiological recording were described previously (Mameli-Engvall et al., 2006). An incision was made in the midline to expose the skull. A burr hole was drilled above the VTA (coordinates: between 3.5 ± 0.3 mm posterior to bregma and 0.5 ± 0.3 mm lateral to midline; Paxinos and Franklin, 2001). Recording electrodes were pulled with a Narishige electrode puller from borosilicate glass capillaries with outer and inner diameters of 1.50 and 1.17 mm, respectively (Harvard Apparatus). The tips were broken under microscope control and filled with 1.5% Neurobiotin in 0.5% Na-acetate. These electrodes had tip diameters of 1–2 mm and impedances of 4–8 MΩ. They were lowered through the burr hole with a microdrive, and a reference electrode was placed in the subcutaneous tissue. Electrical signals were amplified by a high-impedance amplifier (Molecular Devices) and monitored visually with an oscilloscope (Tektronix, TDS 2002) and audibly through an audio monitor (A.M. Systems). When a single unit was well isolated, the oscilloscope sweep was triggered from the rising phase of the action potential and set sufficiently fast to display the action potential over the entire screen (usually 0.5 ms per unit). Such continuous observation of the expanded action potential provided assurance that the same single unit was being monitored throughout the experiment. The unit activity digitized at 25 kHz was stored in the Spike2 program (Cambridge Electronic Design).

Firing pattern quantification. DA cell firing *in vivo* was analyzed with respect to the average firing rate and the percentage of spikes within a burst [SWBs; the number of SWBs divided by total number of spikes in a given window of 1 min duration (%SWB)]. DA cell firing *in vivo* was analyzed with respect to the average firing rate and the %SWB. Neuron basal activity was defined on the basis of ≥5 min or 500 spikes. The

electrophysiological characteristics of VTA neurons were analyzed in the active cells encountered by systematically passing the microelectrode in a stereotaxically defined block of brain tissue, including the VTA. Its margins ranged from 2.92 to 3.88 mm posterior to bregma, 0.24 to 0.96 mm mediolateral with respect to the bregma point, and 3.7 to 4.7 mm ventral to the cortical surface, according to the coordinates of Paxinos and Franklin (2001). Sampling was initiated on the right side and then on the left side. Each electrode descent was spaced ≥10 μm from the others.

DA cell identification. Extracellular identification of DA neurons was based on their location as well as on the set of unique electrophysiological properties that characterize these cells *in vivo*: (1) a typical triphasic action potential with a marked negative deflection; (2) a characteristic long-duration (>2.0 ms) action potential; (3) an action potential width from start to negative of >1.1 ms; (4) a slow firing rate (<10 Hz) with an irregular single spiking pattern and occasional short, slow bursting activity. These electrophysiological properties distinguish DA from non-DA neurons. The mouse was killed with an overdose of anesthetic. The brain was removed and placed in a 4% paraformaldehyde solution. Sixty-micrometer-thick VTA sections were cut and stained with Neurobiotin and TH antibody (a mouse monoclonal antibody for TH, 1:500 with an incubation >2 d; Sigma-Aldrich), and the recorded neurons were identified by fluorescence microscopy. We also added two criteria: (1) that the recording be stable (i.e., the absence of cell perturbation following our saline solution injection); and (2) that the recorded cells be >4 mm from the surface of the brain. These parameters have been used classically to identify DA cells (Mameli-Engvall et al., 2006).

Electrophysiological response to cocaine and quinpirole. The firing rate and %SWB were evaluated using a 60 s moving window and a 15 s time step. Each cell activity was rescaled by its baseline value averaged during the 3 min period before 10 μl of cocaine (20 mg/kg) or quinpirole (0.25–0.50 mg/kg) in 0.9% NaCl was injected intraperitoneally. For firing frequency, rescaling was defined using $x * 100/xb$ with xb being the baseline firing frequency. The results are presented as mean ± SEM. The effect of cocaine or quinpirole was tested by comparing the maximum firing frequency observed during baseline and after cocaine injection. For each neuron, we determined *xav*, the maximum firing frequency fluctuations before cocaine injections (during the 2.5 min period used as baseline); $xav = \max(x)av - \text{mean}(x)\ av$, and *xap* is the maximum firing frequency after cocaine (or quinpirole) injection (during the 3 min period after drug injection). We used a paired nonparametric Wilcoxon test (Wilcoxon signed-rank test) to compare *xav* and *xap* for firing frequency in the two populations.

Electrophysiological recordings from brain slices. AMPA/NMDA ratio of evoked EPSCs from putative DA neurons of the VTA were obtained with whole-cell voltage-clamp recordings using a CsCl-based internal medium, as described previously (Glangetas et al., 2015). Six-to-seven-week old *Htr2b* $^{+/+}$ control and *Htr2b* $^{-/-}$ mice were anesthetized (ketamine/xylazine) for slice preparation. Horizontal 250 μm slices were prepared in bubbled ice-cold 95% O_2/5% CO_2-equilibrated solution containing the following (in mM): 110 cholineCl, 25 glucose, 25 $NaHCO_3$, 11.6 ascorbic acid, 3.1 Na^+-pyruvate, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$. The slices were then heated for 10 min in the same medium at 32°C. Subsequently, slices were stored at room temperature in 95% O_2/5% CO_2-equilibrated ACSF containing the following (in mM): 124 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgCl_2$. Slices were kept at 32–34°C in a recording chamber and were continuously perfused with 2.5 ml/min ACSF. Whole-cell voltage-clamp recording techniques were used to measure excitatory synaptic responses of the VTA. Synaptic currents were evoked by pulses (60 μs) delivered at 0.1 Hz through a glass pipette placed 200 μm rostral to the patched neurons. Recordings were made in the presence of picrotoxin (100 μM) to block $GABA_A$-receptor-mediated currents. Putative DA neurons were identified as large cells (>30 pF capacitance) in the lateral part of the VTA, which are prone to project to the NAcc (Lammel et al., 2008). Currents were amplified, filtered at 5 kHz, digitized at 20 kHz, and recorded at a holding potential of +40 mV (IGOR PRO, Wavemetrics). Access resistance was monitored by a step of −4 mV (0.1 Hz) and experiments were discarded if the access resistance increased >20%. The internal solution contained the following (in mM): 130 CsCl, 4 NaCl, 2 $MgCl_2$, 1.1 EGTA, 5 HEPES, 2 Na_2ATP, 5 Na^+-creatine-phosphate, 0.6 Na_3GTP, and 0.1 spermine, with a liquid junction potential of −3 mV. AMPA-receptor (AMPAR)-EPSCs were pharmacologically isolated using NMDA-receptor (NMDAR) antagonist APV (50 μM), while NMDAR EPSCs were then calculated by subsequent digital subtraction. The ratio of AMPAR/NMDAR responses was calculated by taking the peak values of these averaged (20 sweeps) currents. All drugs were obtained from Abcam, Tocris Bioscience, and HelloBio. APV was dissolved in water, whereas picrotoxin was dissolved in DMSO (diluted 1000× in the final volume). On-line/off-line analysis was performed using IGOR-6 (Wavemetrics) and Prism (GraphPad). Compiled data are expressed as mean ± SEM.

Immunoblot analysis. Wild-type and mutant mice (2-month-old, age-matched) were killed by decapitation and their brains were immediately dissected out from the skull and frozen on dry ice. Microdiscs of tissue were punched out from frozen slices (500 μm thick) within the striatum using a stainless-steel cylinder (1.4 mm diameter). Samples were homogenized in 1% SDS, equalized for their protein content, and analyzed by Western blot. The antibody dilutions were 1/1000 and 1/500 for antibodies against Golf and D1 receptor, respectively. Antibody for Golf was from our laboratory (Corvol et al., 2001). Antibody for D1 receptor was from Luedtke et al. (1999). Antibodies were revealed by Fluoprobes 682 goat anti-rabbit or mouse IgG (Interchim) at a 1:5000 dilution. The fluorescent immunocomplexes were detected with Odyssey (LI-COR Biosciences). Quantification was performed by measuring the average intensity in regions of interest using the Odyssey software and data were analyzed with the Prism 6 software (GraphPad Software).

Retrograde tracing of VTA or SNc neurons. Drd2-EGFP mice were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg) and fixed in a stereotaxic apparatus. Stereotaxically guided injections were made through holes in the dorsal surface of the cranium. Glass capillary tubes were pulled (HEKA pipette puller PIP5) and tips were broken to 40 μm diameter. Capillaries were filled with Dextran Alexa Fluor 568 (Invitrogen). Pressure injections of tracer (0.1 μl for 10 min) were targeted to the NAcc (80 nl) or dorsal striatum (200 nl) for labeling VTA or SNc neurons, respectively. Micropipettes were left in place 10 min before removal to minimize leakage. The stereotaxic coordinates used for these injections were obtained from the Paxinos and Franklin (2001) atlas adapted and checked in pilot experiments for postnatal day 15 mice (NAcc: +1 mm anteroposterior and +0.6 mm mediolateral relative to bregma; −3.5 mm dorsoventral relative to the dura surface; dorsal striatum: +1 mm anteroposterior and +1.5 mm mediolateral relative to bregma; −2 mm dorsoventral relative to the dura surface). Correct placement site was verified by red-dextran injection. After 10 d, surviving animals were used in the single-cell PCR experiment.

Binding assays. Mice were decapitated and brain regions, including the prefrontal cortex and striatum, were dissected on ice and homogenized with 25 ml of ice-cold buffer containing 50 mM Tris, 5 mM $MgCl_2$, pH 7.4. Homogenates were centrifuged for 20 min at 15,000 × *g*. The pellet was resuspended and centrifuged under the same condition three times. To the final suspension (0.2–0.6 mg/ml) was added for 1 h, [^{3}H]raclopride (81.3Ci/mmol; PerkinElmer) or [^{3}H]SCH23390 (85.6Ci/mmol; PerkinElmer; 2 nM) for binding the D2 and D1 receptors, respectively. The process was terminated by immersing the tubes in ice-cold buffer followed by rapid filtration through Whatman GF/B filters (Sigma-Aldrich). Radioactivity was measured using liquid scintillation counting. Binding data were analyzed using the iterative nonlinear fitting software GraphPad Prism 6 to estimate dissociation constants (K_D) and maximum number of sites (B_{max}).

Results

Permanent 5-HT$_{2B}$-receptor gene inactivation increases locomotor effects of cocaine

Locomotor activity recorded every 5 min was evaluated after an intraperitoneal injection of cocaine 30 min after the start of the session. Over the first 10 min, cocaine dose-dependently increased locomotor activity in *Htr2b* $^{-/-}$ mice significantly more than in *Htr2b* $^{+/+}$ mice (Fig. 1*a*) at every tested dose (multiple

