Supporting online material

Experimental procedures

Animals

Knockout and wild-type 129/SvEv age-matched adult male mice (12–20 weeks) derived from heterozygote crossings were used in all experiments that included 5-HT_{1A} knockout mice. For all the other experiments except the CUS paradigm, 129/SvEv mice of the same sex and age were purchased from Taconic. BALB/c mice were used in the CUS paradigm due to their ability to react to chronic stress (*S1*). Mice were housed 4–5 per cage in a 12-h (06:00–18:00) light-dark colony room at 22°C with freely available food and water.

Behavioral testing and chronic unpredictable stress procedure

The Novelty-suppressed feeding test was carried out during a 5-min period as previously described (*S2*). Briefly, the testing apparatus consisted of a plastic box, 50x50x20 cm. The floor was covered with approximately 2 cm of wooden bedding. Twenty-four hours prior to behavioral testing, all food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the center of the box. An animal was placed in a corner of the maze and a stopwatch was immediately started. The measure of interest (chewing) was scored when the mouse was sitting on its haunches and biting with the use of forepaws. Immediately after this test, mice were transferred to their home cage and the amount of food consumed in 5 min was measured (home cage food consumption). In knockout experiments, mice were genotyped after behavioral testing to remove the possibility of investigator bias. Mice were always tested during the light period.

The stress regimen used is a variant of the chronic mild stress procedures (*S3*). Mice were subjected to various and repeated unpredictable stressors for a period of five weeks (*S4*). The different stressors used were: alterations of the bedding (repeated sawdust changing, removal of sawdust, damp sawdust, substitution of

sawdust with 37 C water); cage tilting (45°); predator sounds (15 min.); cage shift (mice were positioned in the empty cage of another male); light to dark or dark to light shifts (four light/dark successions of 30min every 24 hours); reversal of the light/dark cycle. The fluoxetine treatment (10 mg/kg/day, i.p.) started at the beginning of the third week. The state of the coat was assessed at the end of the stress regimen (end of fifth week). The total score resulted from the sum of the score of seven different body parts: head, neck, dorsal coat, ventral coat, tail, forepaws and the hindpaws. For each of the seven body areas, a score of 0 was given for a well-groomed coat and 1 for an unkempt coat. This index has been previously pharmacologically validated (*S4*). The grooming latency was assessed at the end of the sixth week. This test consisted in squirting 200 µl of a 10% sucrose solution on the mouse's snout. The latency to start grooming was then recorded. Each measure was scored by an experimenter blind to the treatment conditions.

Cued Fear Conditioning

Fear conditioning took place in a mouse conditioning chamber enclosed by a sound-attenuating cubicle. Stimulus presentation was controlled by a programmable audio generator. On the training day mice were placed in the conditioning chamber and, following a two-minute habituation period, presented with three acoustic tone CSs (4.5 kHz, 20 sec, 85 dB) that co-terminated with a footshock US (0.7 mA, 1 sec). The CS/US pairings occurred 120, 290 and 400 seconds after placing the animals in the chamber. 24 hours later, mice were placed in a novel conditioning chamber containing different spatial and sensory cues, and presented with the CS after 90 seconds. Freezing (characteristic crouching posture, and cessation of all but respiration-related motion) was used as an index of conditioned fear (*S5*) and scored as the percentage of time spent freezing for 20 seconds preceding and 20 seconds during the CS.

Drugs

Fluoxetine, imipramine, desipramine and haloperidol were dissolved in tap water

at a concentration of 80, 160, 160, and 8 mg/L, respectively. These concentration were established based on the average drinking amount and average weight of the mice used in the study to achieve a final dose of 10 (fluoxetine), 20 (imipramine and desipramine) and 1 (haloperidol) mg/kg/day. HPLC analysis demonstrated that steady serum levels of fluoxetine, nor-fluoxetine, imipramine and desipramine had been achieved by 5 days after the beginning of the oral regimen (data not shown). Vehicle animals received tap water. 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) was dissolved in sterile saline (0.9% NaCl) at a concentration of 4.5 mg/ml, and delivered through Alzet osmotic mini-pumps implanted subcutaneously 28 days prior to behavioral testing. The final average dose delivered was 1mg/kg/day. Sham mice were implanted with mini-pumps containing sterile saline.

