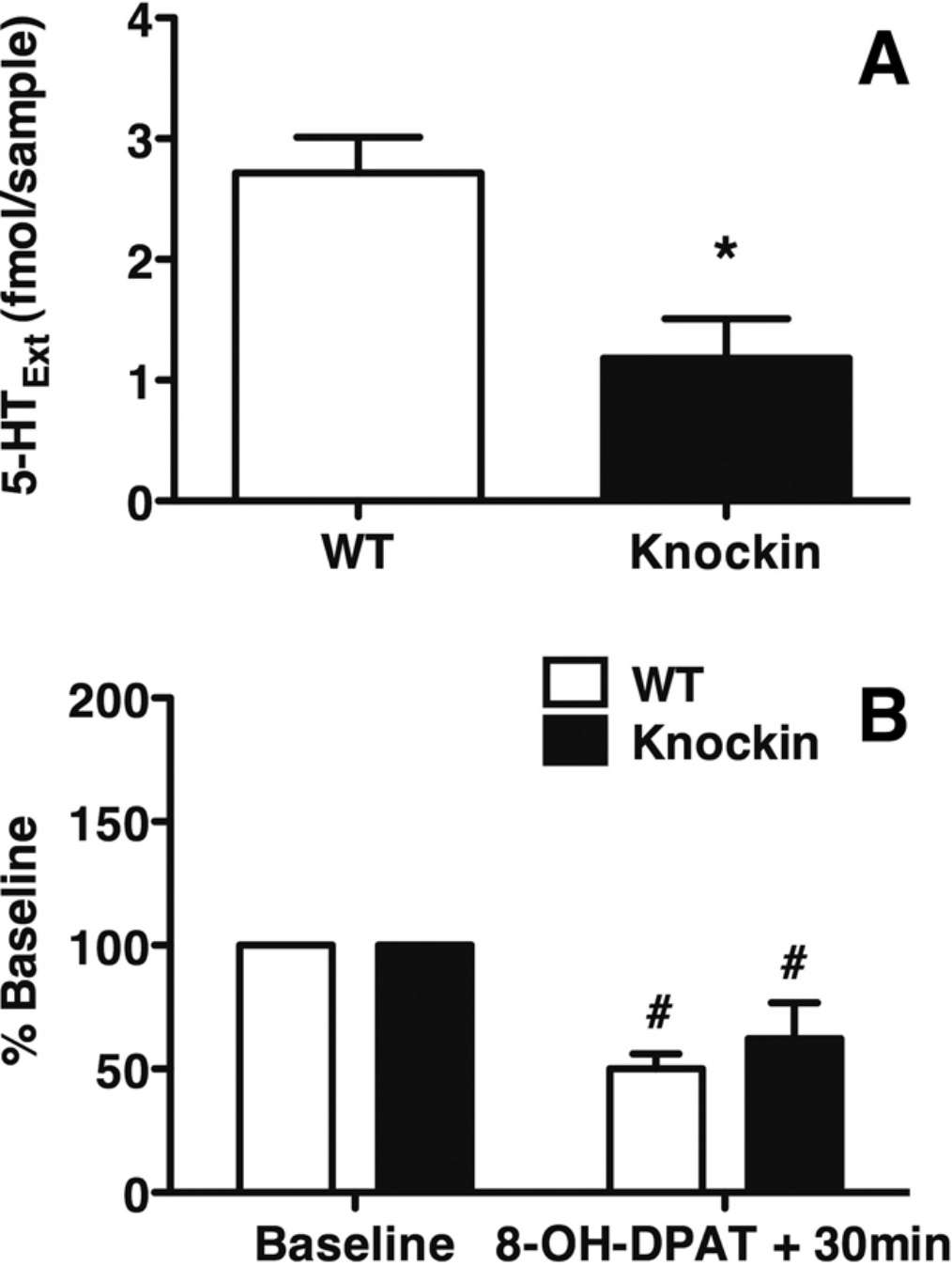
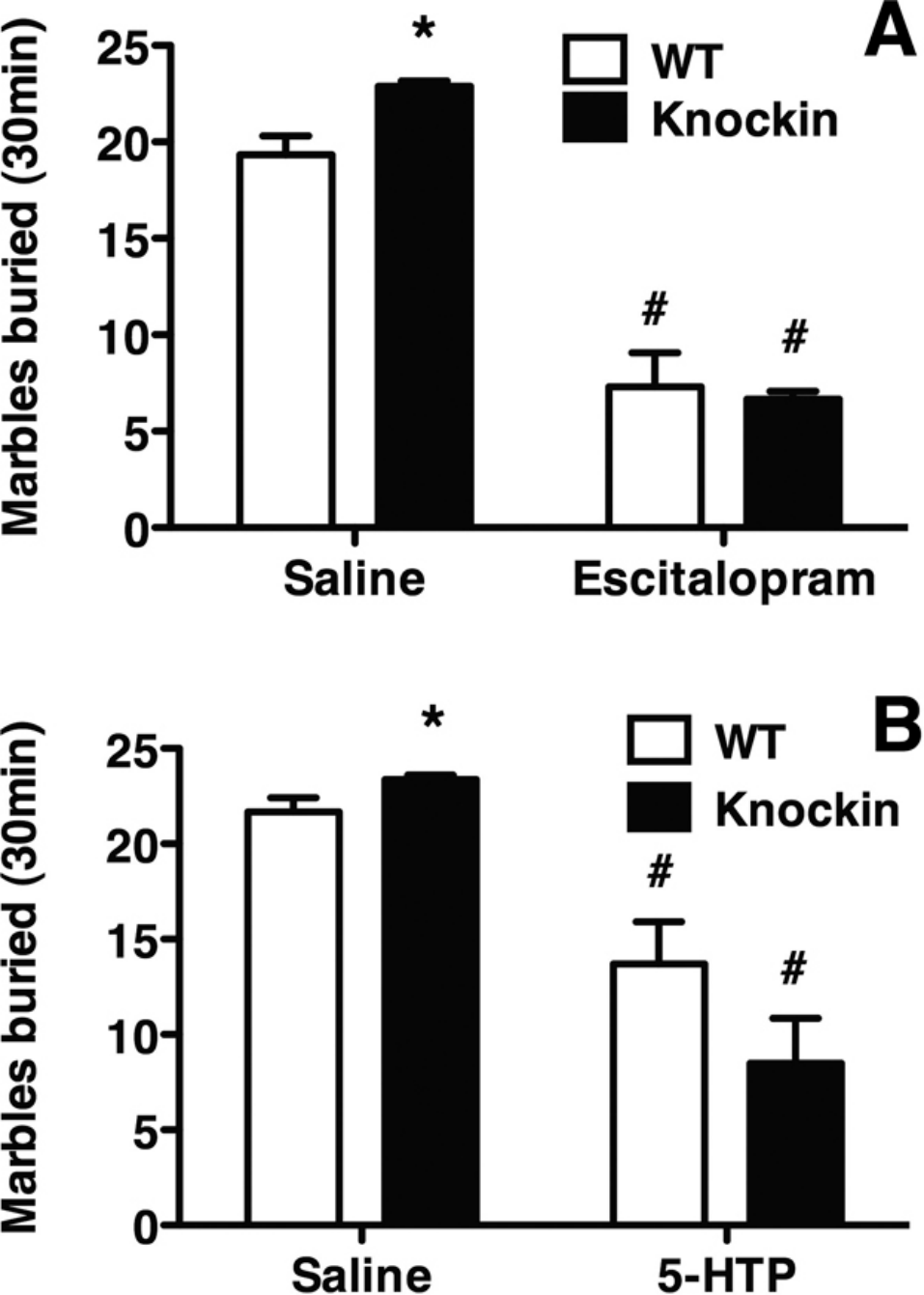