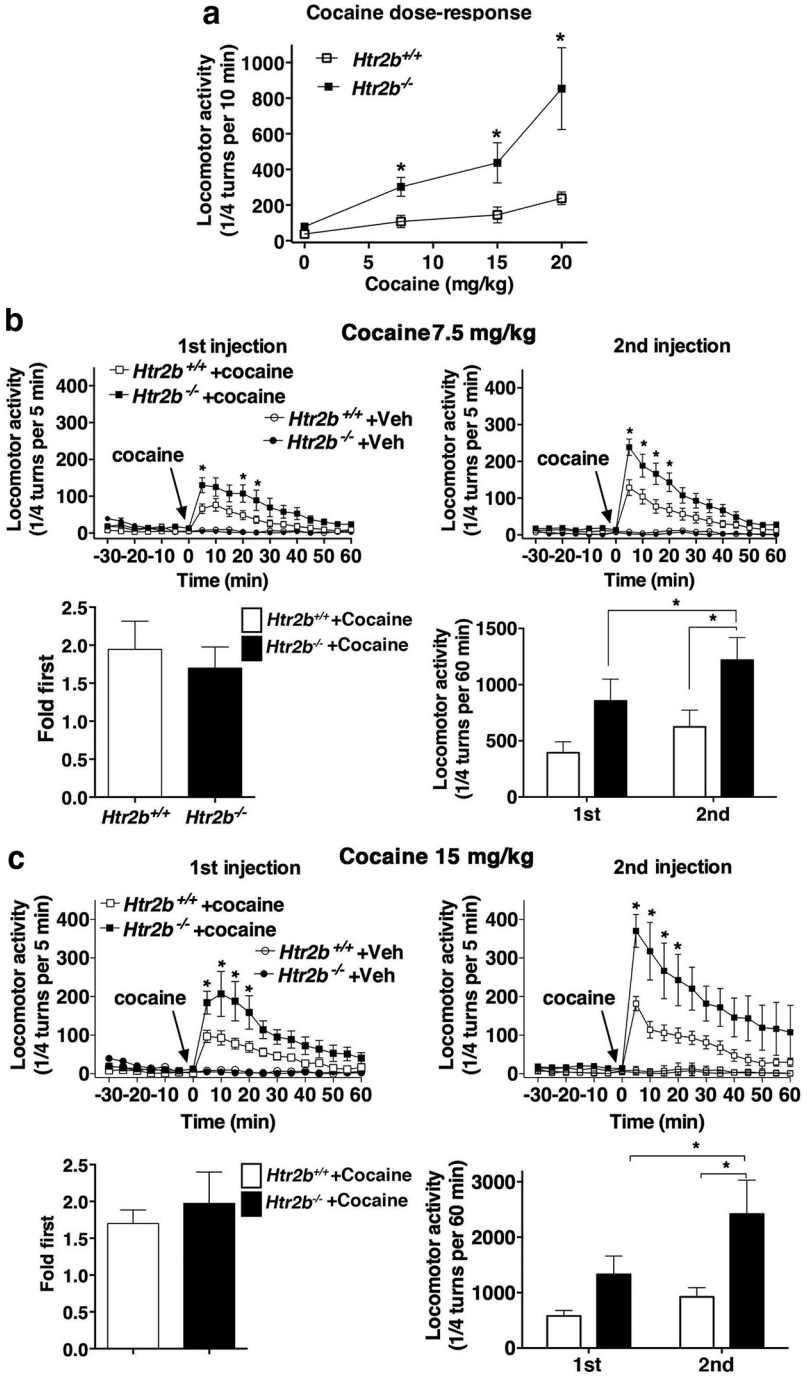

Figure 1. Dose-dependent effects of 5-HT_{2B}-receptor knock-out on cocaine responses. ***a***, Cocaine dose-dependent locomotor activity. The locomotor activity was recorded every 5 min. Cocaine was injected after 30 min of habituation. Over the first 10 min, cocaine dose-dependently increased the locomotor activity significantly more in *Htr2b* $^{-/-}$ mice (black square) than in *Htr2b* $^{+/+}$ mice (white square; means ± SEM). Data were analyzed by multiple *t* tests (*Htr2b* $^{-/-}$: $n = 8$ mice, vehicle; 8 mice, cocaine 7.5 mg/kg; 9 mice, cocaine 15 mg/kg; 3 mice, cocaine 20 mg/kg; *Htr2b* $^{+/+}$: $n = 8$ mice, vehicle; 9 mice, cocaine 7.5 mg/kg, 9 mice, cocaine 15 mg/kg, 5 mice, cocaine 20 mg/kg; $^*p < 0.05$). ***b***, Higher locomotor response to cocaine injection (7.5 mg/kg) of *Htr2b* $^{-/-}$ mice. An injection of cocaine (7.5 mg/kg) increased significantly more the locomotor activity in *Htr2b* $^{-/-}$ (cocaine, black square, $n = 12$) than in *Htr2b* $^{+/+}$ mice (cocaine, white square, $n = 12$; first injection, left, arrow indicates cocaine injection $t = 0$), while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ mice (black circle, $n = 8$) or in *Htr2b* $^{+/+}$ mice (white circle, $n = 8$). Data were analyzed by two-way RM ANOVA. The stimulant locomotor effect of a challenge dose of cocaine 7 d later (second injection, right, arrow indicates cocaine injection $t = 0$) was significantly higher in *Htr2b* $^{-/-}$ mice than in *Htr2b* $^{+/+}$ mice, while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ mice or in *Htr2b* $^{+/+}$mice. The increase in cocaine-induced locomotor activity at the second injection was similar in respect to the first (Fold first; left, $n = 12$–12; unpaired *t* test). Total locomotor activity recorded over 60 min after a second injection (right) was significantly higher in *Htr2b* $^{-/-}$ mice (black bars) compared with *Htr2b* $^{+/+}$ mice (white bars) and compared with the first injection, as analyzed using two-way RM ANOVA for cocaine (means ± SEM; *Htr2b* $^{-/-}$: $n = 12$ mice; *Htr2b* $^{+/+}$: $n = 12$ mice); Bonferroni *post hoc* tests were applied to each graph, $^*p < 0.05$. ***c***, Higher locomotor response to cocaine injection (15 mg/kg) of *Htr2b* $^{-/-}$ mice. An injection of cocaine (15 mg/kg) increased locomotor activity significantly more in *Htr2b* $^{-/-}$ mice (cocaine, black square, $n = 12$) than in *Htr2b* $^{+/+}$ mice (cocaine, white square, $n = 12$; first injection, left, arrow indicates cocaine injection $t = 0$), while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ mice (black circle, $n = 8$) or in *Htr2b* $^{+/+}$ (white circle, $n = 8$) mice. Data were analyzed by two-way RM ANOVA. The stimulant locomotor effect of a challenge dose of cocaine 7 d later (second injection, right, arrow indicates cocaine injection $t = 0$) was also significantly higher in *Htr2b* $^{-/-}$ mice compared with *Htr2b* $^{+/+}$ mice, while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ mice or in *Htr2b* $^{+/+}$ mice. The increase in cocaine-induced locomotor activity at the second injection was similar in respect to the first (Fold first; left; *Htr2b* $^{-/-}$: $n = 12$; *Htr2b* $^{+/+}$: $n = 12$ mice; unpaired *t* test. Total locomotor activity recorded over 60 min after a second injection (right) was significantly higher in *Htr2b* $^{-/-}$ mice (black bars) compared with *Htr2b* $^{+/+}$ mice (white bars) and compared with the first injection, as analyzed using two-way RM ANOVA (*Htr2b* $^{-/-}$: $n = 12$ mice; *Htr2b* $^{+/+}$: $n = 12$ mice; means ± SEM). Bonferroni *post hoc* tests were applied to each graph, $^*p < 0.05$.

unpaired *t* test, 7.5 mg/kg $t_{(51)} = 2.196$, $p = 0.033$; 15 mg/kg $t_{(51)} = 3.407$, $p = 0.0013$; 20 mg/kg $t_{(51)} = 4.619$, $p < 0.0001$). We then evaluated in more detail locomotor effects of cocaine injection at 7.5 and 15 mg/kg. A first injection of cocaine (7.5 mg/kg) induced a stronger increase in locomotor activity in *Htr2b* $^{-/-}$ mice than in *Htr2b* $^{+/+}$ mice (Fig. 1*b*; two-way RM ANOVA, significant effect of genotype, $F_{(1,22)} = 6.04$, $p = 0.022$, and time, $F_{(18,396)} = 23.80$, $p < 0.0001$, with interactions, $F_{(18,396)} = 2.08$, $p = 0.0061$). Bonferroni's post-test showed a significant increase at 5, 20, and 25 min. Similar results were obtained with cocaine 15 mg/kg (Fig. 1*c*; significant effect of genotype, $F_{(1,22)} = 5.22$, $p = 0.032$, and time, $F_{(18,396)} = 23.93$, $p < 0.0001$, with interactions, $F_{(18,396)} = 3.15$, $p < 0.0001$). Bonferroni's post-test showed a significant increase at 5–20 min. Behavioral sensitization can be measured by enhancement of drug-evoked locomotor responses following repeated drug exposure or injections performed a week apart (Stewart and Badiani, 1993; Valjent et al., 2010). Following a second cocaine injection, at the same dose, 7 d after the first, *Htr2b* $^{-/-}$ mice exhibited higher cocaine-induced locomotor response than *Htr2b* $^{+/+}$ mice (7.5 mg/kg; Fig. 1*b*; significant effect of genotype, $F_{(1,22)} = 6.07$, $p = 0.022$, and time, $F_{(18,396)} = 49.84$, $p < 0.0001$, with interactions, $F_{(18,396)} = 5.42$, $p < 0.0001$). Bonferroni's post-test showed a significant increase at 5–20 min. Similar results were obtained with cocaine 15 mg/kg

(Fig. 1*c*; significant effect of genotype, $F_{(1,22)} = 5.62$, $p = 0.027$, and time, $F_{(18,396)} = 17.84$, $p < 0.0001$, with interactions, $F_{(18,396)} = 3.22$, $p < 0.0001$). Bonferroni's post-test showed a significant increase at 5–20 min. The fold increase in cocaine-induced locomotor activity at the second injection with respect to the first was similar in both genotypes at either 7.5 or 15 mg/kg dose (7.5 mg/kg; Fig. 1*b*; unpaired *t* test, $t_{(22)} = 0.528$, $p = 0.60$; 15 mg/kg; Fig. 1*c*; unpaired *t* test, $t_{(22)} = 0.592$, $p = 0.56$). Two-way RM ANOVA analysis of the total locomotor activity over 60 min showed significant effects of genotype and time of injection (7.5 mg/kg; Fig. 1*b*; main factors genotype, $F_{(1,22)} = 6.44$, $p = 0.019$, and injection time, $F_{(1,22)} = 7.76$, $p = 0.010$; no interactions, $F_{(1,22)} = 0.39$, $p = 0.54$; 15 mg/kg; Fig. 1*c*; main factors genotype, $F_{(1,22)} = 6.02$, $p = 0.022$, and injection time, $F_{(1,22)} = 9.76$, $p = 0.005$; no interactions, $F_{(1,22)} = 2.62$, $p = 0.12$). Bonferroni's post-test showed that total locomotor activity over 60 min after a second injection was significantly higher in *Htr2b*$^{-/-}$ compared with *Htr2b*$^{+/+}$ mice and compared with the first injection. Interestingly, the locomotor activity at the first injection in *Htr2b*$^{-/-}$ was similar to that at the second in sensitized *Htr2b*$^{+/+}$ mice.