BrdU Labeling, Immunohistochemistry and Imaging

To assess the effect of antidepressant or irradiation treatments on the number of BrdU-positive cells, mice were administered BrdU (4x75 mg/kg i.p., dissolved in saline, every 2 hr and sacrificed 24 hr after the last BrdU injection. To determine cell survival and phenotype after the various antidepressant treatments, fluoxetine, imipramine and vehicle-treated mice were allowed to survive for 28 days after the last BrdU injection. After anesthesia with ketamine/xylazine (100 mg/kg and 7 mg/kg respectively), mice were transcardially perfused (cold saline for 2 min, followed by 4% cold paraformaldehyde/0.1 M phosphate buffer for 5 min) and brains were collected for immunohistochemistry. All brains were post-fixed overnight in 4% paraformaldehyde at 4°C, then cryoprotected in 30% sucrose and stored at 4°C. Serial sections (35 µm) were cut through the entire hippocampus (plates 41-61; Franklin and Paxinos, 1997) or the SVZ (plates 27-40) on a cryostat and stored in PBS with 0.1% NaN_3. For DAB staining, sections were slide-mounted and boiled in citric acid, pH 6.0, for 5 min, rinsed with PBS, and treated with 0.01% trypsin in Tris/CaCl2 for 10 min. Brain sections were incubated for 30 min with 2N HCl and blocked with 5% NGS. Sections were then incubated over night at room temperature with anti-mouse BrdU (1:100). After

washing with PBS, sections were incubated for 1 hr with secondary antibody (1:200 biotinylated goat anti-mouse) followed by amplification with an avidin-biotin complex. The staining was visualized with DAB. For fluorescent double-labeling, the DNA-denaturation procedure consisted of the following steps: 2 hr incubation in 50% formamide-2X SSC at 65°C, 5 min rinse in 2X SSC, 30 min incubation in 2N HCl at 37°C, and 10 min rinse in 0.1 M boric acid, pH 8.5. Sections were then incubated in TBS-0.1% Triton X-100 (Tx)-5% normal donkey serum (NDS) for 30 min, followed by anti-rat BrdU primary antibody (1:100) in TBS/NDS/Tx for 24 hrs at 4°C in combination with either a mouse monoclonal antibody to NeuN (1:500) or a rabbit polyclonal antibody to GFAP (1:800). The fluorescent secondary antibodies used were biotin-conjugated donkey anti-rat (1:200) plus streptavidin Cy2 (1:200), donkey anti-mouse Cy3 (1:500), or donkey anti-rabbit Cy3 (1:500). Fluorescent images were taken with a confocal laser scanning system coupled to an inverted microscope. For each image acquisition, a Kalman average of five frames was used. For quantification of BrdU labeling a stereological procedure was used as previously described (*S6*). In the irradiation experiments, SVZ was conducted at interaural level 3.34, just within the area covered by the lead shield. For quantification of BrdU/NeuN or BrdU/GFAP double-labeled cells, at least 50 BrdU-positive cells per animal were analyzed using Z-plane sectioning (1 μm steps).

Irradiation

Mice were anesthetized with ketamine and xylazine, placed in a stereotaxic frame and exposed to cranial irradiation using a Siemens Stabilopan X-ray system operated at 300 kVp and 20 mA. Animals were protected with a lead shield that covered the entire body, but left unshielded a 3.22 X 11-mm treatment field above the hippocampus (interaural 3.00 to 0.00). A second shield was constructed, with a sliding opening of the same dimensions as the first, to selectively irradiate either the SVZ or the cerebellum (interaural 3.00 to 6.00 and

0.00 to –3.00 respectively). Positioning of the opening of each shield above the targeted area was confirmed surgically. Dosimetry was done using a Capintec Model PR06G electrometer ionization chamber and Kodak Readypack Radiographic XV films. The corrected dose rate was approximately 1.8 Gy per min at a source to skin distance of 30 cm. The procedure lasted 2 min and 47 sec, delivering a total of 5 Gy. Three 5 Gy doses were delivered on day 1, 4, and 8. To assess the effects of this procedure at different time points, irradiated and sham mice were injected with BrdU on day 11, 15 or 27 and all animals were sacrificed on day 28. A cumulative 15 Gy dose was the minimum necessary to produce a reduction of cell proliferation greater than 85% in the dentate gyrus, which lasted for at least two months.

