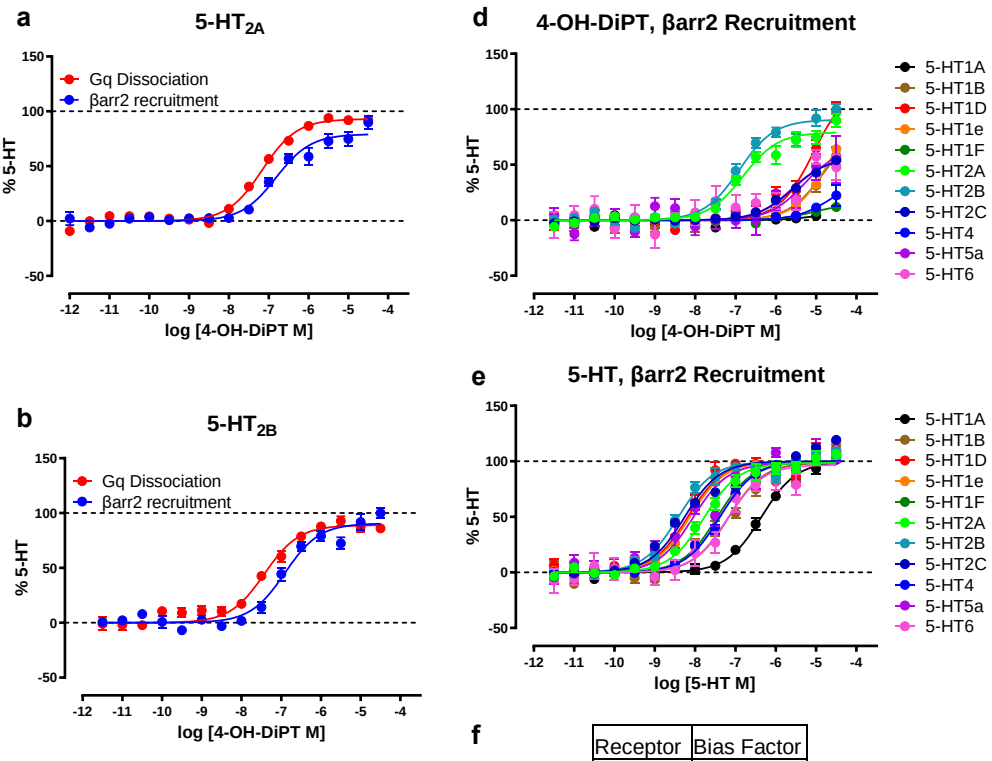
Supplementary Figures



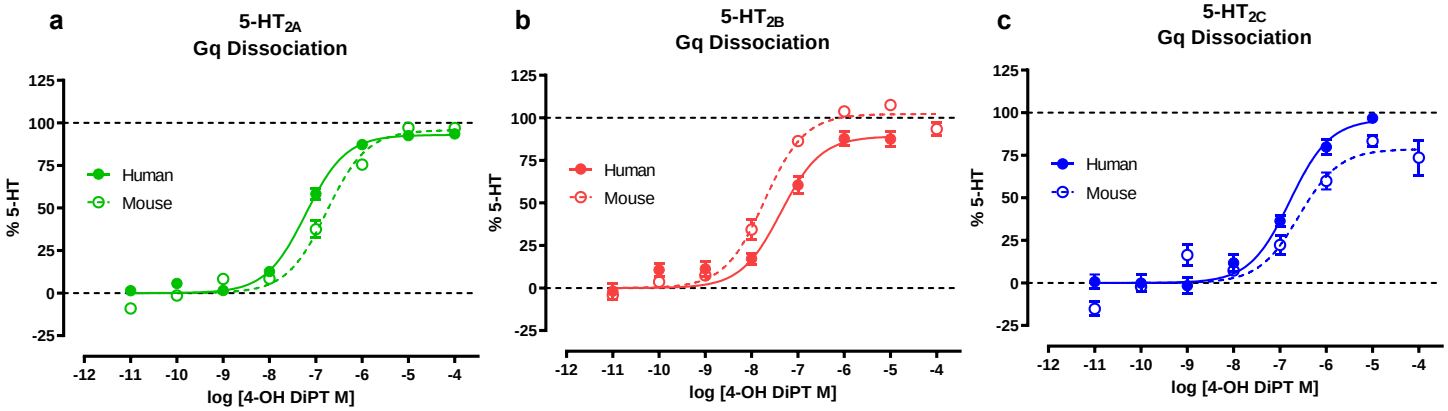
f

Receptor	Bias Factor
5-HT_{1A}	N.C.
5-HT_{1B}	N.C.
5-HT_{1D}	2.5
5-HT_{1e}	N.C.
5-HT_{1F}	N.C.
5-HT_{2A}	0.83
5-HT_{2B}	0.88
5-HT_{2C}	0.70
5-HT_{4}	N.C.
5-HT_{5a}	N.C.
5-HT_{6}	1.1
5-HT_{7a}	N.C.

