Supplemental Experimental Procedures

Animals

All mice were maintained according to the EC directive 86/609/CEE, on a 12 light: 12 dark schedule (lights on at 8:00), and housed in groups of 3-5 of the same genetic background and sex. Food for laboratory mice (SAFE A03, France; 3200 kcal/kg, moisture 12%, proteins 21%, lipids 5%, carbohydrates 52%, fibers 4%, and mineral ash 6%) and tap water were available ad libitum except during experiments. Behavioral studies were carried out in the afternoon (14:00–20:00). Mice were moved to the testing room in their home cage at least 5 days prior to testing to allow for habituation to the environment and stayed there until the end of the experiments.

Reagents

Fluoxetine hydrochloride (Biotrend, Switzerland); Paroxetine hydrochloride hemydrate, (±) 8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OH-DPAT), and BW723C86 hydrochloride (Tocris) were dissolved in 0.9% NaCl. RS127445 hydrochloride (Tocris) was dissolved in dimethyl sulfoxide (DMSO) 1% solution for i.p. injections or 50% for s.c. pumps. 5-Bromo-2'deoxyuridine (BrdU) (Sigma, B9285) was dissolved in 0.9% NaCl at 50°C, and the pH was set at 7.4 with NaOH 10M. All the drugs were injected intraperitoneally (i.p.) in a volume of 0.1 ml/10 g body weight of the animals, with the exception of RS127445 chronically treated mice, which were implanted with osmotic pumps delivering either the drug or its vehicle.

Novelty-Suppressed Feeding Test

The NSF paradigm is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the center of a brightly lit arena. Briefly, the testing apparatus consisted of a plastic box, 37 cm x 57 cm x 10 cm, directly illuminated by a white light. The floor was covered with 2 cm of sawdust. Eighteen hours before the test, food was removed from mice cages. At the time of testing, a single pellet of a familiar food was placed in the center of the box. An animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to start eating (defined as the mouse sitting on its haunches and biting the pellet with the use of forepaws) was recorded for a 5-min period (1), and immediately after, each mouse was put back in its cage, and food consumption in the home cage was measured during 15 min. Mice did not receive the injection corresponding to their experimental group prior to performing the NSF test so as not to introduce a bias in the feeding behavior. The apparent baseline variability in latency to feed in the NSF test can be explained by the fact that, even though experiments are conducted in controlled conditions, (including constant illumination and background noise), many factors may influence the reaction to a novel environment. Despite this variability, mice with non functional 5-HT_{2B} receptors were systematically compared to WT mice in each set of experiments.

Microdialysis in freely moving mice

Microdialysis was performed as already published by our laboratory (2-4). Briefly, microdialysis cannulae were implanted in the ventral hippocampus. According to (5), stereotaxic coordinates in mm for ventral hippocampus were AP -2.9, L + 2.8, DV -1.5 from bregma and the top of the skull; and for raphe nucleus, were AP -4.5, L -1.0 and DV -4.4 with a lateral angle of 20° from bregma and the top of the skull. Probes were inserted into the guide cannula 3 to 5 days after implantation and perfused at a constant rate of 1µl/min with artificial CSF (aCSF). Dialysates were collected every 20 min. All measurements were obtained from freely moving mice 120 min after the beginning of perfusion, by which time a steady state was achieved (stabilization period). Absolute baseline 5-HT levels were then determined from the first 5 samples (100 minutes). Mice were next injected with paroxetine (2 mg/kg, i.p.) during the recording phase. RS127445-pretreated WT mice received an injection of this antagonist 15 min before paroxetine. Dialysate samples were determined by high-pressure liquid chromatography (HPLC) coupled to a coulometric detector (Coulochem III; ESA Inc., Chelmsford, USA).

Serotonin content
Tissue levels of 5-HT, were determined using HPLC with coulometric detection. Mice were decapitated and brain regions including the raphe nucleus and hippocampus were dissected on ice and homogenized in 500 µl of a cold extraction solution (HCl 0.1 N). Samples were then centrifuged at 15,000 g for 30 min at 4°C and the supernatants were filtered with cellulose centrifugal filter (Microcon YM-30; Millipore, USA). Diluted supernatants (1:200) were injected into the HPLC system previously described and with the same conditions.

Binding assays
Mice were decapitated and brain regions, including the raphe nucleus, prefrontal cortex, ventral tegmental area, dorsal striatum, locus coeruleus and the hippocampus were dissected on ice and homogenized with 25 ml of ice cold buffer containing 50mM tris, 5mM MgCl2, pH 7.4. Homogenates were centrifuged for 20min at 15 000x 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 one hour, [^{3}H]paroxetine (21.3Ci/mmol; Perkin Elmer; USA) or [^{3}H]citalopram (85.6Ci/mmol; Perkin Elmer; USA) (2 nM) and paroxetine (1nM- 10µM) for SERT binding or [^{3}H]8-OHDPAT (187 Ci/mmol; Perkin Elmer; USA) and WAY 100635 (1nM- 10µM) for 5-HT_{1A} receptor binding. The process was terminated by immersing the tubes in ice cold buffer followed by rapid filtration through Whatman GF/B filters. Radioactivity was measured using liquid scintillation counting. Binding data were analyzed using the iterative non-linear fitting software GraphPad Prism 4.0 to estimate dissociation constants (K_D) and maximum number of sites (B_{max}).