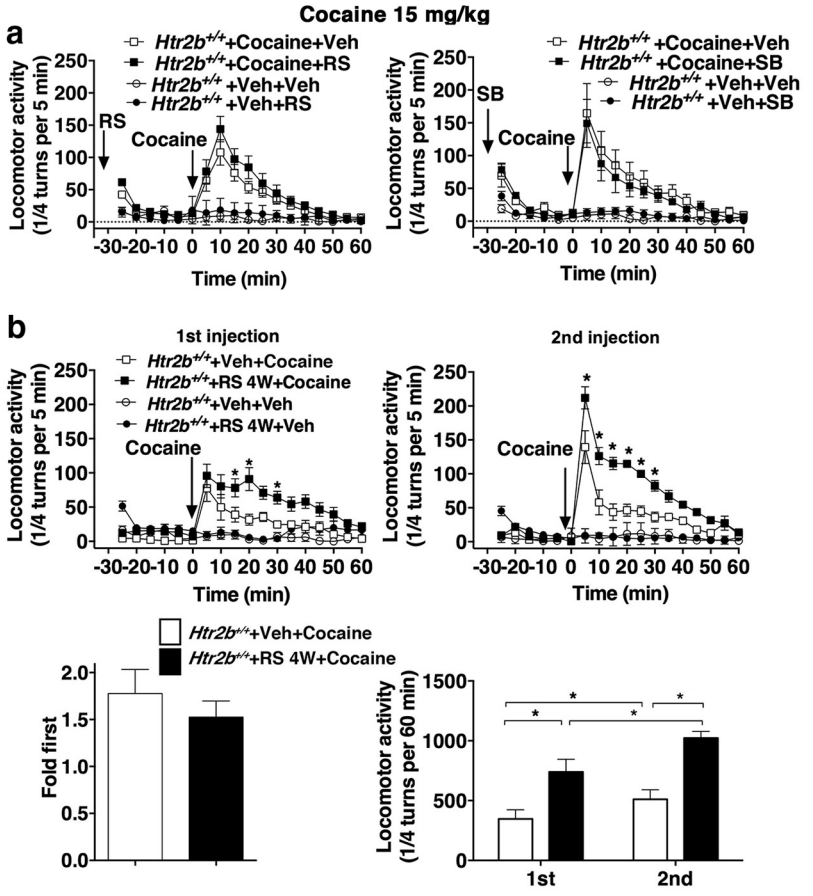

Figure 2. Effects of 5-HT$_{2B}$-receptor pharmacological blockade on cocaine response. ***a***, Lack of effect of an injection of 5-HT$_{2B}$-receptor antagonists on cocaine-induced locomotion. Locomotor activity was the same in response to saline vehicle (Veh, hollow circle, $n = 8$ mice) as to a selective 5-HT$_{2B}$-receptor antagonist (RS127445 0.5 mg/kg-RS, solid circle, $n = 8$ mice; left) or after 15 mg/kg of cocaine intraperitoneally coinjected with vehicle (Veh, hollow square, $n = 12$ mice) or with RS127445 (0.5 mg/kg-RS, solid square, $n = 11$ mice; left) in *Htr2b*$^{+/+}$ mice. Similarly, no locomotor difference was found following saline vehicle (Veh, hollow circle, $n = 7$ mice) or a 5-HT$_{2B/2}$-receptor antagonist (SB206553 3 mg/kg-SB; right; solid circle, $n = 7$ mice) or after 15 mg/kg of cocaine intraperitoneally coinjected with vehicle (Veh, hollow square, $n = 7$ mice) or with SB206553 (3 mg/kg-SB; right; solid square, $n = 7$ mice) in *Htr2b*$^{+/+}$ mice. Data were analyzed using two-way RM ANOVA for cocaine (means ± SEM). ***b***, Mice exposed 4 weeks to RS127445 show increased locomotor responses to cocaine. Locomotor activity was not different in response to saline vehicle (Veh, hollow circle, $n = 8$ mice) or to 4 weeks of treatment with RS127445 (RS127445 1 mg/kg-RS 4W, solid circle, $n = 7$ mice). However, an injection of cocaine (15 mg/kg) increased significantly more locomotor activity in mice treated for 4 weeks with RS127445 (cocaine, solid square, $n = 8$ mice) than in vehicle-treated *Htr2b*$^{+/+}$ mice (cocaine, hollow square, $n = 8$ mice; first injection, left, arrow indicates cocaine injection $t = 0$), as shown by two-way RM ANOVA. The stimulant locomotor effect of a challenge dose of cocaine 7 d later was also significantly higher in *Htr2b*$^{+/+}$ mice treated for 4 weeks with RS127445 (cocaine, solid square, $n = 8$ mice; second injection, right, arrow indicates cocaine injection $t = 0$) compared with vehicle-treated *Htr2b*$^{+/+}$ mice (cocaine, hollow square, $n = 8$ mice), while no locomotor difference was found following saline vehicle (Veh, hollow circle, $n = 8$ mice) or mice treated for 4 weeks with RS127445 (RS127445 1 mg/kg-RS 4W, solid circle, $n = 8$ mice). The increase in cocaine-induced locomotor activity at the second injection was similar in respect to the first (Fold first; first injection: $n = 8$ mice; second injection: $n = 8$ mice; left; unpaired *t* test). Total locomotor activity recorded over 60 min after a first injection of cocaine and a second injection (right) was significantly higher in mice treated over 4 weeks with RS127445 compared with vehicle-treated *Htr2b*$^{+/+}$ mice and compared with the first injection, as analyzed using two-way RM ANOVA (RS127445: $n = 8$ mice; vehicle: $n = 8$ mice; means ± SEM). Bonferroni *post hoc* tests were applied to each graph, $^*p < 0.05$.

Permanent pharmacological 5-HT$_{2B}$-receptor blockade increases locomotor effects of cocaine

We used intraperitoneal injection of 5-HT$_{2B}$-receptor antagonists in wild-type mice to confirm these effects. A single intraperitoneal injection of the highly selective and potent 5-HT$_{2B}$-receptor antagonist RS127445 (0.5 mg/kg; Bonhaus et al., 1999) did not modify basal locomotion (Fig. 2*a*). Similar absence of differential effects was obtained with the 5-HT$_{2B/2C}$-receptor antagonist SB206553 (3 mg/kg; Fig. 2*a*). Interestingly, we saw no difference in cocaine-induced (15 mg/kg) locomotion in *Htr2b*$^{+/+}$ mice, compared with vehicle-treated mice (Fig. 2*a*), after a single intraperitoneal injection of RS127445 (0.5 mg/kg) or after a single intraperitoneal injection of the 5-HT$_{2B/2C}$-receptor antagonist SB206553 (3 mg/kg; Fig. 2*a*). We then evaluated whether the enhanced locomotor response to cocaine in *Htr2b*$^{-/-}$ mice was developmentally mediated or due to long-term neuroadaptations following permanent inhibition of the receptor activity in adult *Htr2b*$^{+/+}$ mice. Treatment for 4 weeks with RS127445 (1 mg/kg/d) by subcutaneous release with miniosmotic pumps (Launay et al., 2002) in adult mice did not modify basal locomotion (Fig. 2*b*). However, treatment for 4 weeks with RS127445 (1 mg/kg/d) significantly enhanced cocaine-induced (15 mg/kg) locomotor response after a first injection (Fig. 2*b*; two-way RM ANOVA, significant effect of RS127445, $F_{(1,14)} = 11.61$, $p = 0.0042$, and time, $F_{(18,252)} = 21.12$, $p < 0.0001$, with interactions, $F_{(18,252)} = 1.77$, $p = 0.029$) compared with vehicle-treated mice. Bonferroni's post-test showed a significant increase at 15, 20, and 30 min. A second cocaine injection, at the same dose 7 d after the first, resulted in a greater increase in locomotion among the chronically RS127445-treated *Htr2b*$^{+/+}$ compared with the vehicle-treated *Htr2b*$^{+/+}$ mice (Fig. 2*b*; two-way RM ANOVA, significant effect of RS127445, $F_{(1,14)} = 25.19$, $p = 0.0002$, and time, $F_{(18,252)} = 78.29$, $p < 0.0001$, with interactions,

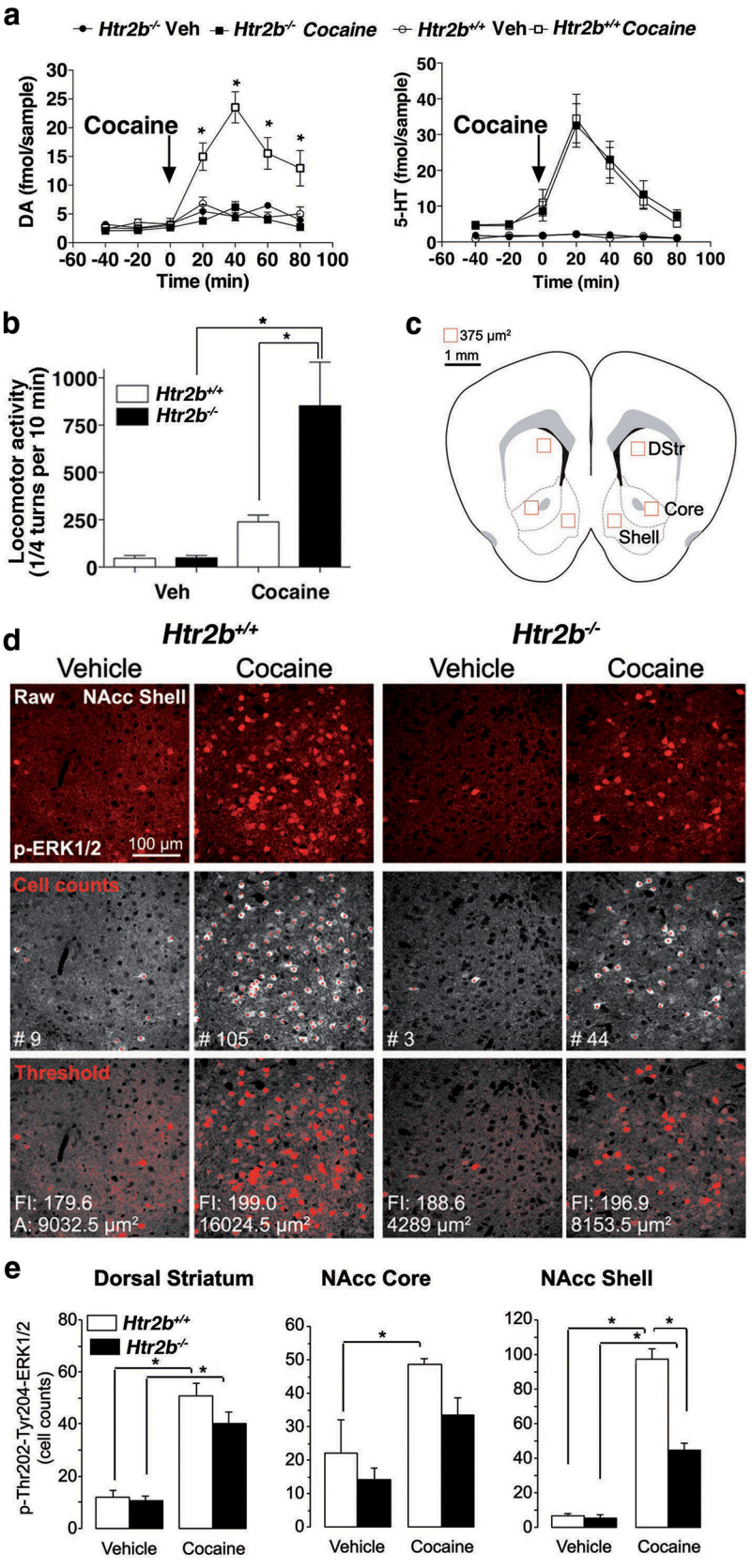

Figure 3. Effect of 5-HT$_{2B}$-receptor knock-out on cocaine-dependent striatal activation. ***a***, Lower extracellular DA but identical 5-HT accumulation in NAcc of cocaine-treated *Htr2b* $^{-/-}$ mice. The 5-HT and DA extracellular concentrations were assessed by microdialysis in the NAcc of awake mice. After cocaine (20 mg/kg) injection ($t = 0$), extracellular DA was significantly more elevated in *Htr2b* $^{+/+}$ (hollow square, $n = 7$ mice) compared with *Htr2b* $^{-/-}$ (solid square, $n = 8$ mice) or saline control (solid and hollow circles; *Htr2b* $^{-/-}$: $n = 4$ mice; *Htr2b* $^{+/+}$: $n = 5$ mice), as analyzed by two-way RM ANOVA. No difference was found in NAcc 5-HT extracellular concentrations in *Htr2b* $^{+/+}$ (hollow square) and *Htr2b* $^{-/-}$ (solid square) mice, as analyzed using two-way RM ANOVA for cocaine (means ± SEM). Bonferroni's *post hoc* tests were applied to each graph. *$p < 0.05$ versus *Htr2b* $^{+/+}$ cocaine. ***b–g***, Reduced ERK1/2 phosphorylation in ventral striatum, but not in dorsal striatum, of cocaine-treated *Htr2b* $^{-/-}$ mice. ***b***, Locomotion was first recorded during the first 10 min after saline (vehicle) or cocaine (20 mg/kg) injection. Locomotor activity was significantly higher in *Htr2b* $^{-/-}$ mice (black) than in *Htr2b* $^{+/+}$ mice (white), as analyzed using two-way ANOVA (*Htr2b* $^{-/-}$: $n = 3$ mice; *Htr2b* $^{+/+}$: $n = 5$ mice; means ± SEM). Bonferroni's *post hoc* tests were applied to each graph, *$p < 0.05$. Immediately after 10 min of locomotor recording, the brains were fixed and sectioned. pERK1/2-immunopositive neurons were counted. ***c***, Location of the images within the striatal tissue (red squares). ***d***, Raw images obtained in the confocal microscope (top), cell counts performed on each image (middle, red dots) and segmentation on each image through signal thresholding (bottom). ***e***, Quantification revealed significantly fewer cells positive for pERK1/2 in the NAcc shell of cocaine-treated *Htr2b* $^{-/-}$ (black) compared with *Htr2b* $^{+/+}$ (white) mice (*Htr2b* $^{-/-}$: $n = 4$ mice; *Htr2b* $^{+/+}$: $n = 4$ mice), as analyzed using two-way ANOVA. No difference between genotype was found in the NAcc core or dorsal striatum. Scale bars, 100 μm. Bonferroni's *post hoc* tests were applied to each graph, *$p <$ 0.05.

$F_{(18,252)} = 8.32$, $p < 0.0001$). Bonferroni's post-test showed a significant increase at 5–30 min. The fold increase in cocaine-induced locomotor activity at the second injection with respect to the first was similar (Fig. 2*b*; unpaired *t* test, $t_{(14)} = 0.808$, $p = 0.43$). Two-way RM ANOVA analysis of the total locomotor activity over 60 min showed significant effects of genotype and time of injection (main effects of RS127445, $F_{(1,14)} = 17.43$, $p = 0.0009$, and injection time, $F_{(1,14)} = 31.59$, $p < 0.0001$, no interactions, $F_{(1,14)} = 2.26$, $p = 0.155$). Bonferroni's post-test showed that total locomotor activity over 60 min after a second injection was significantly higher in mice treated over 4 weeks with RS127445 compared with vehicle-treated *Htr2b* $^{+/+}$ mice and compared with the first injection. Together these results suggest that permanent inactivation of the 5-HT$_{2B}$ receptor leads to increased sensitivity to cocaine as measured by locomotor responses, independently of putative consequences of the receptor knock-out during embryonic development.