TUNEL Analysis

Analysis of apoptotic cells was carried out by TUNEL-labeling followed by Nissl counter staining, as previously described (*S7*). Adult male mice received an X-ray dose of either 0 or 15Gy, and were sacrificed 10hrs later by transcardial perfusion with saline followed by 4% PFA. Brains were post-fixed overnight, cryoprotected in a 30% sucrose solution, and serial sectioned at 20 µm using a cryostat. Sections containing the hippocampus were slide mounted and allowed to dry. TUNEL-labeling was done using the Apoptag cell death detection kit. Briefly, sections were rinsed in PBS and then treated with Proteinase K (20 µg/ml in PBS) for 30 minutes at room temperature. After rinsing in PBS, endogenous peroxidase quenching was done by immersing sections in 3% H2O2 for 15 min. Sections were again rinsed with PBS before incubating with terminal deoxynucleotide transferase in the presence of digoxygenin-dUTP, for 2hrs at 37 C. The reaction was stopped by immersing slides in stop/wash buffer for 30 min at 37 C. After PBS rinse, sections were incubated with anti-digoxygenin peroxidase for 30 min at room temperature. Sections were rinsed, incubated with diaminobenzidine substrate, and observed under a microscope until the reaction was complete. Sections were then counterstained with thionin to visualize basophilic chromatin clumps characteristic of apoptosis.

Stereology

Morphological analyses to assess the effects of irradiation on Nissl-stained granule cells were performed as previously described (*S8*). Briefly, the total number of Nissl-stained cells from 6 sham and 6 irradiated mice was counted monolaterally in the dentate gyrus, through its entire rostro-caudal axis, by using an unbiased method of cell counting (optical fractionator (*S8*)). Throughout the experiment, the investigator was blind to the treatment status. For this analysis, a Zeiss Axioplan-2 microscope equipped with a Zeiss planapochromat 100X oil objective and a CCD camera is used to generate digitized images that will be collected and analyzed on a Micron Millennia computer using the software Stereo Investigator™. Every sixth section throughout the entire extent of the dentate gyrus was counted. The volume was then calculated, taking into account the frequency of sections (1:6) and their thickness (35 μm), according to the Cavalieri principle (*S8*).

Corticosterone Test

To establish baseline levels of corticosterone, blood samples were collected by making a small incision on the tip of the mouse tail. Twenty four hours later the same animals were subjected to an Open Field (*S9*), and 15 min later animals were scarified and trunk blood was drawn. Serum corticosterone concentrations were measured with an ^{125}I-RIA kit.

Electrophysiological recordings

Hippocampal slices were obtained from sham and irradiated mice four weeks after the beginning of the X-ray procedure. Transverse slices (thickness 400 μm), prepared as previously described (*S10*), were maintained in an interface chamber at 29 ºC. and perfused with ACSF solution (124.0 mM NaCl, 4.4 mM KCl, 1.0 mM Na2HPO4, 25.0 mM NaHCO3, 2.0 mM CaCl2, 2.0 mM MgSO4, 10 mM glucose) continuously bubbled with 95% O_2 and 5% CO_2. Slices were

permitted to recover for at least 90 min before recordings. Field excitatory postsynaptic potentials (fEPSP) were evoked by a bipolar concentric platinum-iridium electrode and recorded by low-resistance (3-5 MW) pipettes filled with ACSF solution. Both the stimulating and the recording electrodes were placed in CA1 *stratum radiatum* (SR). Basal synaptic transmission was assayed by plotting the stimulus voltages (V) against slopes of fEPSP to generate input-output relationships. For LTP experiments, baseline stimulation was delivered every minute (0.01 ms pulse duration) at an intensity corresponding to approximately 35% of that used for the maximum evoked response. Baseline responses were recorded for 15 min prior to the LTP-inducing stimulation to assure stability of the response. LTP was induced using theta-burst stimulation (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 15 seconds).