Supplementary Figure 1. β-arrestin 2 Recruitment for 4-OH-DiPT at the 5-HTome. a-c. G protein dissociation and β-arrestin 2 recruitment induced by 4-OH-DiPT at 5-HT_{2A} (**a**), 5-HT_{2B} (**b**), and 5-HT_{2C} (**c**). Data represent mean ± s.e.m. from at least three independent experiments performed in triplicate, all normalized to 5-HT. **d-e.** and β-arrestin 2 recruitment induced by 5-HT (**d**) and 4-OH-DiPT (**e**) at the 12 human 5-HT GPCRs. **f.** Table of bias factors calculated with respect to the reference ligand, 5-HT and G protein activity (Bias factor = 10^ΔΔLog(E_{MAX}/ EC_{50}). N.C. not calculated; due to no activity or undetermined EC_{50} and E_{MAX} parameters.

	5-HT						4-OH-DiPT					
Receptor	EC50, nM	pEC50	SEM	Emax	SEM	Log(E_{MAX}/EC_{50})	EC50, nM	pEC50	SEM	Emax	SEM	Log(E_{MAX}/EC_{50})
5-HT1A, GoB	3.65	8.44	0.08	100	N.D.	8.44	1147	5.94	0.15	69.8	5.9	5.78
5-HT1A, β-Arrestin 2	438	6.36	0.05	100	N.D.	6.36	N.A.					
5-HT1B, GoB	0.640	9.19	0.12	100	N.D.	9.19	597	6.22	0.17	95.4	8.1	6.20
5-HT1B, β-Arrestin 2	67.4	7.17	0.07	100	N.D.	7.17	> 10,000	< 5.00	N.D.	N.D.	N.D.	N.D.
5-HT1D, GoB	1.80	8.75	0.14	100	N.D.	8.75	496	6.31	0.22	64.1	6.7	6.11
5-HT1D, β-Arrestin 2	6.55	8.18	0.07	100	N.D.	8.18	8827	5.05	0.11	123.8	12.0	5.15
5-HT1e, GoB	0.967	9.01	0.08	100	N.D.	9.01	852	6.07	0.13	79.0	5.3	5.97
5-HT1e, β-Arrestin 2	7.39	8.13	0.02	100	N.D.	8.13	> 10,000	< 5.00	N.D.	N.D.	N.D.	N.D.
5-HT1F, GoB	3.24	8.49	0.08	100	N.D.	8.49	557	6.25	0.16	59.1	4.7	6.03
5-HT1F, β-Arrestin 2	35.5	7.45	0.03	100	N.D.	7.45	N.D.	N.D.	N.D.	< 20%	N.D.	N.D.
5-HT2A, Gq	5.31	8.28	0.03	100	N.D.	8.27	68.5	7.16	0.04	93.0	1.4	7.13
5-HT2A, β-Arrestin 2	16.7	7.78	0.06	100	N.D.	7.78	153	6.82	0.08	79.2	2.6	6.71
5-HT2B, Gq	1.05	8.98	0.05	100	N.D.	8.98	37.5	7.43	0.07	89.0	2.3	7.38
5-HT2B, β-Arrestin 2	3.84	8.42	0.06	100	N.D.	8.42	123	6.91	0.07	90.6	2.7	6.87
5-HT2C, Gq	0.159	9.80	0.05	100	N.D.	9.80	180	6.75	0.07	95.0	2.8	6.72
5-HT2C, β-Arrestin 2	5.52	8.26	0.05	100	N.D.	8.26	2598	5.59	0.04	56.5	1.4	5.34
5-HT4, Gs	7.82	8.11	0.03	100	N.D.	8.11	N.A.					
5-HT4, β-Arrestin 2	41.4	7.38	0.03	100	N.D.	7.38	N.A.					
5-HT5a, GoB	18.6	7.73	0.13	100	N.D.	7.73	243	6.62	0.18	56.3	4.5	6.36
5-HT5a, β-Arrestin 2	8.82	8.06	0.11	100	N.D.	8.05	5074	5.30	0.30	65.8	14.9	5.11
5-HT6, Gs	20.4	7.69	0.09	100	N.D.	7.69	697	6.16	0.19	54.7	5.3	5.90
5-HT6, β-Arrestin 2	63.5	7.20	0.12	100	N.D.	7.20	2415	5.62	0.31	56.0	11.0	5.37
5-HT7a, Gs	7.64	8.12	0.07	100	N.D.	8.12	N.D.	N.D.	N.D.	< 20%	N.D.	N.D.