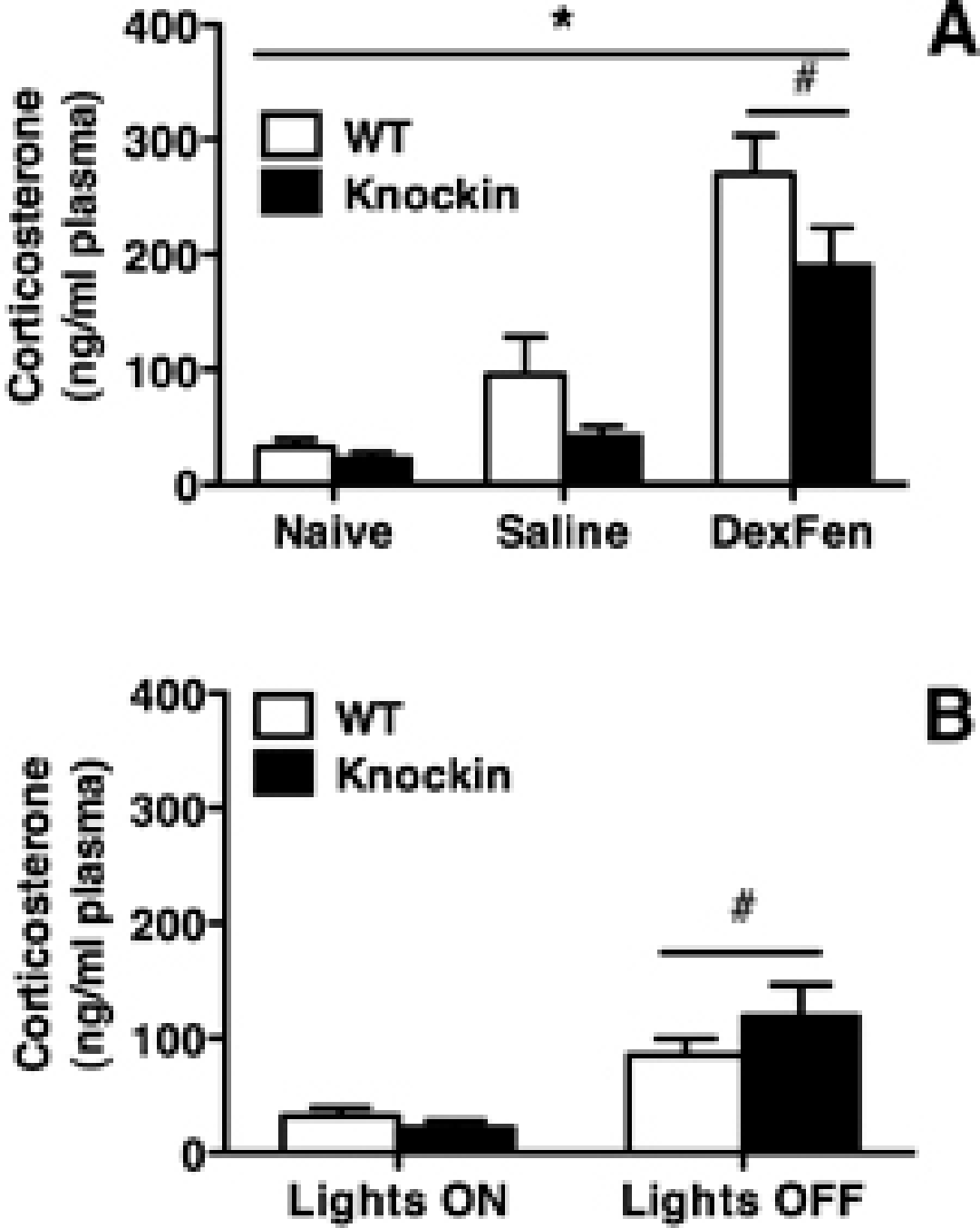
