SUPPLEMENTAL FIGURES AND LEGENDS

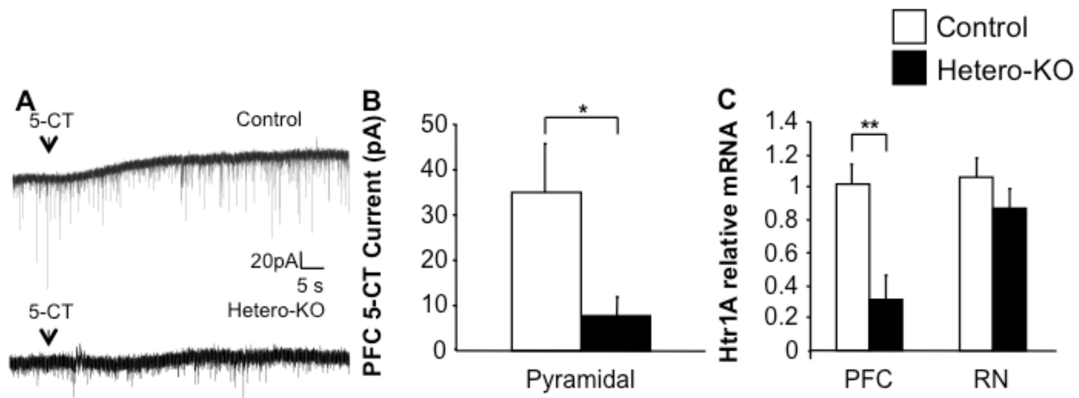

Figure S1. Related to Figure 1 and Table 1. (A) Representative current traces taken from the mPFC of Hetero-KO mice and their controls in response to 5-CT. **(B)** Mean outward current in response to 100 nM 5-CT was decreased in Hetero-KO mice (ANOVA for main effect of group: $F_{(1,19)}=5.266$; $p<0.05$; n=10-11/group). **(C)** Decreased 5-HT_{1A} mRNA in the PFC but not in the raphe nucleus (RN) in Hetero-KO mice when compared to their controls (ANOVA for main effect of group: PFC: $F_{(1,6)}=13.602$; $p<0.01$; RN: $F_{(1,8)}=1.192$; $p=0.307$; n=4-5/group). Data are represented as mean±SEM. *$p\leq 0.05$, **$p<0.01$ compared to control mice.

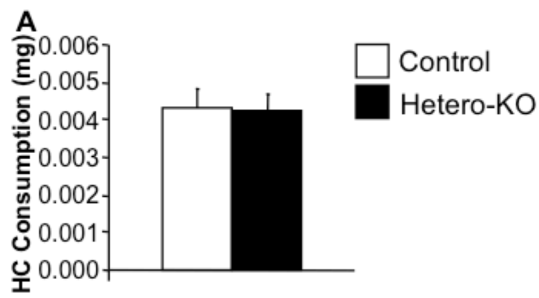

Figure S2. Related to Figure 1. (A) No difference was observed between control and Hetero-KO groups in home cage consumption after the NSF test. (ANOVA for main effect of group: $F_{(1,26)}=0.008$; $p=0.9291$; n=10-18/group). Data are represented as mean±SEM.