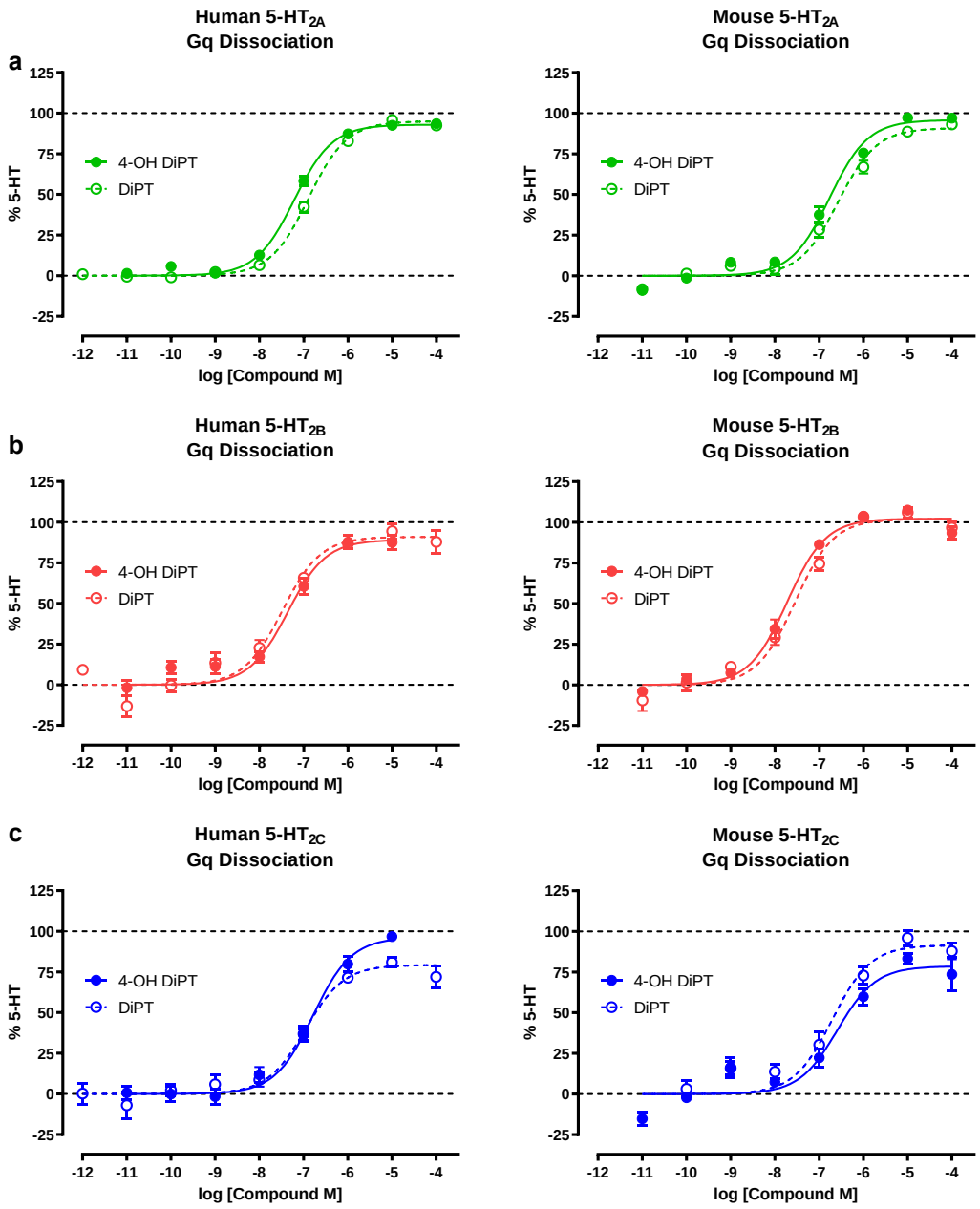
Table S1. 5-HT GPCR Parameter Estimates for 5-HT and 4-OH-DiPT. All parameter estimates for BRET G protein dissociation and β-arrestin recruitment assays. All data represent mean and s.e.m from at least n=3 independent experiments. N.A. no detectable activity; N.D. not determined.