The lack of 5-HT$_{2B}$ receptors reduces ventral striatal cocaine-induced DA outflow and ERK1/2 phosphorylation

To further investigate the consequences of the loss of 5-HT$_{2B}$-receptor expression, we compared monoamine extracellular concentrations after cocaine injection in *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice first measured by microdialysis. In awake *Htr2b* $^{-/-}$ mice, a dose of cocaine (20 mg/kg), which increased locomotor activity (Figs. 1*a*, 3*b*),

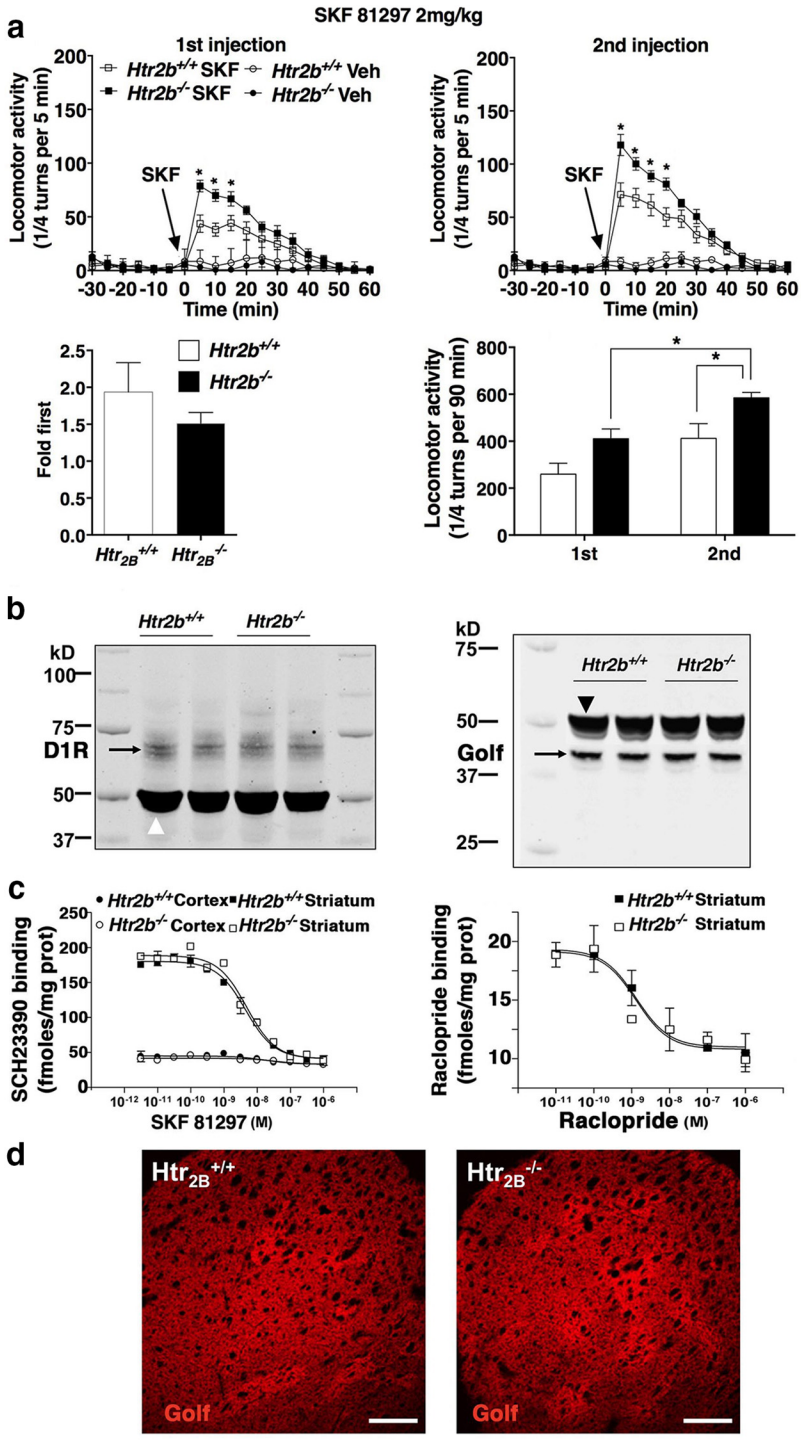

Figure 4. Assessment of DA system in *Htr2b* $^{-/-}$ mice. ***a***, Increased locomotor activity in response to DA-receptor D1 agonist in *Htr2b* $^{-/-}$ mice. An intraperitoneal injection of D1 agonist SKF81297 (SKF, 2 mg/kg) increased locomotor activity significantly more in *Htr2b* $^{-/-}$ mice (SKF, solid square, $n = 8$) than in *Htr2b* $^{+/+}$ mice (SKF, hollow square, $n = 8$ mice; first injection, left, arrow indicates cocaine injection $t = 0$), while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ (Veh, solid circle, $n = 8$ mice) or in *Htr2b* $^{+/+}$ (Veh, hollow circle, $n = 8$ mice), as analyzed using two-way RM ANOVA. The stimulant effect of a challenge dose of SKF81297 7 d later (second injection, right, arrow indicates cocaine injection $t = 0$) was also significantly higher in *Htr2b* $^{-/-}$ (SKF, solid square, $n = 8$) compared with *Htr2b* $^{+/+}$ mice (SKF, hollow square, $n = 8$; second injection, left, arrow indicates cocaine injection $t = 0$), while no locomotor difference was found following vehicle in *Htr2b* $^{-/-}$ (Veh, solid circle, $n =$ 8 mice) or in *Htr2b* $^{+/+}$ (hollow circle, $n = 8$ mice). The increase in SKF81297-induced locomotor activity at the second injection was similar in respect to the first (Fold first; left; first injection: $n = 8$; second injection: $n = 8$; unpaired t test). Total locomotor activity recorded over 60 min after a second injection (right) was significantly higher in *Htr2b* $^{-/-}$ (black bars) compared with *Htr2b* $^{+/+}$ mice (white bars) and compared with the first injection, as analyzed using two-way RM ANOVA for cocaine (means ± SEM; *Htr2b* $^{-/-}$: $n = 8$ mice; *Htr2b* $^{+/+}$: $n = 8$ mice). Bonferroni's *post hoc* tests were applied to each graph, $*p < 0.05$. ***b–d***, Similar D1-receptor and D2-receptor expression level in *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice. ***b***, Western blots from *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mouse striatum proteins (2 examples for each genotype) were revealed using antibody against D1 receptor (left, black arrow) or using Gαolf antibody (right, black arrow). Normalization (using tubulin antibody; arrowhead) revealed no significant difference between genotypes ($n = 4$ mice per genotype; Student's t test). ***c***, Radioligand binding assays with the selective D1-receptor ligand [^{3}H]SCH23390 on membranes prepared from the cortex and striatum showed no differences between *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice (B_{max}: *Htr2b* $^{+/+}$ cortex 18 ± 2.7 fmol/mg of proteins; *Htr2b* $^{-/-}$ cortex 14 ± 2.5 fmol/mg of proteins; *Htr2b* $^{+/+}$ striatum 211 ± 6 fmol/mg of proteins; *Htr2b* $^{-/-}$ striatum 223 ± 7.4 fmol/mg of proteins; left). Radioligand binding assays with the selective D2-receptor ligand [^{3}H]raclopride on membranes prepared from striatum showed no differences between *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice (B_{max}: *Htr2b* $^{+/+}$ striatum 11.4 ± 5.6 fmol/mg of proteins; *Htr2b* $^{-/-}$ striatum 11.4 ± 5.7 fmol/mg of proteins, 3 independent experiments in duplicate; right). ***d***, Immunohistochemistry with a selective antibody directed against Gαolf protein on striatum slices showed no pattern differences between *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice, respectively (scale bars, 200 μm).

elicited a significantly lower increase (2.5-fold) in DA extracellular levels in the NAcc compared with *Htr2b* $^{+/+}$ mice, in which extracellular DA concentration increased 10-fold (Fig. 3*a*; two-way RM ANOVA, significant effect of genotype, $F_{(1,13)} = 43.29$, $p < 0.0001$, and time, $F_{(6,78)} = 26.00$, $p < 0.0001$, with interactions, $F_{(6,78)} = 1.77$, $p < 0.0001$). Bonferroni's post-test showed that the cocaine-dependent DA level was significantly lower in *Htr2b* $^{-/-}$ mice compared with *Htr2b* $^{+/+}$ mice at 20–80 min. At the same dose, no difference in extracellular cocaine-dependent 5-HT accumulation in the NAcc was observed (Fig. 3*a*). Striatal ERK1/2 phosphorylation is a marker of DA-dependent D1-receptor stimulation and an essential component of signaling pathways initiating synaptic plasticity and long-term behavioral effects of drugs of abuse (Girault et al., 2007). Cocaine injection (20 mg/kg) significantly increased locomotor activity for the first 10 min (Fig. 3*b*; two-way ANOVA, significant effect of genotype, $F_{(1,10)} = 9.23$, $p < 0.0125$, and treatment, $F_{(1,10)} = 24.02$, $p = 0.0006$, with interactions, $F_{(1,10)} = 9.06$, $p = 0.013$). Bonferroni's post-test showed that total locomotor activity over 10 min was significantly higher in *Htr2b* $^{-/-}$ mice compared with *Htr2b* $^{+/+}$ mice and compared with the vehicle injection. Ten minutes after cocaine injection, we quantified the number of pERK1/2-immunoreactive neurons in various areas of the striatum of *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice (Fig. 3*c–e*). Activated neurons in vehicle and cocaine conditions displayed globally an equivalent amount of pERK1/2 staining, and

immunoreactive areas of pERK1/2 (not illustrated). Two-way ANOVA showed a significant effect of genotype ($F_{(1,10)} = 40.23$, $p < 0.0001$) and treatment ($F_{(1,10)} = 234.2$, $p < 0.0001$), with interaction between cocaine treatment and genotype ($F_{(1,10)} = 36.34$, $p < 0.0001$) only in the NAcc shell (Fig. 3*e*). Bonferroni's post-test showed that cocaine induced a significantly lower number of pERK1/2-immunopositive neurons in the NAcc shell of *Htr2b*$^{-/-}$ than in *Htr2b*$^{+/+}$ mice (fourfold vs 10-fold). By contrast, the increase in the number of pERK1/2-immunopositive neurons in the NAcc core and dorsal striatum did not differ between genotypes (Fig. 3*e*; two-way ANOVA, no effect of genotype in core, $F_{(1,10)} = 4.57$, $p = 0.058$, or dorsal striatum, $F_{(1,9)} = 1.705$, $p = 0.22$). Bonferroni's post-test showed pERK1/2-immunopositive neurons were significantly increased by cocaine in both *Htr2b*$^{-/-}$ and *Htr2b*$^{+/+}$ mice. These results agree with microdialysis data for the whole NAcc. Altogether, these results suggest that reduced extracellular accumulation of DA blunts cocaine-dependent activation of the ERK1/2 pathway in medium spiny neurons of the NAcc shell from *Htr2b*$^{-/-}$ mice without modification of activity in the dorsal striatum.