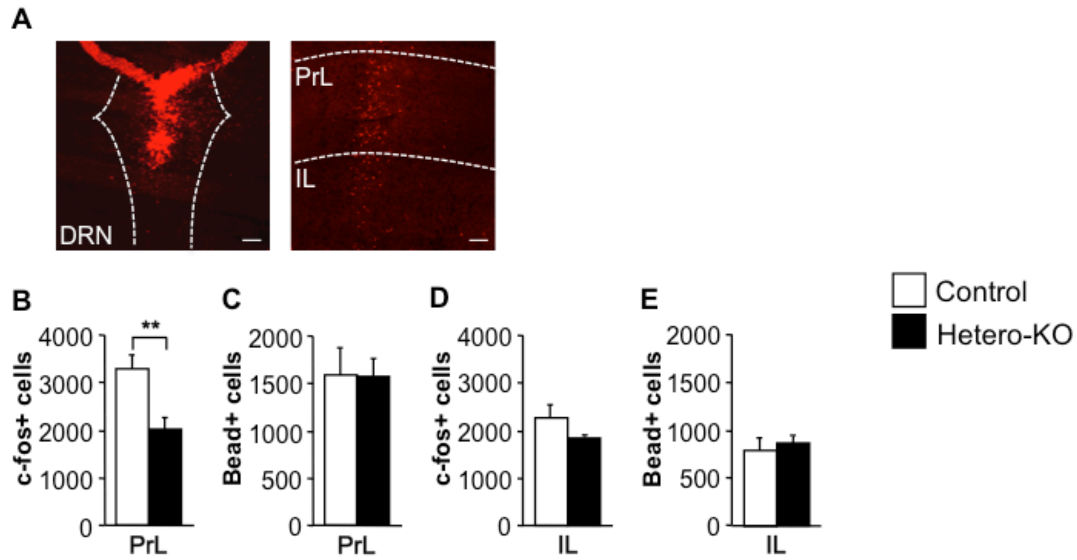

Figure S3. Related to Figure 2. (A) Sample image of immunofluorescence at the retrobead injection site in the DRN, and retrobead localization in the mPFC. **(B)** Decreased number of stress induced c-fos+ cells **(C)** along with no changes in the number of beads in the PrL of Hetero-KO mice when compared to their respective controls after injection of retrobeads into the DRN (ANOVA for main effect of group: c-fos: $F_{(1,6)}=12.457$; $p<0.05$; beads: $F_{(1,6)}=0.001$; $p=0.972$; n=4/group). **(D,E)** No differences were detected in the number of stress induced c-fos+ cells or number of bead+ cells in the IL when comparing Hetero-KO to control mice. (ANOVA for main effect of group: c-fos: $F_{(1,6)}=2.672$; $p=0.1532$; beads: $F_{(1,6)}=0.183$; $p=0.6836$; n=4/group). Data are represented as mean±SEM. *$p\leq 0.05$, **$p<0.01$ compared to control mice.