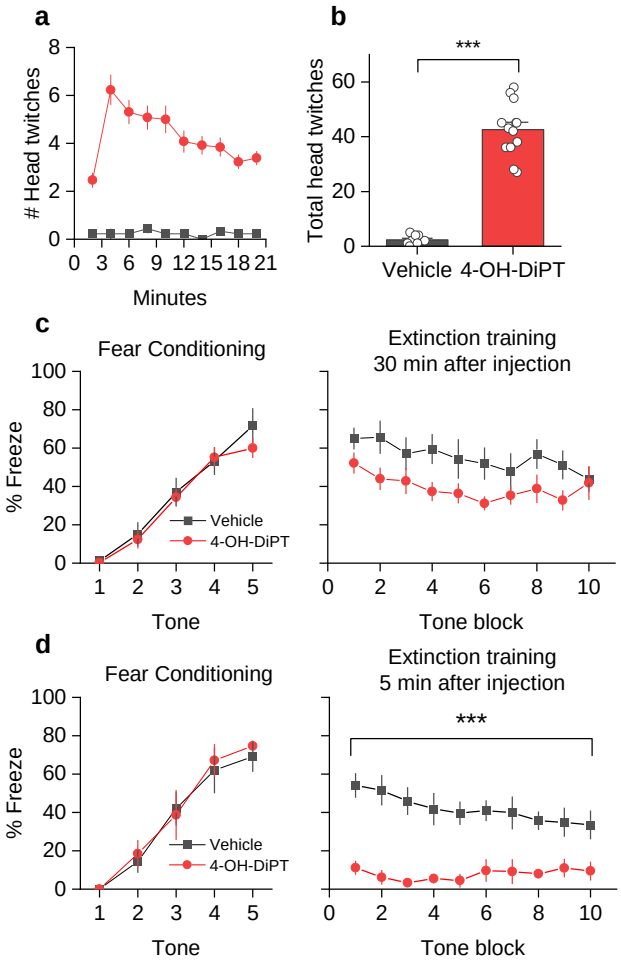
d

	5-HT						4-OH-DiPT					
Receptor	EC50, nM	pEC50	SEM	Emax	SEM	Log(E_{MAX}/EC_{50})	EC50, nM	pEC50	SEM	Emax	SEM	Log(E_{MAX}/EC_{50})
Human 5-HT2A, Gq	5.31	8.28	0.03	100	N.D.	8.27	68.5	7.16	0.04	93.0	1.4	7.13
Human 5-HT2B, Gq	1.05	8.98	0.05	100	N.D.	8.98	37.5	7.43	0.07	89.0	2.3	7.38
Human 5-HT2C, Gq	0.159	9.80	0.05	100	N.D.	9.80	180	6.75	0.07	95.0	2.8	6.72
Mouse 5-HT2A, Gq	11.6	7.94	0.04	100	N.D.	7.94	172	6.76	0.06	95.8	2.1	6.75
Mouse 5-HT2B, Gq	0.980	9.01	0.05	100	N.D.	9.01	18.9	7.72	0.06	102	1.92	7.73
Mouse 5-HT2C, Gq	0.544	9.26	0.08	100	N.D.	9.26	257	6.59	0.15	78.6	4.4	6.49

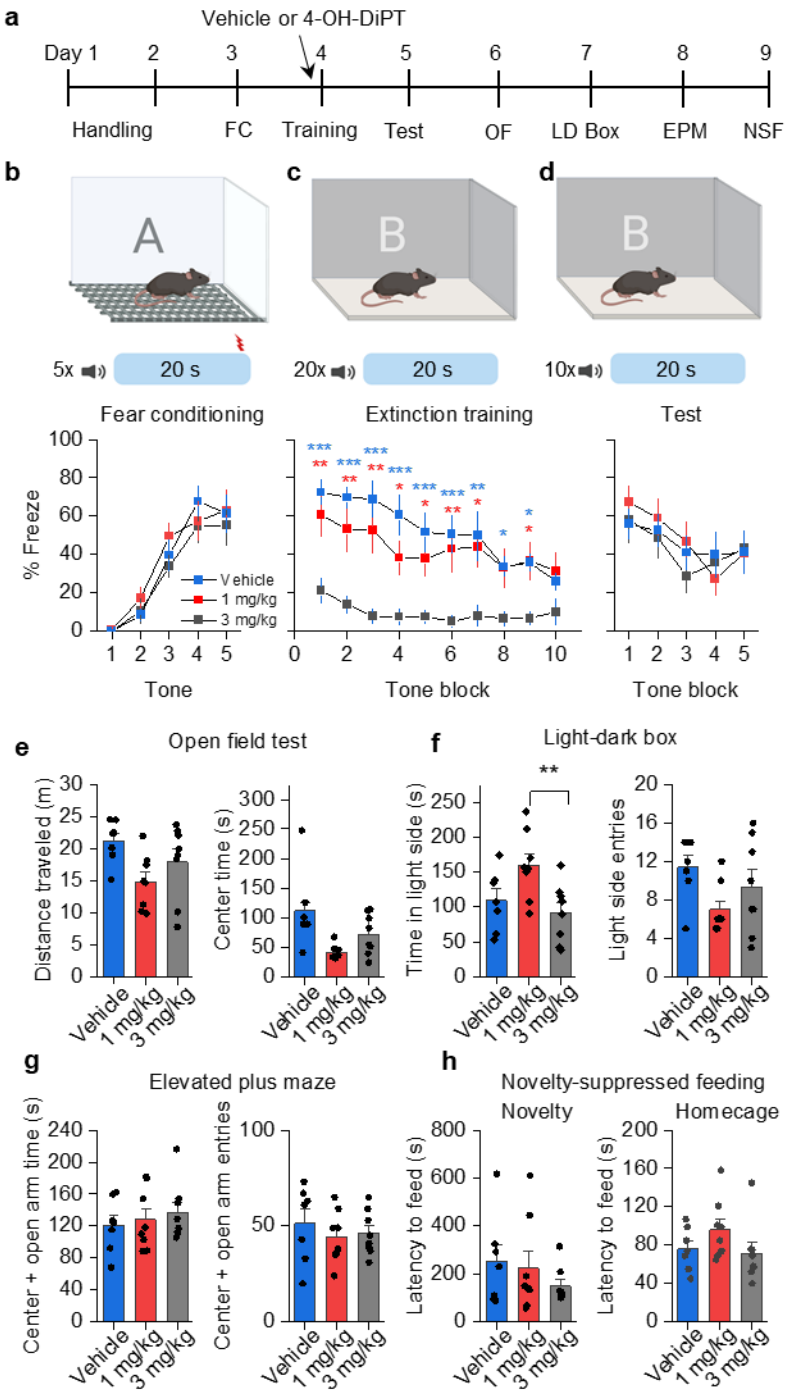
Supplementary Figure 2. 4-OH-DiPT induced Gq protein dissociation at mouse and human $5HT_2$ receptors. 4-OH-DiPT induced Gq protein dissociation at human and mouse $5HT_2$ receptors: 5-HT_{2A} (**a**), 5-HT_{2B} (**b**), and 5-HT_{2C} (**c**). Parameter estimates for BRET G protein dissociation (d). Data represent mean ± s.e.m. from at least three independent experiments performed in triplicate, all normalized to 5-HT.