The lack of 5-HT$_{2B}$ receptors increases locomotor effects of D1 agonist

Since cocaine-dependent ERK1/2 activation relies on D1-receptor-expressing striatal neurons, we assessed the locomotor response to D1-receptor agonist SKF81297 (2 mg/kg). Two-way RM ANOVA showed a significant effect of genotype ($F_{(1,14)} = 5.30$ $p = 0.037$) and time ($F_{(18,252)} = 59.93$, $p < 0.0001$) with interactions ($F_{(18,252)} = 4.34$, $p < 0.0001$; Fig. 4*a*). Bonferroni's post-test showed that SKF81297 increased locomotor activity significantly more in *Htr2b*$^{-/-}$ than in *Htr2b*$^{+/+}$ mice at 5–15 min. A second SKF81297 injection, 7 d after the first, increased more locomotion in *Htr2b*$^{-/-}$ than in *Htr2b*$^{+/+}$ mice (Fig. 4*a*; significant effect of genotype, $F_{(1,14)} = 5.95$, $p = 0.029$, and time, $F_{(18,252)} = 100.4$, $p < 0.0001$, with interactions, $F_{(18,252)} = 5.58$, $p < 0.0001$). Bonferroni's post-test showed that SKF81297 increased locomotor activity significantly more in *Htr2b*$^{-/-}$ mice than in *Htr2b*$^{+/+}$ mice at 5–20 min. The fold increase in cocaine-induced locomotor activity at the second SKF81297 injection with respect to the first was similar in both genotypes (Fig. 4*a*; unpaired *t* test, $t_{(14)} = 1.00$, $p = 0.33$). Two-way RM ANOVA analysis of the total locomotor activity over 60 min showed significant effects of genotype and time of injection (Fig. 4*a*; main effects of genotype, $F_{(1,14)} = 12.30$, $p = 0.0035$, and of injection time, $F_{(1,14)} = 13.19$, $p = 0.0027$, with no interactions, $F_{(1,14)} = 0.057$, $p = 0.82$). Bonferroni's post-test showed that total locomotor activity over 60 min after a second injection of SKF81297 was significantly higher in *Htr2b*$^{-/-}$ mice compared with *Htr2b*$^{+/+}$ mice and compared with the first injection. Nevertheless, basal expression of D1 and D2 receptors in *Htr2b*$^{-/-}$ mice did not differ from those of *Htr2b*$^{+/+}$ mice (Fig. 4*b*,*c*), and neither did Gαolf expression (Fig. 4*b*,*d*). Moreover, there was no difference in striatal dopamine-transporter, serotonin-transporter, and norepinephrine-transporter expression between *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice (Doly et al., 2008; Banas et al., 2011; Diaz et al., 2012). These results suggest that D1 receptors are sensitized in *Htr2b*$^{-/-}$ mice without modification of receptor, Gαolf, or transporter expression.

Mesoaccumbens DA neurons express 5-HT$_{2B}$ receptors

To understand how 5-HT$_{2B}$ receptors could affect cocaine-induced effects in the NAcc, we evaluated their expression in VTA DA neurons. In the absence of reliable antibody against 5-HT$_{2B}$ receptors in mice, we performed single-cell RT-PCR by extracting cytoplasmic RNA of single identified DA neurons expressing GFP from brain slices of *Drd2-EGFP* mice. We found that among D2-positive and TH-positive neurons of the VTA, 40% expressed *Htr2b* mRNA (Fig. 5*a*). The two primary efferent fiber projections of DA neurons from the VTA are the mesocortical and mesolimbic pathways, innervating the prefrontal cortex and NAcc, respectively (Lammel et al., 2014). We anticipated that among the VTA neurons expressing *Htr2b*, some would send projections to the NAcc and dorsal striatum. To visualize these cells, we stereotactically injected red-dextran into the NAcc shell (Fig. 5*b–d*) or dorsal striatum (Fig. 5*e*,*f*) of *Drd2-EGFP* mice and traced retrograde transport. We found that DA neurons projecting to the NAcc shell originated from the parabrachial pigmented area of the VTA (Fig. 5*c*,*d*), as previously reported (Ikemoto, 2007). Strikingly, by performing single-cell RT-PCR on double-labeled neurons (Fig. 5*b*), we found that all double-labeled (*Drd2-EGFP* and NAcc shell-injected red-dextran) neurons expressed 5-HT$_{2B}$ receptors ($n = 13$ of 13). These data support the notion that 5-HT$_{2B}$ receptors are selectively expressed in mesolimbic DA neurons sending axons to the NAcc shell.

The conditional knock-out of 5-HT$_{2B}$-receptor genes in DA neurons increases locomotor effects of cocaine

To functionally validate the 5-HT$_{2B}$-receptor expression in DA neurons, we generated conditional knock-out mice by inserting recombination sites (loxP) flanking the *Htr2b* first coding exon (*Htr2b*$^{fl/fl}$; Fig. 6*a–c*). We crossed mice expressing the Cre recombinase under a DA transporter promoter (*Dat-Slc6a3*; *Dat-Cre*$^{+/0}$) with these *Htr2b*$^{fl/fl}$ mice, generating *Htr2b*^{DAKO}. We verified the proper recombination by colocalization of *Dat-GFP* with TH-positive DA neurons (Fig. 6*b*,*c*). A first injection of cocaine (20 mg/kg) induced a stronger increase in locomotor activity in *Htr2b*^{DAKO} mice than in control *Htr2b*$^{fl/fl}$ littermates (Fig. 6*d*; two-way RM ANOVA, significant effect of genotype, $F_{(1,18)} = 4.03$, $p = 0.05$, and time, $F_{(16,288)} = 12.09$, $p < 0.0001$, with interactions, $F_{(16,288)} = 3.27$, $p < 0.0001$). Bonferroni's post-test showed a significant increase in locomotion at 5–25 min. The locomotor effect of a challenge dose of cocaine 7 d later (second injection) was also significantly enhanced in *Htr2b*^{DAKO} mice compared with *Htr2b*$^{fl/fl}$ control mice (Fig. 6*d*; two-way RM ANOVA, significant effect of genotype, $F_{(1,18)} = 10.63$, $p = 0.004$, and time, $F_{(16,288)} = 22.16$, $p < 0.0001$, with interactions, $F_{(16,288)} = 4.90$, $p < 0.0001$). Bonferroni's post-test showed a significant increase at 5–30 min. The fold increase in cocaine-induced locomotor activity at the second injection with respect to the first was similar in both genotypes (Fig. 6*d*; unpaired *t* test, $t_{(18)} = 0.324$, $p = 0.75$). Two-way RM ANOVA analysis of the locomotion over 60 min showed significant effects of genotype ($F_{(1,18)} = 10.31$, $p = 0.0048$) and time of injection ($F_{(1,18)} = 23.82$, $p = 0.0001$) with interactions ($F_{(1,18)} = 3.74$, $p = 0.069$). Bonferroni's post-test showed that total locomotor activity recorded over 60 min after a second injection was significantly higher in *Htr2b*^{DAKO} mice compared with *Htr2b*$^{fl/fl}$ littermates and compared with the first injection (Fig. 6*d*). Thus, mice that underwent selective *Htr2b* inactivation in DA neurons display increased locomotor responses to cocaine. These data confirmed that inactivation of the receptor in DA neurons is, at least in part, responsible for locomotor effects and potentially for modulation of DA-neuron activity observed in *Htr2b*$^{-/-}$ mice.

The lack of 5-HT$_{2B}$ receptors modulates VTA DA cell excitability

We next tested the hypothesis that 5-HT$_{2B}$ receptors expressed by VTA DA neurons directly modulate activity of these neurons. First, we recorded electrophysiological activity *in vivo* in *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice. Mice were anesthetized and electrophysi-

ologically active cells encountered in a stereotactically defined block of brain tissue including the VTA were recorded; only cells that met all criteria for VTA DA neurons were recorded. Those criteria were as follows: (1) a typical triphasic action potential with a marked negative deflection; (2) a characteristic long-duration (>2.0 ms) action potential; (3) an action potential width from start to negative of >1.1 ms; (4) a slow firing rate (<10 Hz) with an irregular single spiking pattern (tonic) and occasional short, slow-bursting (phasic) activity (Grace and Bunney, 1984a, b; Gonon, 1988; Mameli-Engvall et al., 2006). Juxtacellular single-unit recordings were obtained in anesthetized *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice (Fig. 7*a*). Activities of DA cells were then characterized using the firing rate and the percentage of spikes within a burst (%SWB). DA cells fired at an average rate of 1.6 ± 0.2 Hz in *Htr2b*$^{+/+}$ and 1.8 ± 0.1 Hz in *Htr2b*$^{-/-}$ mice (*Htr2b*$^{+/+}$: $n = 57$ cells; *Htr2b*$^{-/-}$: $n = 58$ cells). The mean percentage of burst firing in individual cells ranged from 0 to 16% in *Htr2b*$^{+/+}$ and 0 to 60% in *Htr2b*$^{-/-}$ mice, and was significantly higher in *Htr2b*$^{-/-}$ (5.2 ± 1.5%) than in *Htr2b*$^{+/+}$ (1.4 ± 0.5%) mice (Fig. 7*a*; unpaired *t* test, Welch-corrected, $t_{(66)} = 2.27$, $p = 0.026$).

After baseline recordings, the same mice were intraperitoneally injected with cocaine and the evoked modification of firing rate was analyzed. Cocaine (20 mg/kg) decreased the firing rate by 62.3% in *Htr2b*$^{+/+}$ mice, while this decrease was significantly greater (90.9%) in *Htr2b*$^{-/-}$ mice (Fig. 7*b*; unpaired *t* test, Welch-corrected, $t_{(16)} = 2.73$, $p = 0.015$). The decrease in firing rate lasted for >30 min after drug delivery and was not always followed by a return to preinjection levels in *Htr2b*$^{-/-}$ mice. However, systemic intraperitoneal injection of the D2 agonist quinpirole (0.25–0.5 mg/kg) produced a similar decrease of firing rate in *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice (Fig. 7*c*). Our results indicate that long-term blockade of 5-HT$_{2B}$ receptor leads to a stronger cocaine-dependent inhibition of DA neuron firing rates and supports the hypothesis that 5-HT modulates VTA DA cell excitability via 5-HT$_{2B}$ receptors without modification of D2 autoreceptor function.