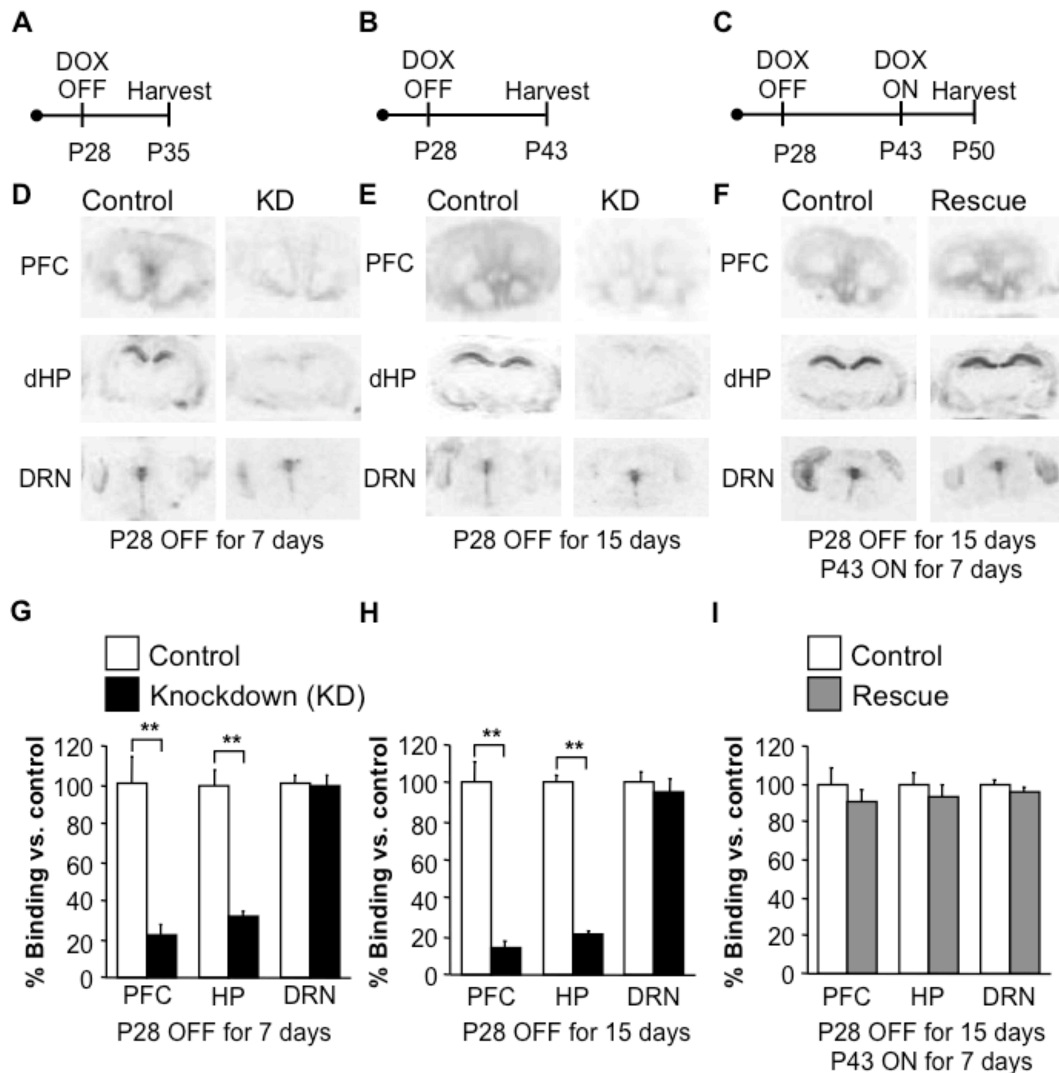

Figure S4. Related to Figure 4. 5-HT_{1A} heteroreceptor expression was knocked down (KD) at different time points for quantitative ^{125}I-MPPI autoradiography studies: **(A)** DOX was removed at P28 and brains harvested at P35 (KD), **(B)** DOX removed at P28 and brains harvested at P43 (KD) and **(C)** DOX was removed between P28 and P43 and brains harvested at P50 (Rescue). **(D,E)** Matched autoradiograms show 5-HT_{1A} receptor binding sites across the PFC, dHP and DRN in coronal sections, comparing KD mice and **(F)** Rescue mice to controls. **(G)** Removal of DOX at P28 results in a 77.7% reduction in 5-HT_{1A} receptor levels in the PFC and a 67.8% reduction in the dHP along with no changes in the DRN when compared to controls at P35 (ANOVA for main effect of DOX: PFC: $F_{(1,16)}$=25.049, p<0.01; dHP: $F_{(1,16)}$=71.967, p<0.01; DRN: $F_{(1,15)}$=0.003, p=0.9584). **(H)** After 14 days (P43), removal of DOX at P28 results in a 85.6% reduction in the PFC, 79.7% in the dHP and no changes in the DRN when compared to their controls (ANOVA for main effect of DOX: PFC: $F_{(1,16)}$=60.830, p<0.01; dHP: $F_{(1,16)}$=315.271, p<0.01; DRN: $F_{(1,10)}$=0.115, p=0.7419) **(I)** Removal of DOX between P28 and P43 results in indistinguishable 5-HT_{1A} receptor binding sites at P50 (ANOVA for main effect of DOX: PFC: $F_{(1,16)}$=0.788, p=0.3879; dHP: $F_{(1,16)}$=0.591, p=0.4534; DRN: $F_{(1,13)}$=1.075, p=0.3187) (n=2–3/group). For ease of visualization images from each region are depicted at different scales. Data are represented as mean±SEM. *$p \leq 0.05$, **$p<0.01$.

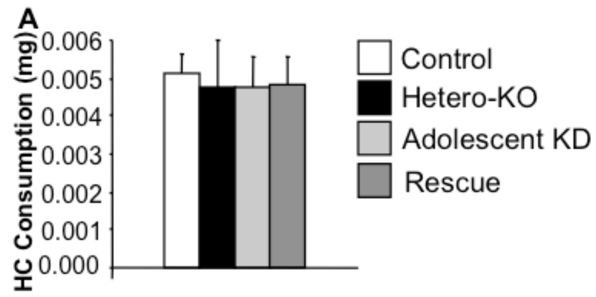

Figure S5. Related to Figure 4. (A) No differences were observed between Control, Hetero-KO, Adolescent KD and Rescue groups in home cage consumption after the NSF test. (ANOVA for main effect of group: $F_{(3,48)}$=0.051; p=0.9846; n=11-15/group). Data are represented as mean±SEM.