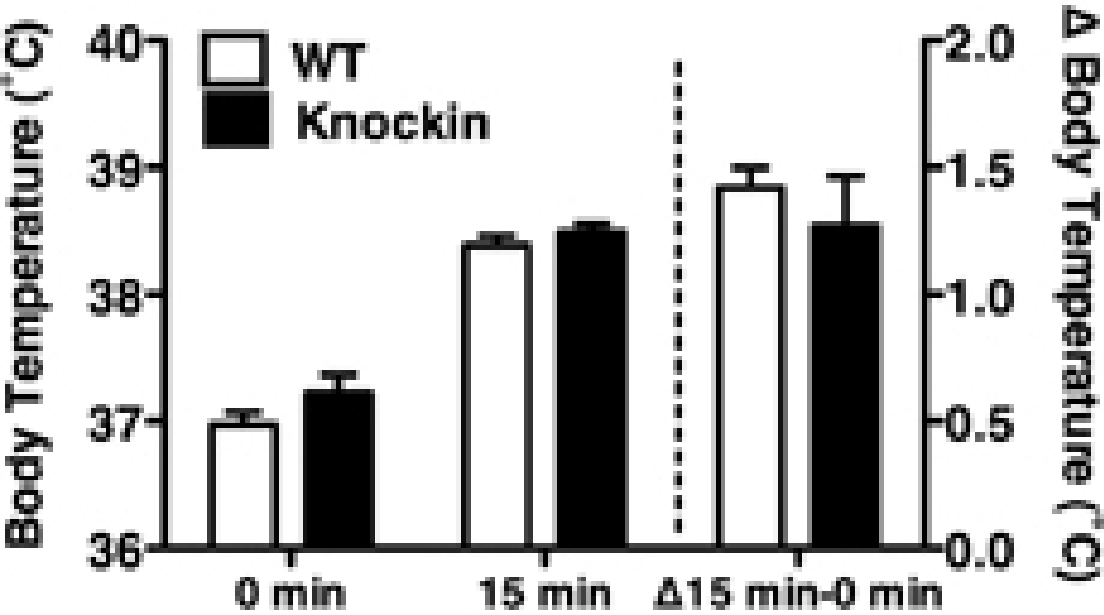
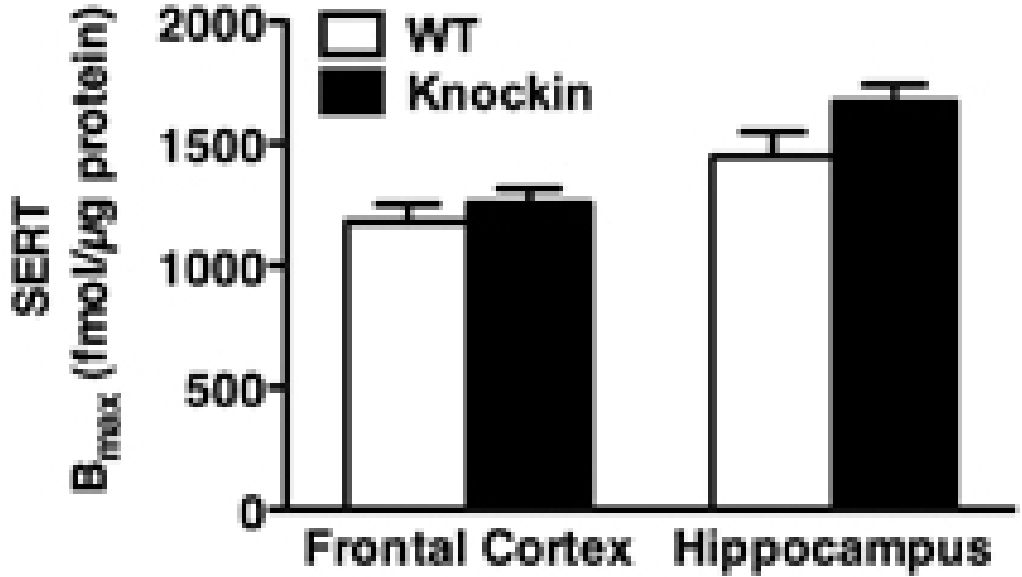