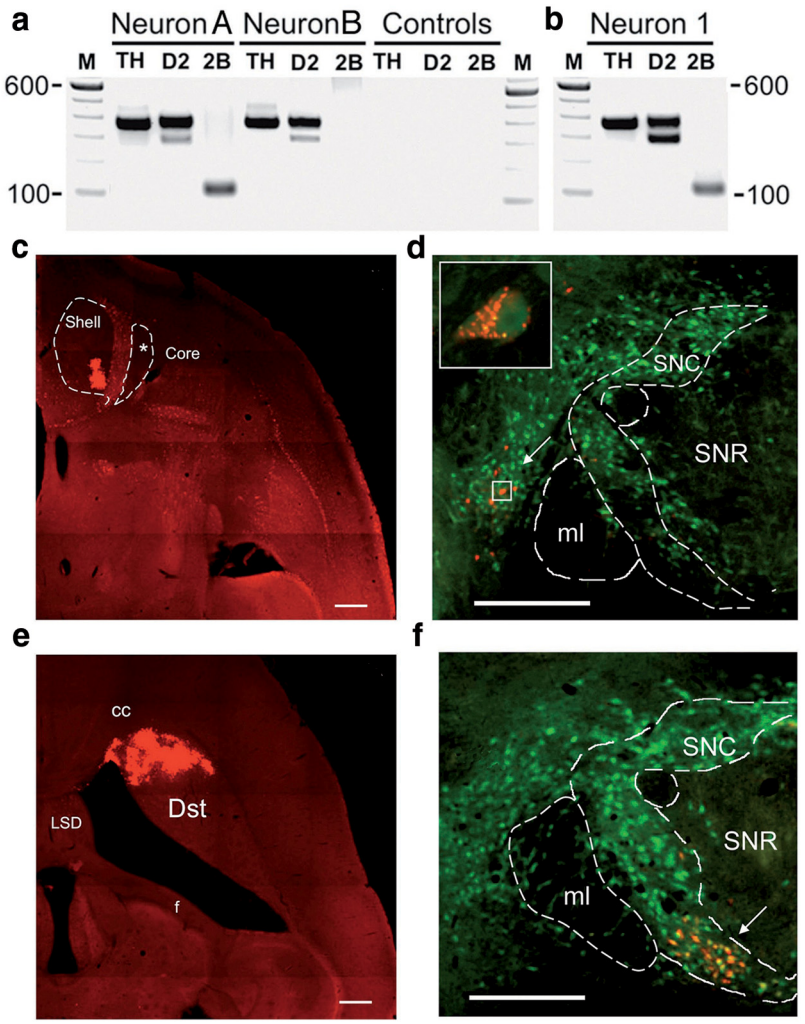

Figure 5. The 5-HT$_{2B}$ receptors are expressed by mesolimbic DA neurons. ***a***, Coexpression of D2 receptors and TH with 5-HT$_{2B}$-receptor mRNA in a subset of individual VTA neurons. RT-PCR was performed on cytoplasmic RNA extracted from individually identified *Drd2-EGFP*-positive VTA neurons. The displayed pattern illustrates a negative neuron (Neuron B) and a positive neuron (Neuron A) expressing 5-HT$_{2B}$ receptor, which is representative of 4 of 10 neurons in the VTA. All neuron classes express TH and D2 receptors. Negative RT-PCR controls (GFP-negative neuron) are also presented. M, Molecular weight marker in base pairs. ***b–d***, VTA neurons expressing 5-HT$_{2B}$ receptor project to the NAcc. ***b***, Stereotactic red-dextran injection in NAcc of *Drd2-EGFP* mice allowed identification, after retrograde tracing, of VTA DA neurons projecting to the NAcc (double-labeled). Cytoplasmic RNA from double-labeled neuron in the VTA was analyzed by single-cell RT-PCR (Neuron 1), which is representative of 13 of 13 VTA neurons expressing 5-HT$_{2B}$ receptor and that project to the NAcc. ***c***, Representative horizontal section, showing NAcc shell stereotactic red-dextran injection site for retrograde tracing experiments using fluorescently red-labeled latex beads. Scale bars, 600 μm. ***d***, Representative horizontal section, showing red-dextran retrograde tracing in VTA of *Drd2-EGFP* mice that identified double-labeled DA neurons projecting to NAcc used for single-cell RT-PCR (arrow and inset). Scale bars, 200 μm. Star indicates the NAcc core nucleus. ***e***, ***f***, Controls of red-dextran injection into dorsal striatum. ***e***, Representative horizontal section, showing dorsal striatum stereotactic red-dextran injection site for retrograde tracing experiments using fluorescently red-labeled latex beads. Scale bars, 600 μm. ***f***, Representative horizontal section, showing red-dextran retrograde tracing in the substantia nigra reticulata (SNR) of *Drd2-EGFP* mice that identify DA neurons projecting to the dorsal striatum (arrow). Scale bars, 200 μm. ml, Medial lemniscus; Dst, dorsal striatum; f, fornix; cc, corpus callosum; LSD, lateral septal nucleus dorsal.

To test whether the lack of 5-HT$_{2B}$ receptors would produce long-lasting synaptic modifications in DA neurons, we used whole-cell patch recordings in acute brain slices. We measured AMPAR-mediated and NMDAR-mediated EPSCs in voltage-clamp mode at +40 mV on horizontal brain slices, which included VTAs from *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice, in DA neurons (large cells, >30 pF capacitance) from the lateral part of VTA. These neurons are prone to project to the NAcc shell. In *Htr2b*$^{-/-}$, the AMPAR/NMDAR ratio was significantly increased compared with that of control mice (*Htr2b*$^{+/+}$: 0.63 ± 0.06, $n = 7$ cells; *Htr2b*$^{-/-}$: 1.01 ± 0.15, $n = 7$ cells; Fig. 7*d*; unpaired *t* test $t_{12} = 2.35$, $p = 0.037$). Altogether, these results indicated that the lack of 5-HT$_{2B}$ receptors promotes an increase in DA neuron bursting *in vivo* properties, a stronger reactivity to cocaine, and a concomitant potentiation of AMPA synaptic transmission of VTA DA neurons measured *ex vivo*.

Htr2b inactivation modifies reinforcing properties of cocaine

To know whether locomotor-activating effects of cocaine are associated with disruptions in primary reinforcing effects of the drug, we tested whether 5-HT$_{2B}$-receptor deletion altered oper-

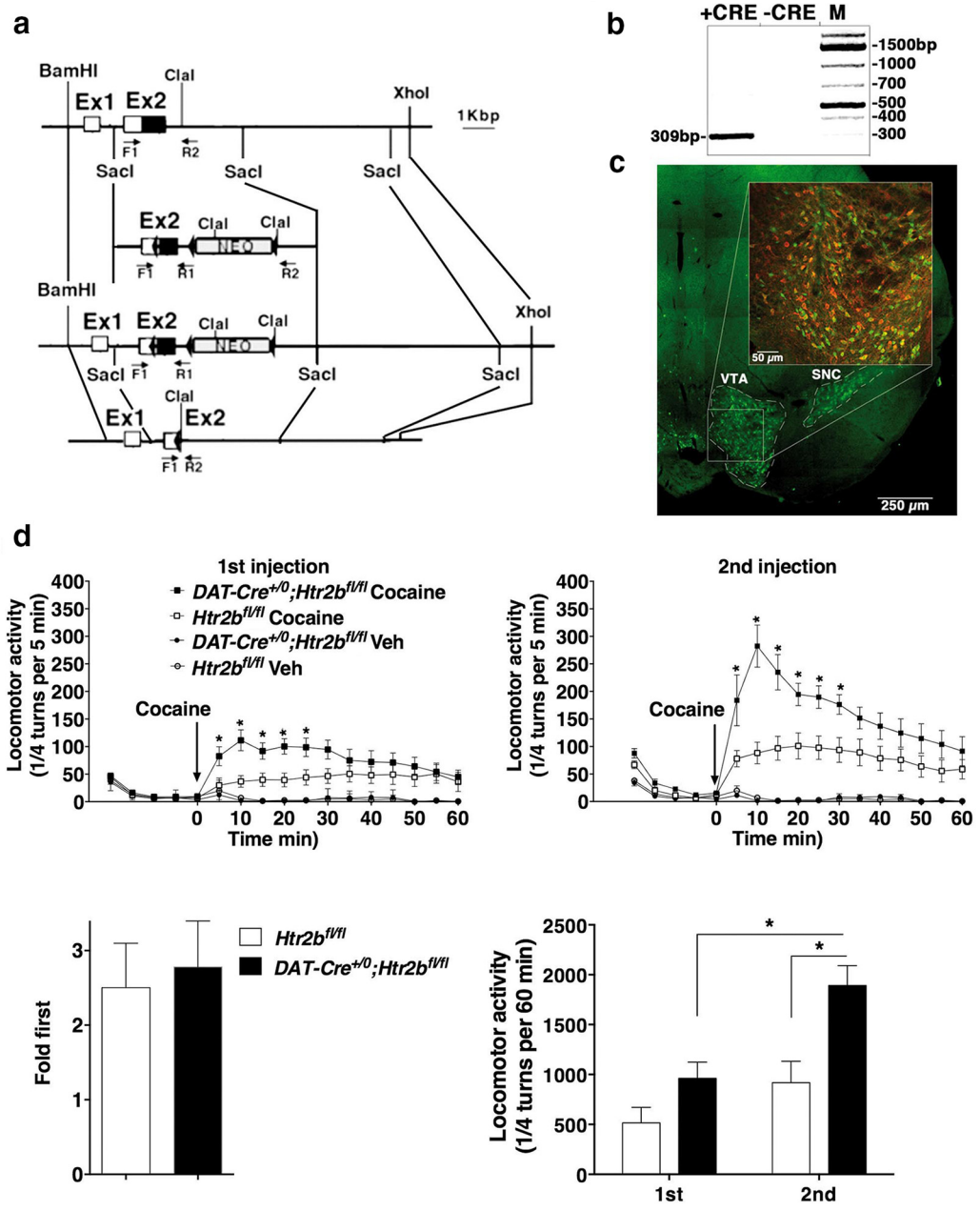

Figure 6. Conditional deletion of 5-HT_{2B} receptors in DA neurons, $Htr2b^{DAKO}$ mutant mice. ***a–c***, Mapping and genotyping of $Htr2b^{DAKO}$ mutant mice. Top, 5-HT_{2B}-receptor locus indicating the positions of exons (Ex1, exon 1; Ex2, exon 2) and restriction sites used for the targeting construct. Middle, Targeting vector designed to flox exon 2 by homologous recombination in genomic DNA generating the targeted locus (below). Exons 1 and 2 are depicted by hollow (untranslated) and solid black boxes (coding) and Neomycin resistance by gray boxes (NEO). Bottom, Sequence-verified structure of the *Htr2b*-null allele (KO) after excision by Cre recombinase of the sequence flanked by LoxP sites (triangles). Horizontal arrows illustrate the position of primers used for genotyping (F1, R1, R2). ***b***, Genomic DNA of VTA from $Htr2b^{DAKO}$ mice was extracted and analyzed by PCR, revealing the effective proper recombination (F1, R2 amplimers, 309 bp). ***c***, Efficient Cre recombination in TH-positive neurons. Immunofluorescence revealed the recombinase-dependent GFP expression in the VTA, which colocalized with the TH antibody staining as seen by confocal microscopy in the coronal section of the VTA of $Dat\text{-}Cre^{+/0}$;*RCE* (*Dat-GFP*) mice (inset). scale bars: 250 μm; inset, 50 μm. ***d***, Restricted 5-HT_{2B}-receptor inactivation to DA neurons leads to increased locomotor response to cocaine. An injection of cocaine (20 mg/kg) increased significantly more locomotor activity in $Htr2b^{DAKO}$ mice ($Dat\text{-}Cre^{+/0}$;$Htr2b^{fl/fl}$ cocaine, solid square, $n = 10$) than in Cre-negative littermate mice ($Htr2b^{fl/fl}$ cocaine, hollow square, $n = 10$; first injection, left, arrow indicates cocaine injection $t = 0$), as analyzed using two-way RM ANOVA, while no locomotor difference was found following vehicle in $Htr2b^{DAKO}$ (Veh, solid circle, $n = 9$) or in $Htr2b^{fl/fl}$ (Veh, hollow circle, $n = 7$). The stimulant locomotor effect of a challenge dose of cocaine 7 d later (second injection, right, arrow indicates cocaine injection $t = 0$) was also significantly higher in $Htr2b^{DAKO}$ mice ($Dat\text{-}Cre^{+/0}$; $Htr2b^{fl/fl}$ cocaine, solid square, $n = 10$) compared with $Htr2b^{fl/fl}$ mice ($Htr2b^{fl/fl}$ cocaine, hollow square, $n = 10$), while no locomotor difference was found following vehicle in $Htr2b^{DAKO}$ mice (Veh, solid circle, $n = 8$) or in $Htr2b^{fl/fl}$ mice (hollow circle, $n = 7$). The increase in cocaine-induced locomotor activity at the second injection was similar in respect to the first (Fold first; first injection, $n = 10$ mice; second injection, $n = 10$ mice; unpaired *t* test). Total locomotor activity recorded over 60 min after a second injection was significantly higher in $Htr2b^{DAKO}$ compared with $Htr2b^{fl/fl}$ littermate mice and compared with the first injection, as analyzed using two-way RM ANOVA for cocaine ($Htr2b^{DAKO}$: $n = 10$ mice; $Htr2b^{fl/fl}$: $n = 10$ mice; means ± SEM). Bonferroni's *post hoc* tests were applied to each graph, $^*p < 0.05$.

ant cocaine self-administration. $Htr2b^{+/+}$ and $Htr2b^{-/-}$ animals were first trained to respond for food pellets under a FR1 schedule of reinforcement (for timeline of self-administration experiments, see Fig. 8*a*). An increase in nose-poke responses was observed during the first sessions leading to a learning-curve pattern in both groups of animals (day, $F_{(7,203)} = 17.9$, $p < 0.001$). Although active responding in the $Htr2b^{+/+}$ group was slightly higher, this difference was not significant (genotype, $F_{(1,29)} = 0.8$,