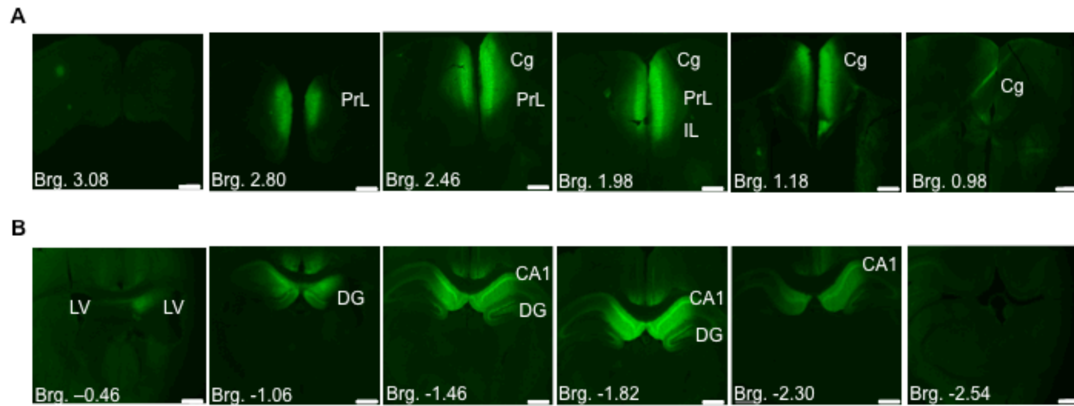

Figure S6. Related to figure 5. Micrographs depicting GFP immunofluorescence in adult mice that were virally infected in adolescence in either the PFC or dHP. **(A)** Representative example of a PFC section stained with GFP in adulthood after injection with AAV9-CaMKIIa-tTs-2A-EYFP-WPRE at P9-P11 into the PFC of Htr1a$^{tetO/tetO}$ mice. Immunofluorescence was detected in cortical areas such as the Prelimbic (PrL), Infralimbic (IL) and cingulate (Cg) regions and restricted to Brg. 2.80-1.18. **(B)** Example of a dHP section stained with GFP in adulthood after injection with AAV9-CaMKIIa-tTs-2A-EYFP-WPRE at P9-P11 into the dHP of Htr1a$^{tetO/tetO}$ mice. Immunofluorescence was largely restricted to the dHP (Brg. (-1.06)-(-2.30)) and detected in areas such as the dentate gyrus (DG) and CA1 regions. LV: Lateral ventricle