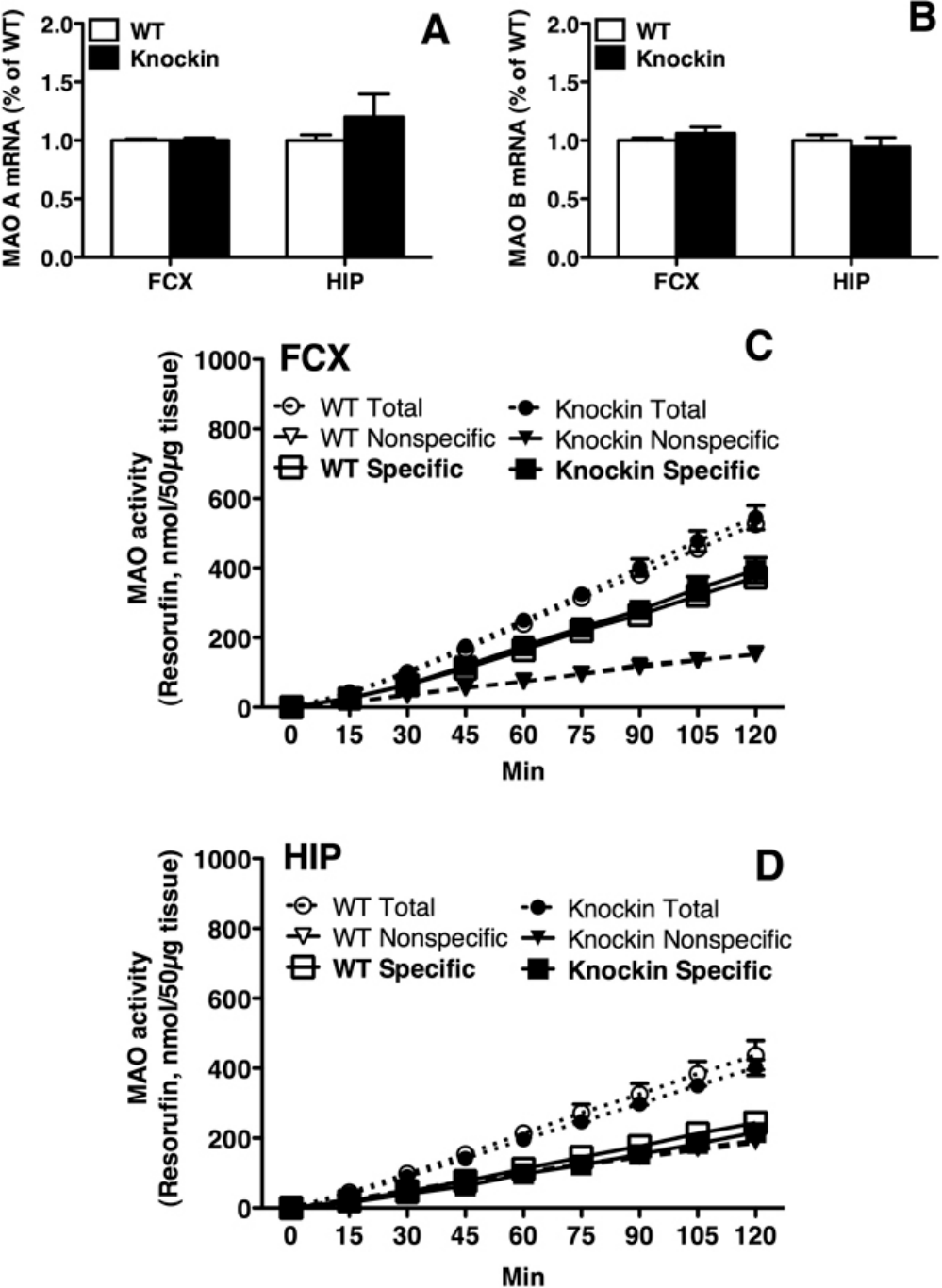