28 days survival

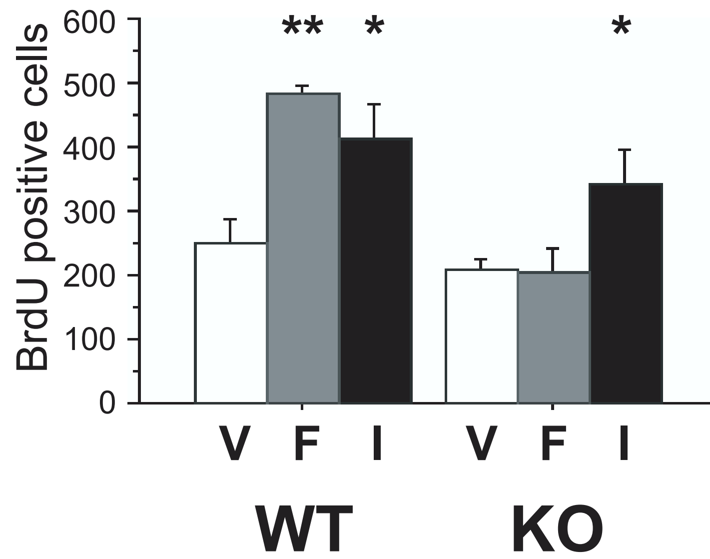
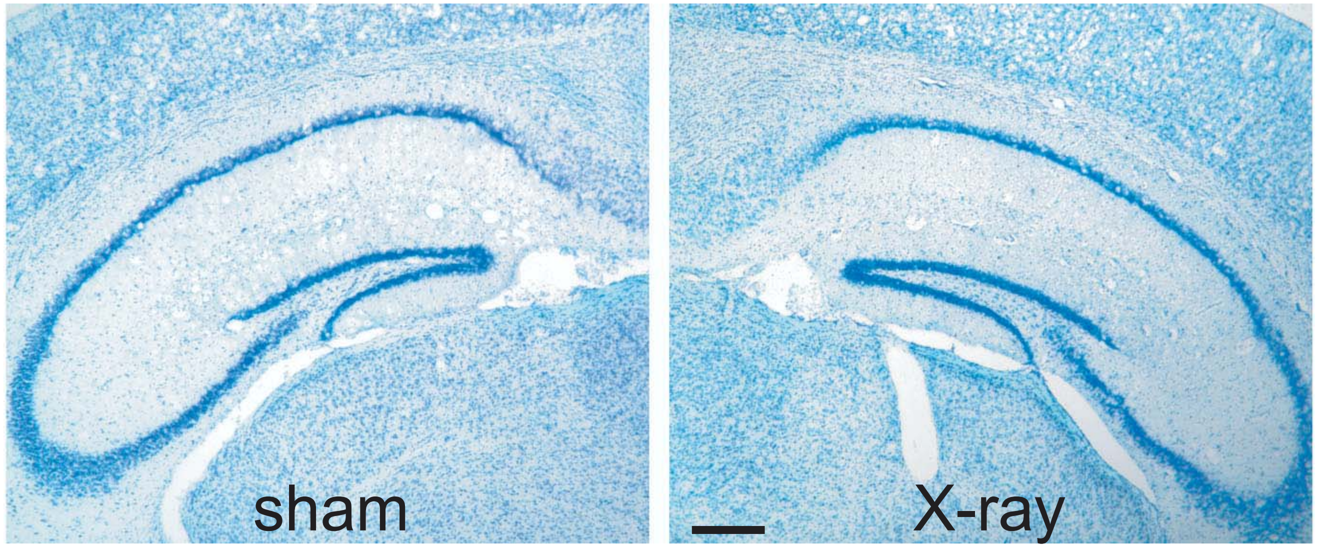


figure S1

A



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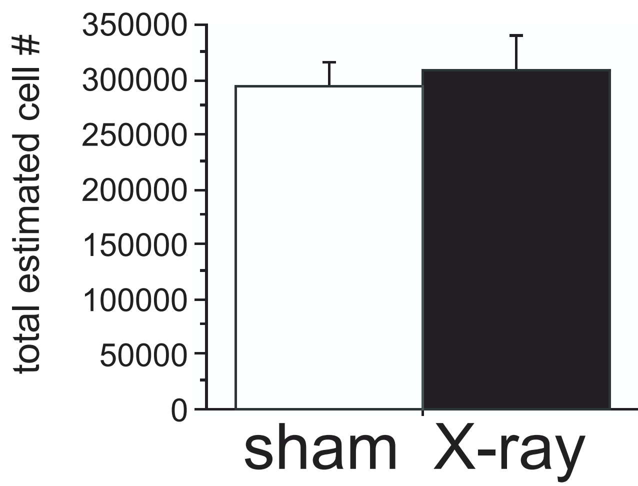