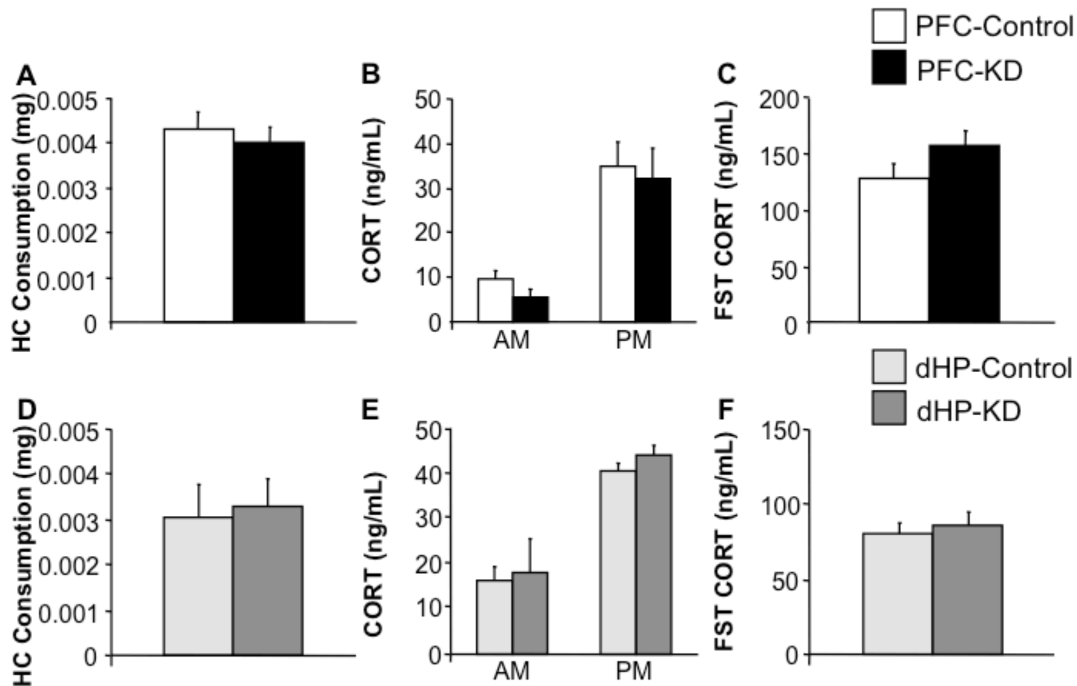

Figure S7. Related to Figure 5. (A) No difference in home cage consumption was observed between PFC-Control and PFC-KD groups after the NSF test. (ANOVA for main effect of group: $F_{(1,27)}$=0.297; p=0.5900; n=14-15/group). **(B)** No difference in basal corticosterone levels was detected between PFC-Control and PFC-KD groups at the onset of either the light or the dark phase (ANOVA for main effect of group: AM: $F_{(1,13)}$=2.199; p=0.1619; PM: $F_{(1,14)}$=0.087; p=0.7727; n=7-8/group). **(C)** Similarly, no difference in corticosterone levels was seen between the two groups after a forced swim stressor (ANOVA for main effect of group: $F_{(1,25)}$=2.685; p=0.1138; n=13-14/group). **(D)** No difference in home cage consumption was observed between dHP-Control and dHP-KD groups after the NSF test. (ANOVA for main effect of group: $F_{(1,20)}$=0.0622; p=0.8055; n=9-13/group). **(E)** No difference in baseline corticosterone levels was detected between dHP-Control and dHP-KD at the onset of either the light or the dark phase (ANOVA for main effect of group: AM: $F_{(1,17)}$=0.048; p=0.8295; PM: $F_{(1,18)}$=1.486; p=0.2386; n=9-10/group) **(F)** No difference in corticosterone levels was detected between the two groups after a forced swim stressor (ANOVA for main effect of group: $F_{(1,20)}$=0.157; p=0.696; n=9-13/group). Data are represented as mean±SEM. *$p \leq 0.05$, **$p<0.01$.

SUPPLEMENTAL MATERIAL AND METHODS

Patch-clamp electrophysiology

Whole-cell voltage clamp recordings were performed in layer 2/3 pyramidal cells and non-pyramidal cells in the medial prefrontal cortex (mPFC). Recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and digitized using a Digidata 1440A acquisition system (Molecular Devices, Sunnyvale, CA) with Clampex 10 (Molecular Devices, Sunnyvale, CA) and analyzed with pClamp 10 (Molecular Devices, Sunnyvale, CA). Following decapitation, 300 µm slices containing the mPFC were incubated in artificial cerebral spinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 2.0 $MgCl_2$, 1.25 NaH_2PO_4, 2.0 $CaCl_2$, 26.2 $NaHCO_3$ and 10.0 D-Glucose, bubbled with oxygen, at 32˚ C for at least 30 minutes. During recording, slices were perfused in 32°C ACSF (with drugs added as detailed below) at a rate of 5 mL/minute. Electrodes were pulled from 1.5 mm borosilicate-glass pipettes on a P-97 puller (Sutter Instruments). Electrode resistance was typically 3–5 MΩ when filled with internal solution consisting of (in mM): 130 K-Gluconate, 5 NaCl, 10 HEPES, 0.5 EGTA, 2 Mg-ATP, and 0.3 Na-GTP (pH 7.3, 280 mOsm). To assay 5-HT_{1A} and $GABA_B$ responsiveness, cells were held at -70 mV in voltage clamp for 1 minute before 100 nM 5-CT or 50 µM baclofen was perfused onto the slice for 5 minutes and the maximal current response during drug perfusion was measured relative to the baseline current. If the magnitude of the response was greater than 2 times the root mean square (RMS) noise, it was considered a significant response. The proportion of cells significantly responding to the drugs under different treatment conditions was compared using a Fischer's exact test. The amplitude of the maximum current response was compared using one-way ANOVA.

Quantitative PCR

As previously described(Garcia-Garcia et al., 2015). Total RNA from the tissues was extracted using TRIzol (Life Technologies, Grand Island, NY, USA). The SuperScript® III First-Strand Synthesis System (Life Technologies, Grand Island, NY, USA) was used to synthesize cDNA, and PCR was performed and quantified using SYBR Green real-time PCR Master Mix (Life Technologies, Grand Island, NY, USA). Data were normalized using GAPDH mRNA levels as a standard. For 5-HT_{1A}, the sense primer was 5'-GACCCTTCCTGTTCACTCA-3' and the antisense primer was 5'-AAAAGCACTGTCCCCTTAA-3'.

Behavioral and physiological studies

All mice used for behavioral testing were male and age matched within 2 weeks. Mice were tested starting at 12–14 weeks of age and the battery of behavioral tests took 4-5 weeks to complete. Baseline anxiety tests were completed before other behavioral tests. Behavioral effects have been seen in multiple cohorts. Data for behavioral tests in Figure 1 comes from a single cohort. Data for corticosterone in Figure 1 come from a separate cohort. For Figure 4 and 5 the same cohorts were used for all the behavioral tests.

Open-field test

Exploration in response to a novel open field was measured as described(Richardson-Jones et al., 2010; Richardson-Jones et al., 2011; Weisstaub et al., 2006) with the following minor modifications: (1) animals were habituated to the testing room for at least 30 min prior to testing, (2) light levels in the open field chambers were maintained at 30–40 lux to encourage exploration of the full environment, (3) animals were placed in a corner of the maze and allowed to explore the center at will, and (4) the test was conducted for a total of 30 min. The center of the arena was defined as a square area occupying the center 50% of the total arena. Dependent measures were total path length (cm), time in the center and percent distance in the center (distance travelled in the center divided by the total distance travelled).