A
5-HT$_{Ext}$ (fmol/sample)
4
3
2
1
0
*
WT
Knockin
B
WT
Knockin
% Baseline
200
150
100
50
0
#
#
Baseline
8-OH-DPAT + 30min


A
Marbles buried (30min)
25
20
15
10
5
0
*
WT
Knockin
#
#
Saline
Escitalopram
B
Marbles buried (30min)
25
20
15
10
5
0
*
WT
Knockin
#
#
Saline
5-HTP


A
Corticosterone (ng/ml plasma)
400
300
200
100
0
*
#
WT
Knockin
Naive
Saline
DexFen
B
Corticosterone (ng/ml plasma)
400
300
200
100
0
WT
Knockin
#
Lights ON
Lights OFF


Body Temperature (°C)
40
39
38
37
36
WT
Knockin
0 min
15 min
Δ15 min-0 min
2.0
1.5
1.0
0.5
0.0
Δ Body Temperature (°C)


SERT
B_{max} (fmol/µg protein)
2000
1500
1000
500
0
WT
Knockin
Frontal Cortex
Hippocampus


A
WT
Knockin
MAO A mRNA (% of WT)
2.0
1.5
1.0
0.5
0.0
FCX
HIP
B
WT
Knockin
MAO B mRNA (% of WT)
2.0
1.5
1.0
0.5
0.0
FCX
HIP
C
FCX
MAO activity
(Resorufin, nmol/50µg tissue)
1000
800
600
400
200
0
WT Total
WT Nonspecific
WT Specific
Knockin Total
Knockin Nonspecific
Knockin Specific
0
15
30
45
60
75
90
105
120
Min
D
HIP
MAO activity
(Resorufin, nmol/50µg tissue)
1000
800
600
400
200
0
WT Total
WT Nonspecific
WT Specific
Knockin Total
Knockin Nonspecific
Knockin Specific
0
15
30
45
60
75
90
105
120
Min

		WT		Tph2 knockin	
Experiment	**Figure**	**males**	**females**	**males**	**females**
Microdialysis, frontal cortex	1B, 4A-F	4	5	4	6
Microdialysis, hippocampus	1F, 4G-L	4	5	5	5
Microdialysis, frontal cortex, K+ depol.	1C	3	5	4	6
Microdialysis, hippocampus, K+ depol.	1G	4	5	5	5
Microdialysis, frontal cortex, escitalopram	1D	9	8	8	10
Microdialysis, hippocampus, escitalopram	1H	7	7	6	5
Marble burying	1I	4	5	3	5
Tissue 5-HT and 5-HIAA, neonatal	1J	2	4	4	5
Tissue 5-HT and 5-HIAA, adult	1K	4	4	4	3
CSF 5-HIAA, microdialysis	2A	3	3	3	2
Fenfluramine prolactin	2B				
Naïve		5	5	4	4
Saline		3	4	3	5
Fenfluramine		5	4	4	4
8-OH-DPAT hypothermia	2C	4	6	5	5
5-HT2A receptor binding	2D	3	4	3	4
CSF 5-HVA and DOPAC, microdialysis	2E	3	3	3	2
Prolactin diurnal	2F				
Naïve, AM		5	5	4	4
Naïve, PM		4	4	4	4
Clonidine hypothermia	2G	4	5	3	5
5-HT1A receptor binding	2H	4	4	4	4
Head Twitches, Escitalopram + clorgyline	3A	5	5	4	6
Head Twitches, Escitalopram + 5-HTP	3B	4	6	5	5
Head Shakes, DOI	3C	15	16	15	17
5-HT1A receptor autoradiography	3D-E	4	4	4	6
5-HT1A receptor GTPgammaS binding	3F-G	4	5	5	5
Microdialysis, frontal cortex, 8-OH-DPAT	S1	3	3	3	4
Marble burying, escitalopram	S2A				
Saline		4	5	4	4
Escitalopram		5	5	5	5
Marble burying, 5-HTP	S2B				
Saline		5	5	5	6
5-HTP		5	5	5	5
Fenfluramine corticosterone	S3A				
Naïve		5	5	4	4
Saline		3	4	3	5
Fenfluramine		5	4	4	4
Corticosterone diurnal	S3B				
Naïve, AM		5	5	4	4
Naïve, PM		4	4	4	4
Stress-induced hyperthermia	S4	4	6	5	5
SERT binding	S5	5	3	4	4
MAO (same tissue for mRNA and activity)					
MAO A mRNA	S6A	3	4	4	3
MAO B mRNA	S6B	3	4	4	3
MAO activity	S6C-D	3	4	4	3

Table S1. Gender distribution within individual experiments.

Figure S1. The 5-HT_{1A}R agonist 8-OH-DPAT decreased frontal cortex 5-HT_{Ext} levels in WT and Tph2 knockin mice confirming that the 5-HT detected in the microdialysates are neuronally derived. **(A)** Baseline levels of 5-HT detected in microdialysates from WT and Tph2 knockin mice. **(B) %** effect of 8-OH-DPAT (1 mg/kg, i.p.) on 5-HT_{Ext} levels in WT and Tph2 knockin mice 30 min after injection. N = 6-7. Data represent means ± SEM. *, $P < 0.05$, WT vs Tph2 knockin, Student's T-test. #, $P < 0.05$, baseline vs 8-OH-DPAT + 30min, Student's paired T-test (Δ 5-HT_{Ext} levels), within genotype.