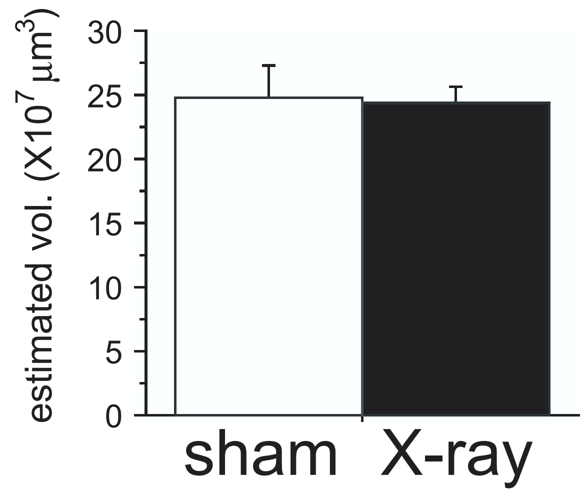
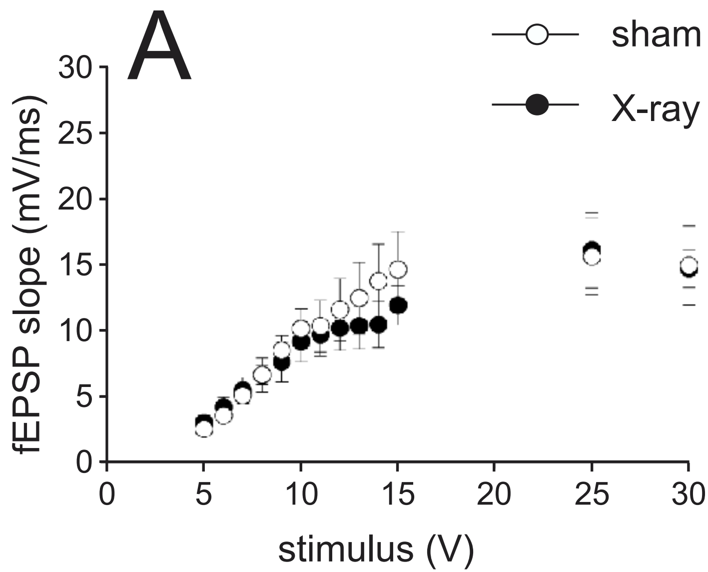
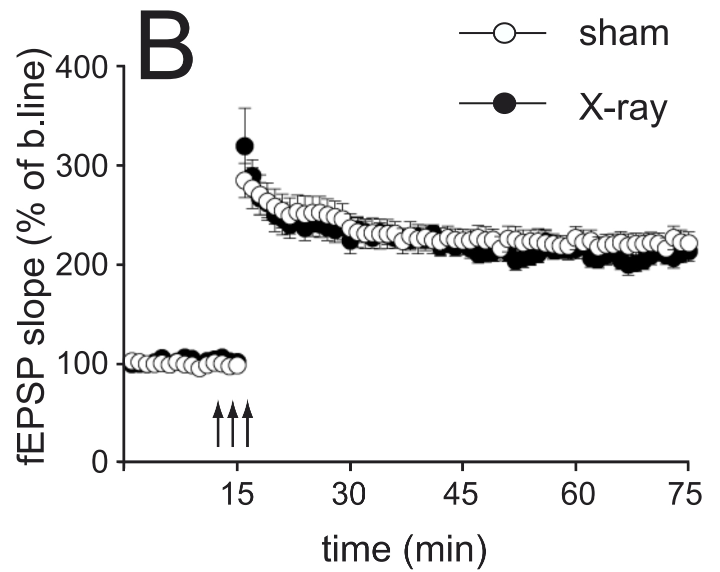
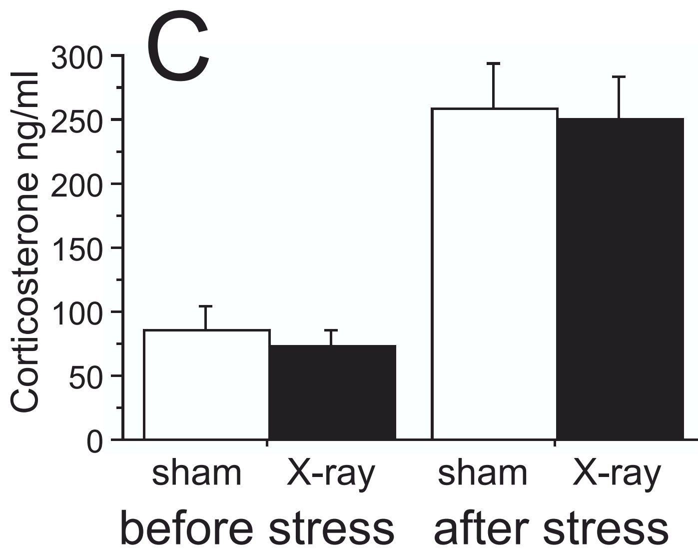