Elevated-plus maze

After animals were habituated to the testing room for at least 30 min, animals were placed into the central area facing one open arm and allowed to explore the maze for 5 min. Testing took place at 90–100 lux. The videos were scored using the behavioral tracking software TopScan (CleverSys Inc, Reston, VA). Dependent measures were total number of entries into the open arms, time in the open arms and percent time in the open arms (time in the open arms divided by the total time).

Novelty suppressed feeding test

Testing was performed as previously described(David et al., 2009; Richardson-Jones et al., 2010). Briefly, animals were food restricted for 24 hours and the latency (dependent measure) to begin chewing a food pellet placed on a white piece of filter paper (12.5 cm diameter) in the center of

brightly lit arena was recorded (40 × 60 cm arena with 2 cm of new corn cob bedding; ~900 lux). The trial was terminated either when an animal began chewing or 300 seconds transpired. Immediately after terminating the trial, animals were placed in their home cage and the amount of food consumed in 5 minutes was measured (home cage consumption), followed by an assessment of post-restriction weight. Percentage body weight lost during food deprivation prior to the testing was assessed to ensure both groups lost similar amounts of weight. Home cage consumption immediately after testing was assessed as a relative measure of hunger.

Fear conditioning

As previously described(Weisstaub et al., 2006). Briefly, mice were trained with a single 20 second tone (CS) that co-terminated with a 0.8 mA, 1 s shock (US), for 2 consecutive days in 8 min sessions. In the first session, the first minute, was scored for basal behavioral “freezing”. During the second day, the mice were exposed to the same context. The first 3 min of the second session were used to measure context related freezing. After the 3 first min, a second round of training was delivered in the same way described above.

On the 3rd day of the experiment (cue test), the mice were handled using different types of gloves, bedding and odor cues. The testing occurred in chambers that were modified by the use of distinct odors, texture and light. The session lasted 6 min and 1 CS was delivered. Conditioning to the tone was assessed during the presentation of the CS.

Cookie test

Testing was performed as previously described(Surget et al., 2011). Briefly, this test requires a device containing three aligned compartments (20 × 20 × 20 cm) that differ only in color (White, grey and black in sequence). Mice were first familiarized with a chocolate cookie 2.5 weeks before the first testing. Animals were faster one hour before testing. At the time of testing, a small amount (≈2 gr,) of chocolate cookie was placed in the center of the black compartment. The mouse was initially placed in the white compartment of the apparatus and must make its way to the black compartment to eat the cookie. Each session of the test lasted 5 minutes. The cookie consumption (# bites) was recorded within the test period. Four sessions of testing were performed within 10 days (inter-test interval: 2 days).

Sucrose Preference

An 8-day sucrose preference protocol was performed as previously described(Garcia-Garcia et al., 2015). On days 1 and 2, mice were presented with two identical bottles filled with water (water/water) for 2 h and 1 h respectively. On days 3 and 4, both bottles contained 1% sucrose solution dissolved in the drinking water (sucrose/sucrose) for 1 h and 30 min respectively. On days 5–8, one bottle was filled with water and the other was filled with 1% sucrose solution for 30 min each day (water/sucrose). The sucrose and water bottles were alternated between days. Preference on each day was calculated as: (weight bottle 1/ (weight bottle 1 + weight bottle 2) ×100).

Modified forced-swim test

Behavioral response to forced swimming (FST) was assayed as described previously(David et al., 2007; Richardson-Jones et al., 2010). After 30 min habituation to the testing room, mice were placed into clear plastic buckets 20 cm in diameter and 23 cm deep filled 2/3 of the way with 26°C water and videotaped from the side for 6 min. Only the last 4 minutes were scored. All animals were exposed to the swim test on two consecutive days. Scoring was done using an automated Viewpoint Videotrack software package (Montreal, Canada), which was validated before by manual scoring. Dependent variables were immobility, swimming and climbing.

Corticosterone AM-PM levels and stress evoked increase of corticosterone levels

Baseline corticosterone levels were collected at the dark-light transition and 12 hours later at the light-dark transition as previously described(Garcia-Garcia et al., 2015). For stress evoked corticosterone, experiments were performed starting at 12.00 where mice were exposed to FST for 6 min and blood was drawn from the submandibular vein 12 min later. Cortiocosterone levels were assessed by ELISA (Enzo Life Sciences) as previously described(Garcia-Garcia et al., 2015).