Figure S2. Enhancing brain 5-HT function decreases marble burying in WT and Tph2 knockin mice. **(A)** Escitalopram (10 mg/kg, i.p. 60 min before testing) decreases marble burying in WT and Tph2 knockin mice. **(B)** 5-HTP (50 mg/kg, i.p. 60 min before testing) decreases marble burying in WT and Tph2 knockin mice. N = 8-11. Data represent means ± SEM. *, $P < 0.05$, saline WT vs saline Tph2 knockin mice, Student's T-test. #, $P < 0.05$, saline vs drug treated mice, Two-way ANOVA and Bonferroni post-hoc test.

Figure S3. Dexfenfluramine evoked and basal plasma corticosterone is normal in Tph2 knockin mice. **(A)** The effect of dexfenfluramine (DexFen) on plasma corticosterone. There was an overall genotype effect ($F_{1,46} = 5.3$, $p = 0.025$) on plasma corticosterone that seemed to be driven by a decreased corticosterone response to the injection procedure *per se* in Tph2 knockin mice. No treatment × genotype interaction was detected ($F_{2,46} < 1$, $P > 0.4$). N = 6-10. **(B)** Diurnal plasma corticosterone was

significantly higher at the start of the diurnal dark phase compared to the start of the light phase. WT and Tph2 knockin mice had similar plasma corticosterone levels at both time points. N = 6-10. Data represent means ± SEM. *, $P < 0.05$, WT vs Tph2 knockin. $^{\#}$, $P < 0.05$, compared to start of light phase within genotype. Two-way ANOVA and Bonferroni post-hoc test.

Figure S4. Stress-induced hyperthermia is normal in Tph2 knockin mice. Rectal temperature was significantly increased compared to 15 min after the initial rectal probe insertion (0 min) in both genotypes. There were no significant genotype temperature differences at either time point and the Δ change was likewise similar. $^{\#}$, $P < 0.05$ compared to 0 min within genotype. RM-ANOVA and Bonferroni post-hoc test (absolute temperatures) and Student's Paired T-test (Δ temperature changes).

Figure S5. SERT levels in the frontal cortex and hippocampus as detected by saturation binding are unchanged in Tph2 knockin mice. N = 8. Data represent means ± SEM. NS, not significant. Student's T-test.

Figure S6. MAO A and B mRNA levels and MAO mediated 5-HT degradation are unchanged in Tph2 knockin mice. **(A)** MAO A and **(B)** MAO B mRNA levels in frontal cortex (FCX) and hippocampus (HIP) tissue, as detected by PCR. Data normalized to mean of WT. N = 7. **(C-D)** MAO mediated 5-HT degradation in tissue preparations from **(C)** frontal cortex and **(D)** hippocampus. Data represent accumulation of the fluorophore resorufin. Non-specific activity was determined in the presence of MAO inhibitors and

subtracted from total activity to yield specific, MAO mediated, activity. N = 7. Data represent means ± SEM. Student's t-test (MAO mRNA levels) and RM-ANOVA and Bonferroni post-shoc test (MAO activity).

Supplementary methods

HPLC-EC analysis

The HPLC system consisted of a BASi (West Lafayette, IN) LC-4C detector coupled to a BASi LCEC radial flow cell. The potential was set at + 650 mV. Flow was provided by a Shimadzu (Columbia, MD) LC-20AD solvent delivery module. The pump was preceded by an online degasser series 1100 from Agilent (Santa Clara, CA). The chromatograms were analyzed using PowerChrom software (eDAQ, Colorado Springs, CO).

Dialysates. Ten µl of dialysate were separated on a 1 x 100 mm UniJet microbore 3 µm ODS column at a flow rate of 80 µl/min. The mobile phase consisted of 24 mM Na_2HPO_4, 3 mM octanesulfonic acid, 27.4 mM citric acid, 107 µM EDTA and 17-18.5 % (v/v) MeOH, pH adjusted to 4.8 with NaOH. 5-HT eluted around 11 min.

CSF: As for dialysates above, but pH adjusted to 3.6 to retard elution of the acidic monoamine metabolites. DOPAC, 5-HIAA and HVA eluted at 2.4, 3.5 and 4.6 min, respectively.

Tissue: 5-HT and 5-HIAA in 10 µl tissue filtrate were separated on a 1 x 100 mm UniJet microbore 5 µm C-8 column. The mobile phase consisted of 24 mM Na_2HPO_4, 3 mM sodium octyl sulfate, 27.4 mM citric acid, 107 µM EDTA and 17 % (v/v/) MeOH, pH adjusted to 5.1 with NaOH. The flow was set at 100 µl/min. 5-HT and 5-HIAA eluted around 8.5 and 2.5 min, respectively.