Supplementary Figure 3. G protein dissociation induced by 4-OH-DiPT or DiPT at mouse and human 5-HT$_2$ receptors. a-c. G protein dissociation by 4-OH-DiPT or DiPT at human (left) or mouse (right) 5-HT$_{2A}$ (**a**), 5-HT$_{2B}$ (**b**), and 5-HT$_{2C}$ (**c**). Data represent mean ± s.e.m. from at least three independent experiments performed in triplicate, all normalized to 5-HT.

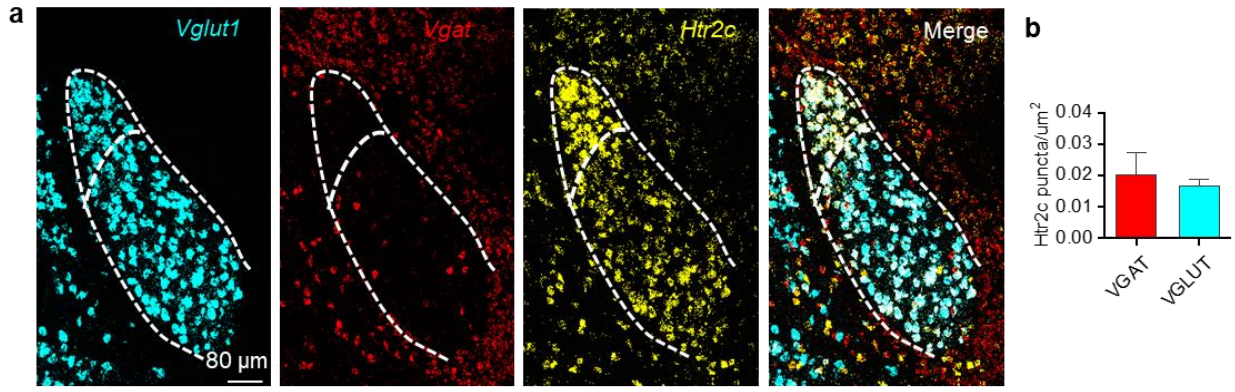


Supplementary Figure 4. The fear suppressing effect of 4-OH-DiPT is time sensitive. a. Average number of head twitches after injection of 4-OH-DiPT or Vehicle. Head twitches were binned in two-minute intervals. **b.** 4-OH-DiPT treated mice had significantly more head twitches than vehicle treated mice ($p < 0.001$, n = 13 4-OH-DiPT treated mice (10 female, 3 male) and 9 vehicle treated mice. **c.** Freezing responses to five 20 second tones followed by a 0.5 mA foot shock (left). Freezing was not significantly different between groups. Twenty-four hours later mice were injected with 4-OH-DiPT (3 mg/kg) or Vehicle 30 minutes before being placed in a new context and presented with 20 tones at 60-80 second pseudo-random intervals (right). Freezing was not not significantly different between treatment groups (two-way RM ANOVA: $F_{1,162} = 0.433$, $p = 0.06$), n= 10 mice per group. **d.** Freezing responses to five 20 second tones followed by a 0.5 mA foot shock in a separate group of mice (left) was not significantly different. Twenty-four hours later mice were injected with 4-OH-DiPT (3 mg/kg) or Vehicle 5 minutes before being placed in a new context and presented with 20 tones at 60-80 second pseudo-random intervals (right). Freezing was significantly reduced in mice treated with 4-OH-DiPT (two-way RM ANOVA: Treatment: $F_{1,117} = 22.257$, $p < 0.001$); Treatment x Tone block: $F_{9,117} = 1.4$, $p > 0.05$) n = 6-9 mice per group.