Retrobead injections

For the retrobead experiment, male mice received a single injection of 0.25 µl of red retrobeads (Lumafluor) into the DRN (x=0.00, y=(+4.7)-(+4.8), z=(-3.1)-(3.2)). 15 days after surgery c-fos expression was induced as previously described by a forced swim stressor (10 min) and mice were perfused 2 hours later(Garcia-Garcia et al., 2013). Brains were postfixed overnight at 4°C, cryoprotected in 30% sucrose and coronal serial sections (35 µm) of the DRN were taken.

Immunohistochemistry

C-fos induction and tissue processing

C-fos expression was induced as previously described by a forced swim stressor (10 min) and mice were perfused 2 hours later(Garcia-Garcia et al., 2013). Mice were transcardially perfused (cold 0.1 M phosphate buffer (pH = 7.4)(PBS) under ketamine/xylazine (100 mg/ml and 20 mg/ml respectively). Brains were removed, post-fixed (24 h), cryo-protected in a 30% sucrose solution (in phosphate buffer) and stored at 4 °C. Serial sections (35 μm) were cut through the entire brain on a cryostat (Leica CM3050 S) and stored in PBS with 0.1% NaN_3.

The expression of stress-induced c-fos in different brain regions including mPFC (infralimbic (IL) and prelimbic (PrL) regions), dentate gyrus in the hippocampus (DG), basolateral amygdala (BLA), lateral wings (LW) and dorsal (DRN) and medial (MRN) areas of the raphe nucleus (RPH) was studied(Morgan et al., 1987). Serial free-floating sections, at 1:4 intervals through the RPH and at 1:6 through the forebrain areas were used.

Immunofluorescence studies

For retrobeads experiments, PFC sections were blocked in 10% Normal donkey serum (NDS) and then were blocked in 10% Normal donkey serum (NDS, Jackson Immunoresearch) with 1% Triton in PBS for 1 hr. and then incubated with rabbit c-fos antibody (1:5000, Calbiochem PC38) overnight at 4 °C. After washing with PBS, sections were incubated for 1 hr with secondary antibody (1:200 biotinylated donkey anti-rabbit, Jackson Immunoresearch) followed by amplification with an avidin (1:200,Cy5, Jackson Immunoresearch) complex and NeuroTrace fluorescent Nissl stain (Invitrogen).

Image processing and quantification

For all experiments, sections were imaged with identical exposure times, and parameters with a confocal microscope (Leica, NY, USA). Induced c-Fos immunoreactivity was assessed at a magnification of 20x and only very intense red stained cells were counted. Each section was assessed for the number of single c-fos+ nuclei, the number of single CamKII, GAD67 or TPH+ cells as well as the number of double-labeled cells. CamKII and GAD67+ cells were identified with by the typical perinuclear halo pattern of fluorescence. Co-localization of induced c-fos immunoreactivity with CamKII, GAD67 or TPH was confirmed stack analysis of the images and evaluating the sections in Z series (ImageJ software). All counts were done by investigators that were blinded to conditions and all sections were counted by more than one investigator.

In vivo anesthetized recordings of 5-HT neurons.

Mice were anaesthetized with chloral hydrate (400 mg/kg i.p) and placed in a stereotaxic frame (using the David Kopf mouse adaptor) with the skull positioned horizontally. The extracellular recordings were performed using single- or five-barreled glass micropipettes (R&D Scientific Glass, USA) for recordings in the DRN. Micropipettes were preloaded with fiberglass strands to promote capillary filling with a 2 M NaCl solution. Single glass micropipettes pulled on a pipette puller (Narishige, Japan) with impedances ranging from 2.5 to 5 mV, were positioned 0.2–0.5 mm posterior to the interaural line on the midline and lowered into the DRN, usually attained at a depth of 2.5–3.5 mm from the brain surface. The DRN 5-HT neurons were then identified according to the following criteria: a slow (0.5–2.5Hz) and regular firing rate and a long- duration, positive action potential.

Intracerebral in vivo microdialysis

Extracellular 5-HT levels were measured by in vivo microdialysis as previously described with minor modifications(Guiard et al., 2008b). Specifically, two concentric dialysis probes were implanted in the DRN and mPFC (outer diameter by active length: 0.3 × 1.6 mm and 0.3 × 2 mm, respectively) of anesthetized mice (chloral hydrate, 400 mg/kg, i.p.). Stereotaxic coordinates were as follows (in mm): DRN: anterior, −4.5; lateral, 0; ventral, 3.0 mm: mPFC: anterior, 1.6; lateral, 1.3; ventral, 1.6 from bregma (Franklin and Paxinos, 1997). Following recovery, probes were continuously perfused with aCSF, and dialysates were collected every 15 min in the mPFC and every 30 min in the DRN for 120 min for analysis by HPLC-amperometry (Guiard et al., 2008a). Baseline 5-HT levels were calculated as the average of the first four samples, ± SEM. Freely moving mice were treated (t = 0) with either a challenge dose of fluoxetine (FLX) (18 mg/kg; i.p.) (BIOTREND Chemicals AG,Wangen, Zurich) or its vehicle, and dialysate samples were collected for a 0–120 min post-treatment period. The limit of sensitivity for 5-HT was 0.5 fmol/sample (signal-to-noise ratio, 2).