figure S2







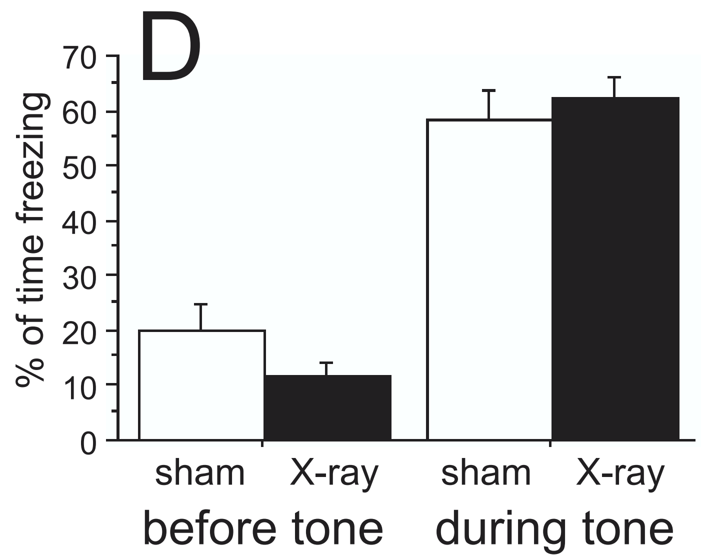


figure S3

Figure legends

Fig. S1 Assessment of BrdU positive cells 28 days after BrdU administration (survival)**.**

A significant proportion of cells generated at the end of the antidepressant treatment survived ≥28 days; differences between treatment groups were maintained. Effect of ADs treatment, ANOVA, $F_{2,16}$ = 10.6, P = 0.001, genotype, ANOVA, $F_{1,16}$ = 11.9, P = 0.032, and interaction between the effect of treatment and genotype, ANOVA, $F_{2,16}$= 6.0, P = 0.01 (n =4-5 mice per group). Significant differences (Fisher post-hoc) between vehicle (V) and fluoxetine (F) or imipramine (I) are indicated by *, $P< 0.05$ or **, $P< 0.01$.

Fig. S2 X-ray Does Not Affect Hippocampal Morphology, Cell number and Total volume.

(A) Nissl-stained sections from sham (left) and irradiated (right) animals at the time of behavioral testing. No gross morphological alterations were apparent. Scale bar, 500µm. **(B)** Stereological analysis revealed no significant effect of irradiation on cell number (mean ±SEM of total estimated cell number per hemisphere) and on total volume of the dentate gyrus (mean ±SEM of estimated volume per hemisphere).

Fig. S3 X-ray does not affect CA3/CA1 physiology, neuroendocrine response to stress, or cued fear conditioning.

(A) Sham (*open circles*) and irradiated (*closed circles)* mice showed similar field input-output relations in Shaffer collateral/CA1 connections (mean ± SEM of fEPSP slope; n=8). **(B)** Summary graphs of LTP shows no difference between Sham and irradiated mice (mean ± SEM of percentage of baseline fEPSP slope; n=8). Arrows indicate the delivery of the theta-burst stimulation. **(C)** Serum corticosterone levels 24 hours before and 15 minutes after open field stress are comparable in sham and irradiated mice (mean ± SEM of serum concentration; n=15 per group). **(D)** Cued fear conditioning leads to a similar fear response in both sham and irradiated mice. The amount of freezing was measured for 20 sec before and for 20 sec during the presentation of the tone (mean ± SEM of the percentage of time spent freezing; n=15 mice per group).

References

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