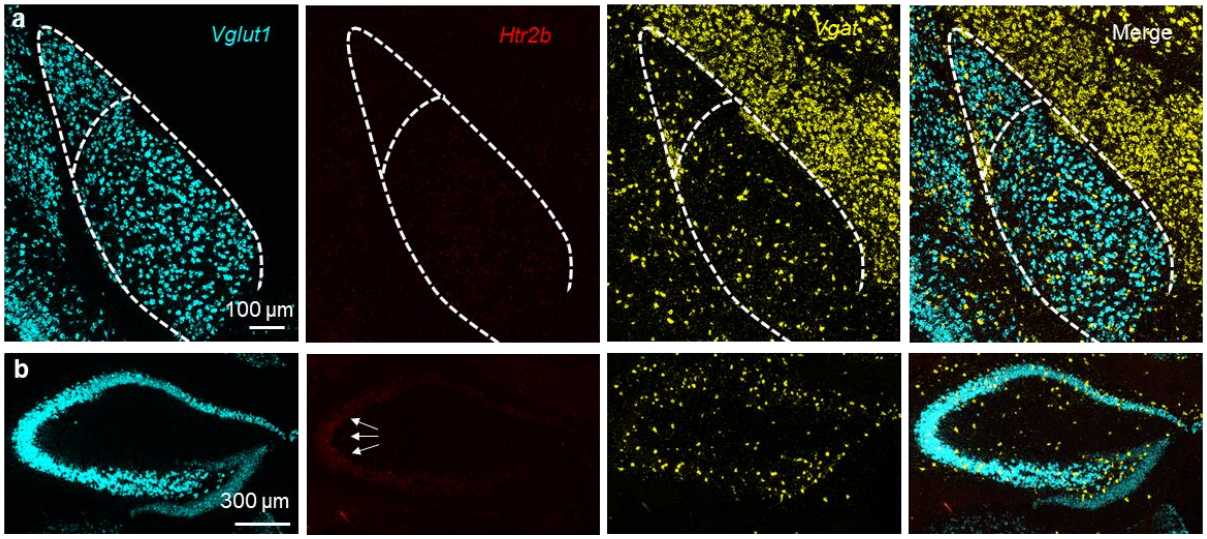


Supplementary Figure 5. 4-OH-DiPT-paired extinction training reduces conditioned fear expression but does not affect avoidance behavior in male mice. a. Timeline of behavioral and electrophysiological experiments; the arrow denotes administration of 4-OH-DiPT or Vehicle. **b.** Freezing responses to five 20 second tones with followed by a 0.5 mA foot shock were not significantly different between treatment groups (two-way RM ANOVA: $F_{2,84} = 0.133$, $p = 0.876$). **c.** Freezing responses to tones with 5 second intervals in a new context 24 hours later. Male mice that received 4-OH-DiPT froze significantly less than vehicle controls (two-way RM ANOVA: 4-OH-DiPT dose, $F_{2,190} = 13.604$, $p < 0.001$;

tone block, $F_{9,190}$ = 8.427, p < 0.001; dose x tone, $F_{18,190}$ = 1.731, p < 0.037; 3 mg/kg vs. 1 mg/kg, red*; Vehicle vs. 3 mg/kg, blue*). **d.** 4-OH-DiPT treatment during extinction training did not affect the overall level of freezing during the extinction test 24 hours later (two-way RM ANOVA: dose, $F_{2,84}$ = 0.127, p = 0.881), n=8-9 mice/group. **e.** There was no difference in the distance traveled (one-way ANOVA: $F_{2,20}$ = 0.127 p > 0.05) or time spent in the center of an open field (Kruskal Wallis ANOVA on ranks: H_2 = 2.124, p > 0.05) between treatment groups. **f**. Mice treated with 1 mg/kg 4-OH-DiPT spent more time in the light side of the LD box than mice that were treated with 3 mg/kg 4-OH-DiPT (one-way ANOVA: $F_{2,20}$ =4.931, p = 0.018; Vehicle vs 3 mg/kg, p > 0.05; Vehicle vs. 1 mg/kg p > 0.05), while entries were the same between groups (one-way ANOVA: $F_{2,20}$ =2.637, p > 0.05). **g.** Mice treated with either 1 or 3 mg/kg 4-OH-DiPT showed no difference in the number of entries (one-way ANOVA: $F_{2,20}$ = 0.658, p > 0.05) or time (one-way ANOVA: $F_{2,20}$ = 0.347, p > 0.05), spent in the center and open arms of the elevated plus maze. **h.** Mice treated with either 1 or 3 mg/kg 4-OH-DiPT showed no difference in the latency to feed in a novel environment (Kruskal Wallis one-way ANOVA: H_2 = 0.887, p > 0.05) or in the home cage (Kruskal Wallis one-way ANOVA: H_2 = 3.568, p > 0.05). N=7-8 mice/treatment group for avoidance behaviors. Data are presented as mean ± s.e.m.