Heteroreceptor Autoradiography

As previously described(Richardson-Jones et al., 2011). Mice were cervically dislocated and decapitated. Brains were extracted, immediately frozen on dry ice, and maintained at −80 °C until sectioning. Brains were cryosectioned at a thickness of 20 μm and thaw mounted on Superfrost Plus slides (Fisher Scientific). Afterwards, sections were maintained at −80 °C until processing. Sections

were processed for 4-(2′-methopxyphenyl)-1-[2′-(n-2″-pyridinyl)-p-[^{125}I] iodobenzamido]ethylpiperazine (^{125}I-MPPI) autoradiography. To determine receptor levels, films were scanned in 32-bit grayscale at 1600 dpi and analyzed using ImageJ software for quantification. For prefrontal cortex (PFC) and hippocampus (HP) matching coronal sections were identified for each brain. The dorsal raphe (DRN) was outlined by hand in a randomly selected control individual, and this outline was then applied to the corresponding section in all the other animals. Levels of 5-HT$_{1A}$ binding were determined by analyzing the region of interest and subtracting from a same-size adjacent "background" region of tissue lacking specific binding, to obtain a normalized luminosity value for each region of interest. Signals were determined to be within the linear range of the film using a standard curve constructed from an ARC146-F 14C standard (ARC). Values are represented in percentage versus respective control. Autoradiograms in figure 4 were enlarged from original size for visualization purpose.

Viral injections and temporal regulation of virally expressed tTS

All surgical procedures were performed under sterile conditions. P9-P10 Htr1a$^{tetO/tetO}$ male mice were anaesthetized with isoflurane, and were placed in a neonatal mouse adaptor stereotax (Stoelting, Wood Dale, IL). Briefly, a small craniotomy was made using anteroposterior (AP) and mediolateral (ML) coordinates from bregma and dorsoventral (DV) coordinates from skull surface (provided in millimeters): mPFC: ML=(±0.1)-(±0.15), AP=(+0.85)-(+1.0), DV=(-1.4)-(-1.5); dHP: ML=(±0.45)-(±0.55), AP=(-0.9)-(-1.0), DV=(-2)-(-2.1). Mice were bilaterally infused in the mPFC or dHP using a pressure injected glass micropipette with 0.1 microL of the AAV9-CaMKIIa-tTs-2A-EYFP-WPRE (2.20e+13vg/mL) virus at 0.025 microL/min. After surgery, all mice were maintained on DOX (ON DOX whole-life-Control). At P28, half of the mice were withdrawn from DOX and maintained on normal chow (OFF DOX starting at P28-KD) (Figure 5). All animals used for behavioral testing were age matched within 2 weeks. Animals were initially tested at 12–14 weeks of age. Baseline anxiety tests were completed before other behavioral tests. At the conclusion of the experiment, viral injection sites were confirmed by immunohistochemistry. For the PFC injections animals with virus-spread limited to the PrL, IL and Cg in both hemispheres were included in the experimental groups. For the dHP injections animals with virus-spread over the dentate gyrus and CA1 in both hemispheres were included in the experimental groups. Animals with virus expression in surrounding areas beyond a few cells in the needle track or with only was hemisphere infected were excluded. In the mPFC intervention out of 33 injected mice 29 mice were included while for dHP intervention all mice were included for behavioral analysis.

Immunohistochemistry to determine the extent of the viral infection

After behavioral experiments were completed, the extent of viral infection was confirmed by visual examination of EYFP fluorescence. Specifically, free-floating coronal serial sections (35 µm) of the mPFC or HP were blocked in 10% NDS for 1 hour at room temperature and incubated in primary antibody overnight at 4°C (1:500, rabbit anti-GFP, Invitrogen). After sections washed in PBS and incubated for 1 hr with secondary antibody (1:200, donkey anti- rabbit Cy2, Jackson ImmunoResearch) and NeuroTrace fluorescent Nissl stain (Invitrogen). Sections were imaged with a confocal microscope (Leica, NY, USA) (Supplementary Figure S6).

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