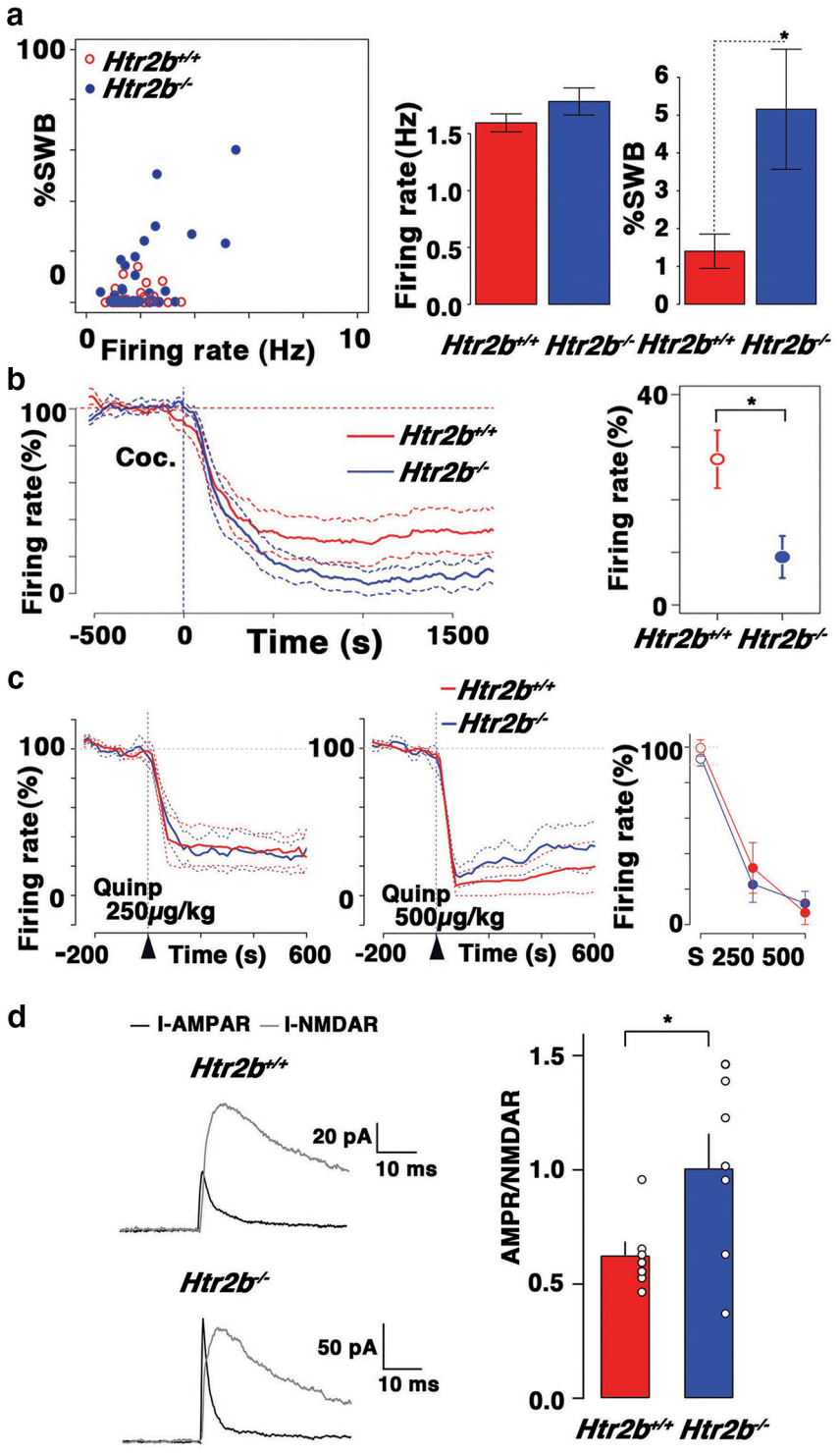

Figure 7. Electrophysiological effects of 5-HT_{2B}-receptor knock-out on VTA DA neurons. ***a***, Changes in basal *in vivo* firing of *Htr2b* $^{-/-}$ VTA DA neurons. After *in vivo* recordings, the mean frequency (Hz) was plotted against the percentage of spikes within a burst (%SWB) of DA neurons in *Htr2b* $^{+/+}$ (red, $n = 57$ cells) and *Htr2b* $^{-/-}$ mice (blue, $n = 58$ cells). Barplot shows no change in the mean frequency (left) and a significant increase in percentage of SWB (right) for the same groups (unpaired *t* test, Welch-corrected). ***b***, Cocaine induced a stronger decrease in the firing rate of VTA DA neurons in *Htr2b* $^{-/-}$ mice. Systemic intraperitoneal injection of cocaine (20 mg/kg) in *Htr2b* $^{+/+}$ (10 mice; red line) and *Htr2b* $^{-/-}$ (9 mice; blue line) mice produced a decreased firing rate. On average, cocaine-induced decrease in firing rate was significantly stronger in *Htr2b* $^{-/-}$ than in *Htr2b* $^{+/+}$ mice (unpaired *t* test, Welch-corrected). The percentage of variation in firing rate (right) is illustrated. ***c***, No change in the quinpirole-dependent decrease in firing rate of VTA DA neurons in *Htr2b* $^{-/-}$ mice. Systemic intraperitoneal injection of the D2 agonist quinpirole (Quinp 250, Quinp 500 μg/kg) in *Htr2b* $^{+/+}$ mice (250 μg/kg: $n = 6$ mice; 500 μg/kg: 5 mice; red line) and *Htr2b* $^{-/-}$ mice (250 μg/kg: $n = 7$ mice; 500 μg/kg: 6 mice; blue line) produced a similar decrease of firing rate. The percentage of variation in firing rate (right) is illustrated for the two concentrations. ***d***, Strengthening of AMPA transmission in the VTA neurons of *Htr2b* $^{-/-}$ mice. Representative sample traces for AMPAR (black) and NMDAR EPSCs (gray) recorded at +40 mV in DA neurons from the lateral part of the VTA. These neurons are prone to project to the NAcc shell of *Htr2b* $^{-/-}$ and *Htr2b* $^{+/+}$ mice. Right, Bar graph and scatter plot illustrate the significant increase in AMPAR/NMDAR ratio in the *Htr2b* $^{-/-}$ versus *Htr2b* $^{+/+}$ mice (unpaired *t* test; *Htr2b* $^{-/-}$: $n = 7$ mice; *Htr2b* $^{+/+}$: $n = 7$ mice).

$p = 0.36$) and the discrimination between active and inactive nose-poke responses was rapidly acquired by both *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice (Fig. 8*b*; hole, $F_{(1,29)} = 144.4$, $p < 0.001$). No statistical differences between genotypes were obtained when data from the acquisition days were analyzed ($F_{(1,29)} = 2.7$, $p = 0.112$; Fig. 8*c*). To avoid interference from food responding to cocaine self-administration, animals were tested following catheter implantation on intravenous saline self-administration until responding rates decreased. The mice included in the statistical analysis (18 *Htr2b* $^{+/+}$ and 13 *Htr2b* $^{-/-}$ mice) showed patent catheters up to the end of the experiment. Three-way RM ANOVA analysis of the acquisition of cocaine (0.250 mg/kg/infusion) self-administration data showed a significant interaction between hole × day ($F_{(7,203)} = 2.58$, $p < 0.05$), but not between hole × genotype ($F_{(1,29)} = 1.51669$, $p = 0.228$), day × genotype ($F_{(7,203)} = 0.69168$, $p = 0.679$), or hole × day × genotype ($F_{(7,203)} = 0.2108$, $p = 0.983$; Fig. 8*d*). Significant main effects of hole ($F_{(1,29)} = 47.19$, $p < 0.001$), day ($F_{(7,203)} = 4.02$, $p < 0.001$), and genotype ($F_{(1,29)} = 4.56$ $p < 0.05$) were revealed. During 8 d of acquisition training, 61% of wild-type and 38.5% of knock-out mice met the stability criteria for cocaine self-administration at this dose. Cocaine intake (mg/kg) during the last 3 d of training at the dose of 0.25 mg/kg/infusion was not significantly different between genotypes (ANOVA: $F_{(1,29)} = 1.61$, $p = 0.213$; Fig. 8*e*). Statistical analysis of cocaine dose–response results (Fig. 8*f*) showed that *Htr2b* $^{-/-}$ mice earned a significantly lower number of cocaine infusions (genotype, $F_{(1,29)} = 5.4$, $p < 0.05$), especially at the dose of 0.125 (genotype, $F_{(1,29)} = 6.3$, $p < 0.05$) and 0.500 mg/kg/infusion (genotype, $F_{(1,29)} = 4.2$, $p < 0.05$) with respect to *Htr2b* $^{+/+}$ mice. Although *Htr2b* $^{-/-}$ animals obtained a lower breaking point in the progressive ratio schedule of the reinforcement test (Fig. 8 *g*), this difference was not significant (genotype, $F_{(1,29)} = 2.1$, $p = 0.162$). Finally, daily liquid intake was analyzed in *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ animals following self-administration experiments. Both groups of mice drank similar amounts of water during a 24 h period (genotype, $F_{(1,29)} = 0.5$, $p =$

0.502; Fig. 8*h*), and both groups exhibited a comparable preference for sucrose-enriched water (genotype, $F_{(1,29)} = 1$, $p = 0.325$; Fig. 8*i*). Although learning ability is similar in *Htr2b*$^{-/-}$ mice as in *Htr2b*$^{+/+}$ mice, they self-administer less cocaine, show a trend toward a lower breaking point, and display no difference in their postcocaine reward systems.

Discussion

Here, we report that mice lacking 5-HT$_{2B}$ receptors totally or exclusively in DA neurons exhibit heightened cocaine-induced locomotor responses. The lack of 5-HT$_{2B}$ receptors induces a reduced cocaine-induced extracellular DA accumulation. It also causes the NAcc shell medium spiny neurons to overreact to cocaine or D1 agonist, without any modification of DA signaling in the dorsal striatum. We describe the selective expression of 5-HT$_{2B}$ receptors in VTA DA neurons sending axons to the NAcc shell. An increase in basal DA neuron bursting *in vivo* properties and a concomitant increase in AMPA synaptic transmission to VTA DA neurons measured *ex vivo* were found in mice lacking 5-HT$_{2B}$ receptors. The lack of 5-HT$_{2B}$ receptors leads to a stronger inhibition of DA neuron firing rates in response to cocaine without modification of D2 autoreceptor function. Finally, the absence of 5-HT$_{2B}$ receptors is associated with decreased cocaine self-administration with a trend toward a lower breaking point in the progressive ratio schedule of reinforcement tests. These data point toward the requirement of 5-HT via 5-HT$_{2B}$ receptors to modulate both DA neurons and their targets.

DA and 5-HT interactions are known to be important in mesolimbic and nigrostriatal DA pathways. The 5-HT-immunoreactive fibers are dense in both the VTA and SNc (Hervé et al., 1987). The 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have previously been shown to modulate the effects of cocaine (Cunningham and Anastasio, 2014). We show here that genetic ablation of 5-HT$_{2B}$ receptors, even restricted to DA neurons, increases cocaine-induced locomotor activity. However, acute pharmacologic inhibition does not reproduce this effect. These data agree with previous neuropharmacological data indicating the inability of 5-HT$_{2B}$-receptor antagonists to affect cocaine responses while acute blockade of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors generated opposing modulatory actions on cocaine-induced activity (Filip et al., 2010; Cunningham and Anastasio, 2014; Devroye et al., 2015). Nevertheless, we show here that a chronic (4 weeks) exposure to a 5-HT$_{2B}$-receptor antagonist is sufficient to increase the locomotor response to cocaine to the same extent as the increase observed in *Htr2b*$^{-/-}$ mice. While 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors generate opposing actions, 5-HT$_{2B}$ receptors likely modulate directly and indirectly cocaine-induced locomotion via DA-dependent circuitry, since we found that 5-HT$_{2B}$ receptors are expressed in VTA DA neurons projecting to the NAcc shell and that their selective elimination in DA neurons by conditional knock-out is sufficient to reproduce the increased locomotor response to cocaine. It is clear that the 5-HT$_{2B}$-receptor mode of action differs from that of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors and must affect different DA neuron subpopulations and/or different effectors.

One of our most striking findings is the dissociation between increased locomotor activity and decreased cocaine-induced extracellular DA accumulation in the NAcc associated with blunted activation of the ERK1/2 pathway selectively in medium spiny neurons of the NAcc shell. Cocaine, like other drugs of abuse, is known to preferentially increase extracellular DA in the NAcc shell compared with the core (Di Chiara and Bassareo, 2007). This preferential action has also been demonstrated during acquisition of cocaine self-administration (Lecca et al., 2007). We identified that 100% of the retrogradely labeled DA neurons in the NAcc shell originating from VTA express 5-HT$_{2B}$ receptors. Interestingly, the reduced ERK1/2 activation of NAcc shell neurons following cocaine injection in *Htr2b*$^{-/-}$ mice does not seem to take place in other striatal areas, such as the core and dorsal striatum, as indicated by the lack of effect on cocaine-induced increase of pERK1/2. The reduction of DA transmission in the NAcc shell upon cocaine injection may result from a heightened sensitivity to the inhibitory effect of cocaine on the firing of DA neurons projecting to the NAcc shell, since we observed that cocaine produced a stronger decrease in the firing rate of VTA DA neurons in *Htr2b*$^{-/-}$ mice. Recent pharmacological studies in rats have shown that acute 5-HT$_{2B}$-receptor antagonist injection had no effect on cocaine-induced DA outflow in the NAcc shell or core, or in the dorsal striatum (Devroye et al., 2015). However, these authors showed that an antagonist injection reduced basal DA levels in the NAcc shell (Devroye et al., 2015), and had no effect in the dorsal striatum (Devroye et al., 2016). These acute effects may be involved in neuroadaptations observed upon chronic antagonist exposure. Although, the cocaine dose of 0.25 mg/kg/infusion has reinforcing effects in *Htr2b*$^{-/-}$ mice, the doses of 0.125 and 0.50 mg/kg/infusion do not. The fact that cocaine does not result in extracellular DA accumulation in the NAcc of these animals may underlie this observation. However, there could also be DA-independent mechanism at work, possibly via 5-HT release, which is unchanged in *Htr2b*$^{+/+}$ and *Htr2b*$^{-/-}$ mice. Thus, the impaired 5-HT/DA balance could explain why *Htr2b*$^{-/-}$ mice do not self-administer cocaine due to aversive properties of still-existing cocaine-evoked 5-HT release.