Supplementary Figure 6. *Htr2c* is expressed in the basolateral amygdala. a. *Vglut1* (cyan), *Vgat* (red), and *Htr2c* (yellow) are expressed in the BLA. **b.** *Htr2c* puncta density is not significantly different in *Vgat* neurons compared to *Vglut1* neurons (p = 0.652, n = 4 BLA sections). Data are presented as mean ± s.e.m.



Supplementary Figure 7. *Htr2b* is not expressed in the basolateral amygdala. a. *Vglut1* (cyan), *Vgat* (yellow) are expressed in the BLA while *Htr2b* (red) is absent. **b.** *Htr2b* puncta are expressed in the hippocampus of the same section (n = 4 sections).

Supplementary Methods

Animals

The Institutional Animal Care and Use Committee at the Medical College of Wisconsin approved all animal maintenance and use protocols. Adult female and male C57BL/6J mice (stock#: 000664; Jackson Laboratory; Bar Harbor, ME) aged 8-16 weeks at the beginning of experiments were housed together and maintained at (23 ± 1 °C) in a humidity-controlled room (40-60%) under an alternating light (14 h)/dark (10 h) cycle with unlimited availability of food and water, unless stated otherwise.

Stereotaxic Surgery

Anesthesia was induced with a SomnoSuite® Low-Flow Anesthesia System using 2.5% isoflurane delivered at a rate of 100ml/min in a 2 x 2-inch induction chamber. Mice were then placed in a robot stereotaxic system (Neurostar; Tübingen, Germany), and held under anesthesia with 2% isoflurane at a rate of 100 ml/min delivered through a nosecone. AAV9-mDlx-NLS-mRuby2 was bilaterally injected at: AP -1.22, ML ± 2.83, DV -4.75, -4.70, and -4.65 (150 nl each). AAV9-mDlx-NLS-mRuby2 was a gift from Viviana Gradinaru (Addgene viral prep #99130-AAV1). AAV injection was performed with a Nanoject III Programmable Nanoliter Injector (Drummond Scientific Company; Broomall, PA) at a rate of 60 nl/min. Injection pipettes were left at the injection site for 5 minutes to ensure complete diffusion of the viral titer from the pipette. Following surgeries mice were given subcutaneous injections of buprenorphine-SR 1 mg/kg. At least 7 days were given between injection and electrophysiological recordings to allow sufficient time for viral gene expression.

Behavior

The timeline of behavioral tests is outlined in Figure 2a and Supplementary Figure 5a. Only one behavioral test was conducted per day.

Fear conditioning, extinction, and recall

All mice were handled for at least 5 minutes for 2 days prior to behavior testing. Mice were placed in a 30 cm x 30 cm x 25 cm chamber (Actimetrics, Wilmette, IL, USA) which was housed in a light and sound attenuating box. A 25 W clear bulb provided illumination within the chamber. FreezeFrame 4.0 Software (Actimetrics) was used to control experiment contingencies and record freezing behavior. On day 1, mice were habituated to the fear conditioning chamber for 2 minutes, followed by 5 auditory cues lasting 20 seconds (75-85 dB tone) that ended with a 0.5 mA foot shock for 1 second. Tones were separated by a 60-80 second pseudorandom interval. After the last tone and shock, mice were left in the chamber for an additional 2 minutes. One day following fear conditioning, mice were administered either 1 or 3 mg/kg 4-OH DiPT or vehicle prior to extinction training. Plastic inserts were sprayed with a solution of 4% acetic acid in water and placed in the chamber to cover the sides and floor. The 25 W clear light bulb was replaced with a 25 W red bulb. Mice were first habituated to the new chamber for 2 minutes before being exposed to 20 auditory cues (75-85 dB, 20 s) with either 60-80 second pseudorandom intervals or 5 seconds intervals. After the last tone mice were left in the chamber for an additional 2 minutes. Twenty-four hours later, a cued extinction test was performed under the same parameters with 10 tones instead of 20. Fear extinction and recall data were analyzed by averaging % of time freezing during two subsequent tone presentations (one tone block).

Anxiety-like behaviors

Mice were habituated to the experimental room for 20 minutes behavior tests. For the open field test, mice were placed in the corner of a 50 x 45 x 30 cm box and allowed to roam freely for 20

minutes. Locomotion was recorded and assessed using ANY-Maze software (Stoelting, Wood Dale, IL). Protocols for the Light Dark Box, Elevated Plus Maze, and Novelty suppressed feeding test were performed as previously described [1] and analyzed with ANYmaze.

Electrophysiology

Acute slice preparation

BLA brain slices were prepared as previously described [1-3]. Briefly, anesthesia was induced with isoflurane, brains were extracted and embedded in 4% low melting point agarose and cut into 250µm thick sections with a VT 1200S (Leica Biosystems; Nussloch, Germany). Slices were cut in an NMDG based solution containing: 92 mM NMDG, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM $CaCl_2 \cdot 2H_2O$, and 7 mM $MgSO_4$ (pH 7.3–7.4 with HCl). After slice cutting, ACSF was progressively spiked into the NMDG solution every 5 min for 20 min at room temperature to gradually reintroduce Na^+ as previously described [4]. Slices were left to recover for at least an additional 30 min in Artificial Cerebrospinal Fluid (ACSF) consisting of: 119 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose. All solutions were saturated with 95% O2 and 5% CO2.