SymDAQ HPLC-tandem-MS analysis

Dialysates were mixed with 4 µl internal standards mix (deuterated forms of the neurotransmitters under study) and then derivatized with the proprietary SymDAQ reagent (BrainLink, Groningen, NL) in the autosampler (SIL-10ADvp, Shimadzu, Japan) by addition of 40 µl of SymDAQ reagent solution to the sample vial. After a reaction time of 2 min, 50 µl of the mixture was injected into the HPLC-MS system. Chromatographic separation was performed on a reversed phase Synergi MAX-RP 100 x 3.0 mm, 2.5 µm particle size, column (Phenomenex, Utrecht, NL) at 35 °C. Sample analytes were separated using a 100 to 0 % gradient of mobile phase A (ultrapure water/acetonitrile (98/2), 0.1% formic acid) to mobile phase B (ultrapure water /acetonitrile (30/70), 0.1% formic acid) at a flow rate of 300 µl/min. A post-column make-up flow of 150 µl/min was added to the flow of the HPLC, which was diverted to the waste for 2.85 min, after which it was switched to the MS for detection of the neurotransmitters. MS analysis was performed using a system consisting of an API 4000 MS/MS detector and a Turbo Ion Spray interface (both from Applied Biosystems, Nieuwerkerk aan den Ijssel, NL). The acquisitions were performed in positive ionization mode, with ionization spray voltage set at 4 kV and a probe temperature of 200 °C. The instrument was operated in multiple-reaction-monitoring (MRM) mode. The collision gas (nitrogen) pressure was held at 2 psig. Data were calibrated and quantified using the Analyst™ version 1.4.2 data system (Applied Biosystems).

Stress-induced hyperthermia

The paradigm is a well-described drug screening assay for anxiolytic-like activity [1]. In brief, mice we left completely undisturbed the night and morning before the experiment

where after two rectal temperature recordings were performed with a 15 min interval using a digital thermometer. The first rectal probe insertion records basal body temperature and serves as a stressor. The second insertion records the temperature response to the first insertion. The Δ temperature change is the anxiety-like measure assessed.

Ligand saturation binding of 5-HT_{1A}R, 5-HT_{2A}R and SERT in brain tissue

Membrane fractions were prepared by three rounds of the following: Homogenization in 10 volumes of ice cold 50 mM Tris, 10 % sucrose, pH 7.4 (Tris-sucrose) using a Tissue-Tearer, incubation at room temperature for 10 min, and centrifugation (30,000 *g*, 20 min, 4 ˚C). The pellet (membrane fraction) from the final centrifugation step was resuspended in a small volume of Tris-sucrose and assayed for protein content using a Bradford assay (BSA standard). Then, membrane fractions were resuspended to 0.4 mg/ml in Tris-sucrose. Fifty µl were incubated with 250 µl binding buffer (for [^{3}H]WAY100635 and [^{3}H]ketanserin, 50 mM Tris, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4; for [^{3}H]citalopram, 50 mM Tris, 150 mM NaCl, 5 mM KCl, pH 7.4), containing radioligand ([^{3}H]WAY100635: 0.08 to 6 nM, in two-fold increments. [^{3}H]ketanserin: 1.6 to 50 nM, in two-fold increments. [^{3}H]citalopram: 0.6 to 20 nM, in two-fold increments). Binding reactions were incubated for 1 h at room temperature, and then harvested onto GF/A or GF/B filters using a cell harvester. The filters were washed three times with ice cold 50 mM Tris, pH 7.4, dried, added to scintillation fluid and counted on a scintillation counter. Non-specific binding for each concentration of radioligand was assessed in reactions

containing unlabeled WAY100635 (1 µM), mianserin (10 µM) or citalopram (1 µM) to block 5-HT_{1A}R, 5-HT_{2A}R and SERT, respectively. Specific radioligand binding (total - non specific) was plotted as a function of radioligand concentration and regressed using Prism 4.0 software (GraphPad , La Jolla, CA) to obtain B_{max}.

5-HT_{1A}R autoradiography

[^{125}I]p-MPPI binding. 5-HT_{1A}R autoradiography using [^{125}I]p-MPPI was performed as described with minor modifications [2]. Briefly, the sections were thawed at 4 ^{0}C under desiccation for 1 h and then pre-incubated in binding buffer (50 mM Tris HCl, pH 7.4, 2 mM $MgCl_2$) at room temperature (RT) for 30 min. Following, the sections were incubated at RT for 2 h in 0.17 nM [^{125}I]p-MPPI for total 5-HT_{1A}R binding and 0.17 nM [^{125}I]p-MPPI with 10 µM NAN-190 to estimate non-specific 5-HT_{1A}R binding. The binding reactions were terminated by 2 × 5 min washes in ice-cold binding buffer, followed by a dip in ice-cold double-distilled (DD) H_2O. The sections were dried at 30 ^{0}C for 1 h and exposed to Kodak Biomax MR film for 48 h (hippocampus/hypothalamus) or 120 h (dorsal raphe). Developed films were photographed using a light box and camera connected to an Alpha Imager EP system (Cell Biosciences, Santa Clara, CA). Densitometry was performed by an experimenter blinded to genotype. Areas of interest were outlined according to the atlas by Franklin and Paxinos [3] and gray tones within the outline determined using Image J software (NIH, Bethesda, MD) and related to [^{14}C] standards (American Radiolabeled Chemicals, St. Louis, MO) for quantification.