Immunohistochemistry
Newborn cells were detected by peroxidase immunostaining of BrdU, an exogenous marker of cell division. Free-floating sections were first incubated overnight in 0.1% H_2O_2. After rinsing, sections were exposed to 2 N HCl for 1 h for deoxyribonucleic acid hydrolysis, immediately rinsed in 1X PBS and blocked in 0.2% gelatin and 0.5% triton in 1X PBS solution for 1 h. Sections were then incubated overnight with the primary antibody (rat anti-BrdU 1:400; AbDserotec OBT0030; clone BU1/75-ICR1) at 4°C, and, after washing, exposed to the secondary biotinylated antibody (goat anti rat, 1:400; Vector) for 2 h at room temperature and to 1:400 Streptavidin-biotinylated horseradish peroxidase complex (Amersham). Sections were incubated in Tris 0.1M-DAB 3%-triton 0.1% solution for 30 min, and finally, 5‰ of H_2O_2 was added for 45 min to reveal peroxidase activity. After rinsing in Tris 0.05 M, sections were mounted and cover-slipped in Mowiol mounting medium. For the endogenous cell cycle marker Ki67, classical peroxidase immunohistochemistry protocols were employed (i.e., the same as that described for BrdU staining but with no HCl- hydrolysis step). Sections were incubated overnight at 4°C with antibodies against Ki67 (rabbit 1:1000; Novocastra: NCL-Ki67p) and, then, incubated with the appropriate secondary biotinylated antibodies (1:400) for 2 h, followed by detection.

Immunofluorescence
Identification of newly formed cells was performed by double labeling cells with neuronal or astroglial markers and BrdU, followed by confocal microscopy. Commercial antibodies were as follows: rat anti-BrdU (1:400; AbDserotec OBT0030), immature neuron markers goat anti doublecortin (1:500; Santa Cruz) and mouse anti-PSA-NCAM (1:250; AbCys: IHCR1010-6); mature neuronal markers IgM mouse anti-calbindin (1:20000; AbCys: CP331) and mouse anti NeuN (1:1000; Santa Cruz); astroglial marker rabbit anti-GFAP (1:800; Dako Cytomation); and rabbit anti-SERT (1:1000; Calbiochem PC177L). Similarly to the protocol described for immunohistochemistry, sections were incubated with the primary antibodies against neuronal or glial markers overnight at 4°C. After rinsing, sections were exposed for 2 h to corresponding secondary fluorescent antibodies (Alexa 488-conjugated antibodies, 1:400) at room temperature. Sections were then fixed with paraformaldehyde 4% for 15 min at room temperature, rinsed, and then the protocol for BrdU staining already mentioned was carried out. After incubation with the secondary goat anti-rat, sections were incubated with Streptavidin-Cy3 (1:400;

Sigma S6402) for 2 h at room temperature. Finally, sections were cover-slipped in antifading mounting medium (mowiol-DABCO 25 mg/ml).

Counting Procedure

The number of BrdU-labeled cells revealed by DAB histochemistry was quantified with a bright-field microscope at 40X magnification, on a series of every sixth section at 300-µm intervals spanning the entire hippocampus. BrdU–labeled cells were counted in the granule-cell layer (GCL) for survival assays and in the subgranular zone (SGZ) (defined as a two-cell soma-wide zone along the base of the GCL) for proliferation studies. BrdU-labeled cells were also counted in the subventricular zone as control for the specificity of antidepressant action. Cells were considered BrdU+ when their nuclei were completely filled with DAB product or showed patches of variable intensity (this dilution pattern is classical for survival assays). The number of BrdU+ cells was estimated by multiplying the total number of cells by 6 (6 sections per series). The quantification of Ki67 was done in the same way as BrdU. In proliferation and survival experiments, the percentage of double-labeled cells (BrdU/ doublecortin - PSA-NCAM – calbindin - NeuN - GFAP) in the GCL was determined at the Institut du Fer à Moulin Imaging facility using a Leica SP2 confocal laser scanning microscope (Leica, Mannheim, Germany). Between 15 and 30 BrdU+ cells per mouse (n = 4 mice/experimental group) were analyzed in their entire z-axis with a 1-µm step to exclude false double-labeling. Double-labeled cells were expressed as percentage of total BrdU+ cells quantified.

References

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