Furthermore, *Htr2c*$^{-/-}$ mice also display increased sensitivity to locomotor stimulant effects of cocaine and exhibit enhanced cocaine-induced elevations of extracellular DA levels in the NAcc, but not in dorsal striatum (Rocha et al., 2002). The drug is more reinforcing in these mice. The 5-HT$_{2C}$ receptor has been shown to be expressed in some VTA neurons, which synthesize and potentially release both DA and GABA and project to the NAcc (Bubar et al., 2011). Since we recently reported a dominance of 5-HT$_{2C}$ over 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors upon coexpression, which was also observed *in vivo* (Moutkine et al., 2017), 5-HT$_{2B}$/5-HT$_{2C}$-receptor heterodimerization may participate in some of these cocaine responses.

Another explanation for the dissociation between the cocaine-induced decrease in DA accumulation, the increase in locomotion, and the reduction in cocaine self-administration is the increased postsynaptic responsiveness in other striatal areas. An important finding is the observation that a delay is necessary to allow adaptations that are responsible for these apparent paradoxical responses, the increased cocaine response being only an indirect consequence of 5-HT$_{2B}$-receptor-dependent reduction in DA tone observed in NAcc and downstream adaptation. Our observation of sensitized locomotor response to D1 agonists in *Htr2b*$^{-/-}$ mice may be the reason for the apparent discrepancy between the reduction of DA transmission in the NAcc shell and increased locomotion. The trend in reduction of reinforcing properties of cocaine as indicated by reduction in the breaking point for cocaine responding in a progressive ratio schedule is consistent with the reduction of DA stimulant effects of cocaine in *Htr2b*$^{-/-}$ mice. These observations suggest that NAcc shell DA acting on D1 receptors (pERK findings) is critical for cocaine reinforcement. This conclusion is consistent with recent results obtained in rats, in which D1 receptors have been silenced by siRNA in the NAcc shell while sparing the core, and which were

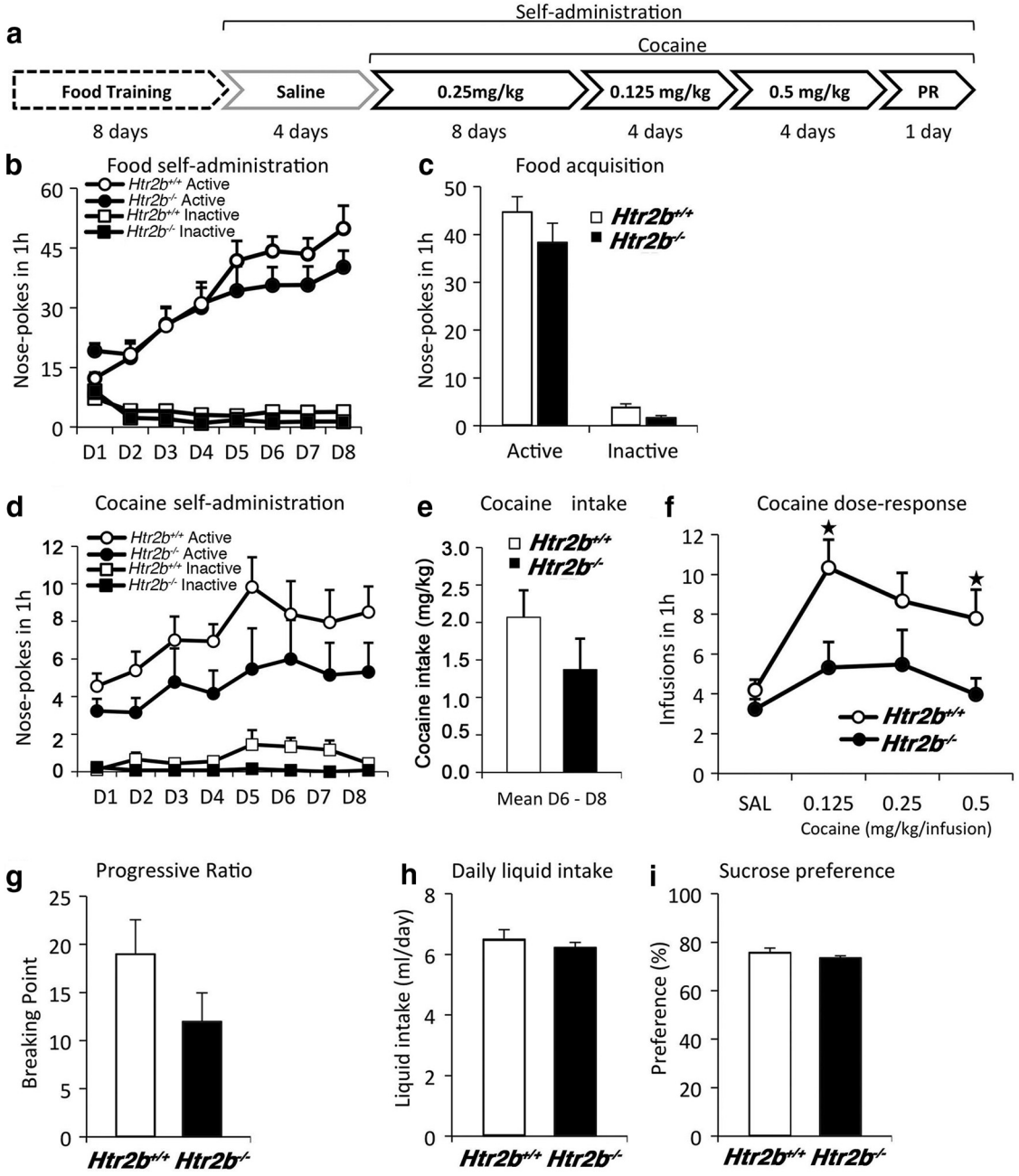

Figure 8. Cocaine self-administration in *Htr2b* $^{-[supi]/-}$ mice. ***a***, Timeline for the self-administration experiments. ***b–c***, Similar training with food pellets in *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice. Mice were first trained to respond to food pellets under FR1 schedule of reinforcement in 1 h daily self-administration sessions until response to food criteria was acquired. A significant increase in nose-poke responses was observed during the first sessions leading to a learning-curve pattern in both groups of mice. Data analyzed by three-way ANOVA showed an effect of the day. ***b***, The discrimination between active and inactive nose-poke responses was rapidly acquired in both *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice. ***c***, No statistical differences between genotypes were obtained when data from the acquisition days were analyzed. ***d–g***, Lower cocaine self-administration in *Htr2b* $^{-/-}$ mice. A saline solution was presented first until a reliable and stable of responses was obtained. ***d***, Mice were then trained to self-administer cocaine at the dose of 0.25 mg/kg/infusion during 8 d on a FR1 schedule of reinforcement. ***e***, *Htr2b* $^{-/-}$ mice showed a nonsignificant decrease in the mean intake of cocaine (mg/kg) during the last 3 d of training. ***f***, In the dose–response curve for cocaine self-administration, *Htr2b* $^{-/-}$ mice earned a significantly lower number of cocaine infusions at the doses of 0.125 and 0.50 mg/kg/infusion ($p < 0.05$). Each point represents the mean of 4 d of responses for each dose. For saline and for the dose of 0.25 mg/kg/infusion, the point shown represents the mean of the last 4 d of training. ***g***, Subsequently, a progressive ratio procedure was performed to test the motivation of mice to work for cocaine at the dose of 0.125 mg/kg/infusion. The trend toward a lower break point in the progressive ratio schedule of the reinforcement test did not reach significance (unpaired *t* test). ***h***, ***i***, No difference in postcocaine reward system between genotypes. ***h***, To evaluate the effects of 5-HT$_{2B}$-receptor deletion on the postcocaine reward system, two bottles of water were first available to individually caged mice and liquid consumption was monitored over 2 consecutive days. ***i***, Then, the liquid from one of the bottles was replaced with a 2% sucrose solution and liquid-intake measurements were performed daily over 3 d. Total liquid intake, as well as sucrose preference, was evaluated in *Htr2b* $^{+/+}$ and *Htr2b* $^{-/-}$ mice. No statistical differences between genotypes were obtained. (*Htr2b* $^{+/+}$: $n = 18$ mice; *Htr2b* $^{-/-}$: $n = 13$ mice; unpaired *t* test).

prevented from the acquisition of cocaine self-administration (Pisanu et al., 2015). Interestingly, excitatory afferents of VTA DA neurons that project to the NAcc are potentiated upon exposure of animals to addictive drugs (Lammel et al., 2012). This induction depends on NMDA and D1 receptors (Ungless et al., 2001), which are activated when DA neurons become active, releasing DA from their dendrites. The NMDARs that drive this induction are located on DA neurons themselves, supporting the

idea that this form of plasticity is a VTA-autonomous process. The ratio of the amplitude of AMPAR/NMDAR-mediated postsynaptic currents, a parameter often used to quantify synaptic strength in acute brain slice preparation, becomes higher than normal upon exposure to addictive drugs (Ungless et al., 2001). Here, we found that permanent ablation of 5-HT_{2B} receptors is associated with higher reactivity to cocaine of VTA DA neurons and higher AMPAR/NMDAR ratio. These data support the notion that the 5-HT_{2B} receptor acts as an important factor preventing drug-evoked synaptic plasticity.

Previous evidence indicated that 5-HT_{2B} receptors could directly modulate 5-HT neurons and indirectly modulate DA neurons. Indeed, genetic (knock-out) or pharmacologic manipulation (antagonist) of 5-HT_{2B} receptors interferes with the effects of molecules that directly target the 5-HT system. These pharmacologic manipulators include serotonin selective reuptake inhibitors, antidepressants, and amphetamine-derived 5-HT releasers 3,4-methylenedoxymethamphetamine (MDMA) and dexfenfluramine (Doly et al., 2008, 2009; Banas et al., 2011; Diaz et al., 2012). For example, the enhanced locomotor response to the psychostimulant MDMA was abolished in *Htr2b*$^{-/-}$ mice (Doly et al., 2008). These mutant mice as wild-type mice following injection of a 5-HT_{2B}-receptor antagonist (RS127445) exhibit neither behavioral sensitization nor conditioned place preference following MDMA (10 mg/kg) injections. Nevertheless, high doses (30 mg/kg) of MDMA induce DA-dependent but 5-HT-independent behavioral effects (Doly et al., 2009). It was also reported that 5-HT_{2B}-receptor-selective antagonists can reduce significantly the DA outflow induced by amphetamine in the NAcc shell, but not in the dorsal striatum (Auclair et al., 2010). We independently found that *Htr2b*$^{-/-}$ mice display higher amphetamine-induced locomotion (Pitychoutis et al., 2015). Together with our present findings, these observations clearly distinguish between a direct action of 5-HT_{2B} receptors on 5-HT neurons that is mimicked by antagonists, from an action at DA neurons that requires long-term inhibition of 5-HT_{2B} receptors and neuroadaptations, i.e., that is not reproduced by direct antagonist injections. This finding is important for genetic polymorphisms that permanently affect gene expression, such as the *HTR2B* (Gln20Ter) mutation (Bevilacqua et al., 2010).

Several parameters remain to be determined to fully explain our findings, e.g., the exact neural circuits that are modified and the time needed for the blockade of 5-HT_{2B}-receptor activity to produce the observed changes in reactivity to cocaine. Nevertheless, this work established for the first time that permanently inactive 5-HT_{2B} receptor is associated with a local hypodopaminergy that paradoxically and ultimately results in increased cocaine psychostimulant responses and a trend toward blunted motivation to the drug. To sum up, the chronic 5-HT_{2B}-receptor inhibition makes mice behave like animals already exposed to cocaine with higher cocaine-induced locomotion associated with changes in DA neuron reactivity.

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