[^{35}S]GTPγS binding. [^{35}S]GTPγS binding was performed as described with minor modifications [4]. Briefly, the sections were thawed at 4 ^{0}C under desiccation for 1 h and subsequently brought to RT. The sections were then pre-incubated for 20 min at RT in binding buffer (50 mM HEPES KOH, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.2 mM DTT and 2 mM GDP) supplemented with 1 µM DPCPX. Nonspecific binding was performed in binding buffer with 1 µM DPCPX, 0.08 nM [^{35}S]GTPγS and 2 mM cold GTPγS. Basal [^{35}S]GTPγS binding was performed in binding buffer with 1 µM DPCPX and 0.08 nM [^{35}S]GTPγS. 5-HT_{1A}R stimulated [^{35}S]GTPγS binding was performed in binding buffer with 1 µM DPCPX, 0.08 nM [^{35}S]GTPγS and 10 µM 8-OH-DPAT. All reactions were carried out at 30 ^{0}C for 1 h. [^{3}H]GTPγS binding was terminated with 2 × 2 min washes in ice-cold 50 mM Tris HCl, pH 7.4, followed by a dip in ice-cold DD H_2O. The sections were dried at 30 ^{0}C for approximately 1 h and exposed to a Kodak BioMax MR film for 96 h (hippocampus/hypothalamus) or 240 h (dorsal raphe). Densitometry was performed as described above for [^{125}I]p-MPPI binding.

Tissue MAO A and B mRNA expression

Reverse-transcriptase PCR and quantitative real-time PCR. Mice were rapidly euthanized by cervical dislocation and the frontal cortex and hippocampus rapidly dissected and frozen on dry ice. Total RNA was prepared from the samples with the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) and converted to single-stranded cDNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Quantitative real-time PCR experiments were performed with the cDNA with a Light Cycler (Roche, Indianapolis, IN) and 1 x SYBR Green universal PCR master mix (Invitrogen, Carlsbad, CA) as described previously [5]. The fluorescence signal was quantified by the LightCycler Data Analysis software (Roche) with the second derivative maximum method. The threshold cycle for each sample was chosen within the linear range. MAO-A and MAO-B mRNA were normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences were the following. MAO-B: Forward, 5'-GAAGTTGAGCGGCTGATACAC-3'; reverse, 5'-GCATCACTGGGAATCTCTTGG-3'; MAO-A: Forward, 5'-GCCCAGTATCACAGGCCAC-3'; reverse, 5'-CGGGCTTCCAGAACCAAGA-3'. GAPDH: Forward, 5'-CATGTTCCAGTATGACTCCACTC-3'; reverse, 5'-GGCCTCACCCCATTTGATGT-3'. Expression levels were calculated from the threshold cycles as $2^{-(MAO-GAPDH)}$. Data were normalized to the mean of WT.

Tissue MAO activity

MAO activity in frontal cortex and hippocampal tissues were assessed employing the Amplex Red Monoamine Oxidase Assay semi-kit (Invitrogen). Amplex red reacts with H_2O_2, a byproduct of MAO activity, producing the stable fluorophore resorufin in a reaction catalyzed by horse radish peroxidase [6]. The assay was optimized to measure MAO activity in brain tissue homogenates using 5-HT as the enzymatic substrate. Tissues were rapidly dissected, frozen on dry ice and stored at -80 ˚C. On the day of experiment, the tissues were weighted frozen and homogenized by sonication in ice cold 50 x (w/v) lysis buffer (50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, 0.5% (v/v)

Triton X-100, pH 7.4). The homogenate was centrifuged (15000 g, 10 min) and the supernatant recovered and further diluted 40 x (v/v) in reaction buffer (50 mM NaH_2PO_4, pH 7.4) to a final sample dilution of 0.5 µg tissue/µl. Triplicates of 100 µl sample (or reaction buffer for blank samples) were incubated for 30 min at 37 ˚C with 0.5 µM of the MAO inhibitors pargyline and clorgyline (to define non-specific activity) or in the absence of MAO inhibitors (total activity) in 96 well plates. Next, 50 µl of 1000 µM (final 250 µM) 5-HT was added to all sample wells. 50 µl of a mixture of Amplex red (800 µM, final 200 µM) and horse radish peroxidase solution (2 U/ml, final 0.5 U/ml) was then added to all sample wells to start the reaction and immediately thereafter fluorescence was determined (t = 0 min). Fluorescence development (λ_{ex} = 544, λ_{em} = 590) of resorufin was measured every 20 min for 120 min using a plate reader (NOVOstar, BMG Labtech, Offenburg, Germany). Concentrations of resorufin formed in the samples were interpolated from a series of resorufin standards (10, 30, 100, 300 and 1000 nmol per 200 µl) included on each plate. MAO activity was expressed as nmol of resorufin formed per 50 µg tissue. Specific (MAO derived) activity was calculated as “total” resorufin produced – “non-specific” resorufin produced.

Supplementary references

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