Electrophysiological recordings

The BLA was visualized under differential interference contrast (DIC) microscopy. Whole cell recordings were made using patch-clamp amplifiers (Multiclamp 700B; Molecular Devices, San Jose, CA). Data was acquired using DigiData 1550B digitizers and analyzed with pClamp 10.7 software (Molecular Devices) or MiniAnalysis (Bluecell Co., Seoul, Korea). Signals were sampled at 10 kHz and filtered at 2kHz. Low resistance glass pipettes (~4 MΩ) were filled with internal solution, for recordings of principal neurons the solution contained (in mM): 90 K-gluconate, 50 KCl, 10HEOES, 0.2 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3Na_2GTP and 10 Na_2-

phosphocreatine; for interneuron recordings, the internal solution contained (in mM): 135 tetramethyl ammonium (TMA)-OH, 10 EGTA, 2 $MgCl_2$, and 40 HEPES, titrated to pH 7.2 with hydrofluoric acid. Some interneuron recordings were performed with a solution containing (in mM): 90 K-gluconate, 50 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine, titrated to pH 7.2 with KOH. sIPSCs were recorded in the presence of AMPA receptor antagonist CNQX (5µM). sIPSCs were measured and analyzed similar to our previous studies [5,6]. sIPSC amplitude and frequency was compared using a 1-minute time block from before and after bath perfusion of 4-OH-DiPT (20µM). For 5-HT receptor blockade experiments, slices were incubated in Ketanserin (10µM) or Volinanserin (150 nM) for at least 10 minutes prior to recording. Interneurons were identified by mRuby expression and a high firing rate in current clamp after a brief depolarization. 4-OH-DiPT was administered to interneurons via a pressure perfusion pencil (Toohey Spritzer, Fairfield, New Jersey). Action potential firing in BLA interneurons was measured for 20 seconds following a 2s pressure perfusion and compared to the 20 s window prior to pressure perfusion. Series resistance was monitored (typically 15-30 MΩ) and data discarded if the resistance changed by more than 20%. Temperature was maintained at ~32 °C with an automatic temperature controller (Warner Instrument s LLC, Hamden, CT).

Histology

Histological preparation was performed as previously described [1]. Briefly, mice were anesthetized with isoflurane before transcardial perfusion of 0.1 M PBS, followed by perfusion of 4% PFA in 0.1M PBS. The brain was removed and post fixed in 4% paraformaldehyde and 4% sucrose-PBS (pH 7.4) overnight at 4 ºC prior to dehydration with increasing sucrose concentrations in 0.1 M PBS (20% and 30%). Coronal forebrain sections (20 µm) were cut with a CM1860 cryostat (Leica Biosystems), washed with 0.1 M PBS and mounted on slides. To

verify viral injection sites, sections were imaged on a VS200 slide scanner microscope (Evident Corporation, Tokyo, Japan).

RNAscope *in situ hybridization*

Mice were transcardially perfused as described above. Brains were immediately removed and embedded in OCT (without post-fixation), frozen on granulated dry ice, and stored at -80°C. Coronal BLA sections (14 µm) were cut on a cryostat (Leica CM1860, Nussloch, Germany). Fluorescent probes targeting *Mus musculus* mRNA (Advanced Cell Diagnostics Inc; Hayward, CA) were incubated according to the manufacturer's directions and imaged with a TCS SP8 confocal microscope (Leica). mRNA expression for *Vglut1*, *Vgat*, *Htr2a, Htr2b*, and *Htr2c* was quantified using the spots and surfaces functions of Imaris (Bitplane, Zürich, Switzerland).

Chemicals

4-OH-DiPT HCl (Item No. 11312), ketanserin tartrate (Item No. 22058), volinanserin and (Item No. 15936) were obtained from Cayman Chemical Company. 5-HT creatine sulfate was purchased from Sigma-Aldrich (St. Louis, MO). For behavioral studies 4-OH DiPT was dissolved in DMSO and diluted in saline (1:5) and kept for up to 1 week prior to use. All other common chemicals were obtained from Sigma-Aldrich.

Statistics

All data are presented as the mean ± SEM. Sample sizes for all behavior experiments were derived using a combination of power analysis (power = 0.8, α = 5%), prior experience, and sample sizes generally used in the field. Tone blocks during fear conditioning or extinction training and test sessions were compared between treatment groups two factor repeated measures ANOVA with Holm-Sidak *post-hoc* analysis. All other experiments were analyzed with either one-way ANOVA, Kruskal-Wallis ANOVA on ranks, unpaired Student's *t* test, Welch's *t*

test (unequal group variance), Mann-Whitney test, or paired *t* tests for before and after drug effects. Results were considered significant at $p < 0.05$.

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