

Supplementary Information

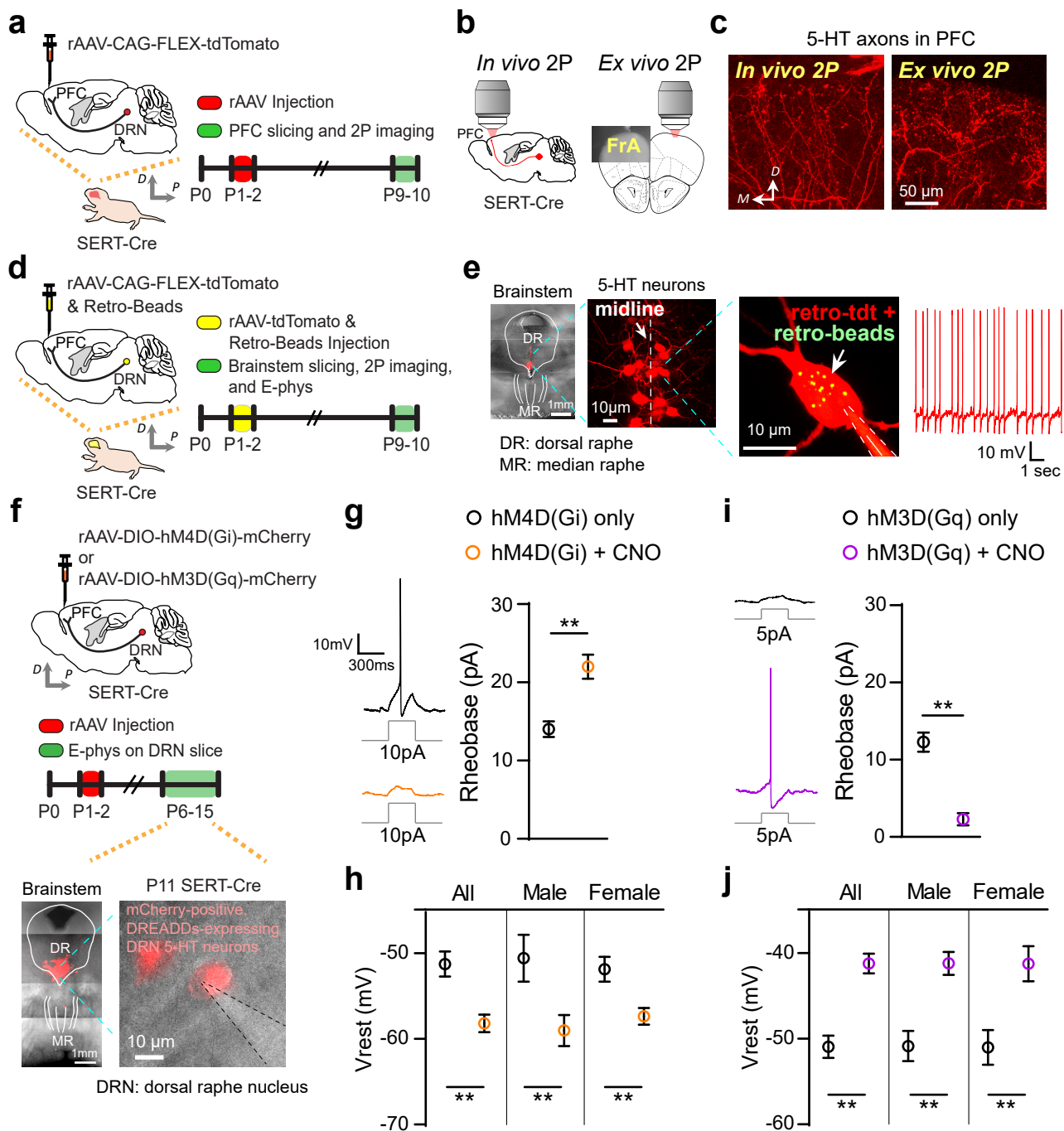
Serotonin modulates excitatory synapse maturation in the developing prefrontal cortex

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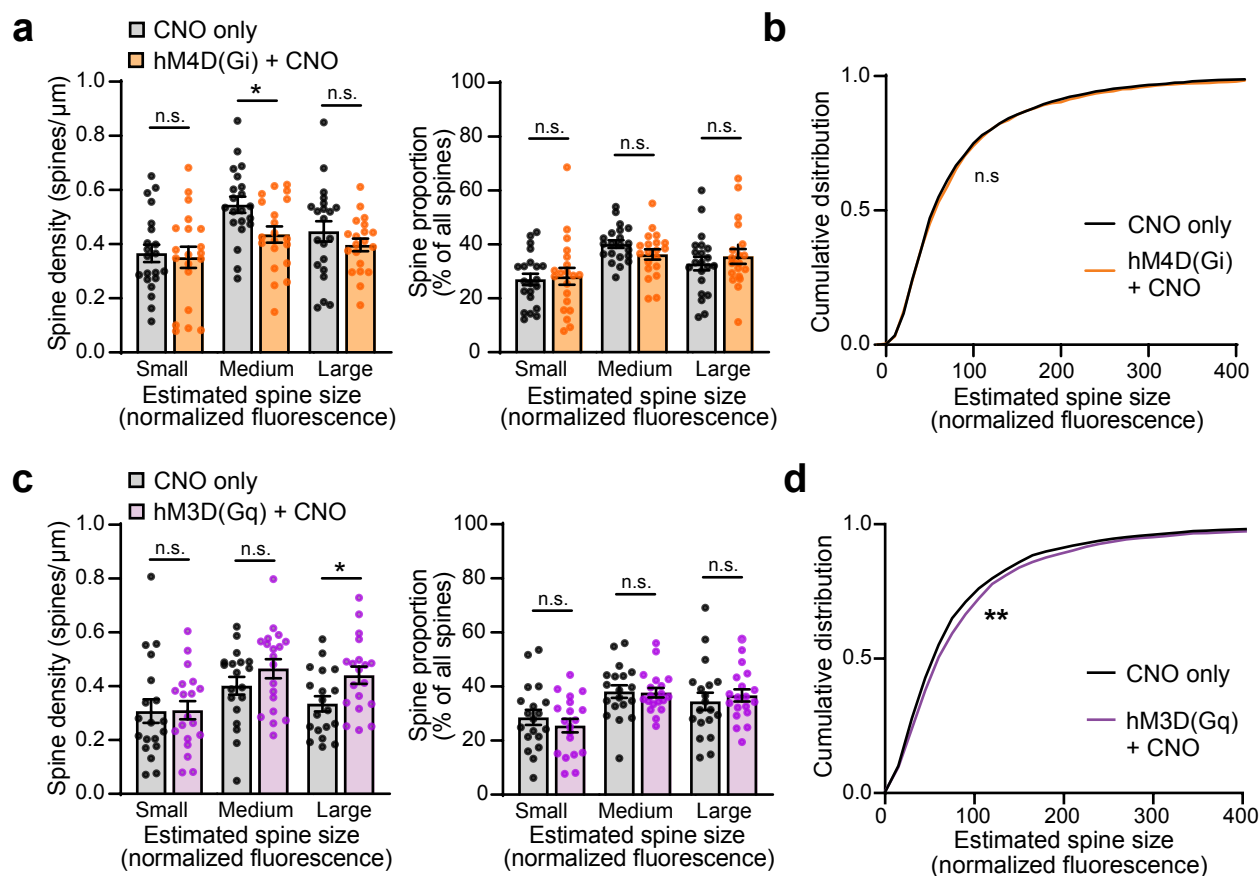
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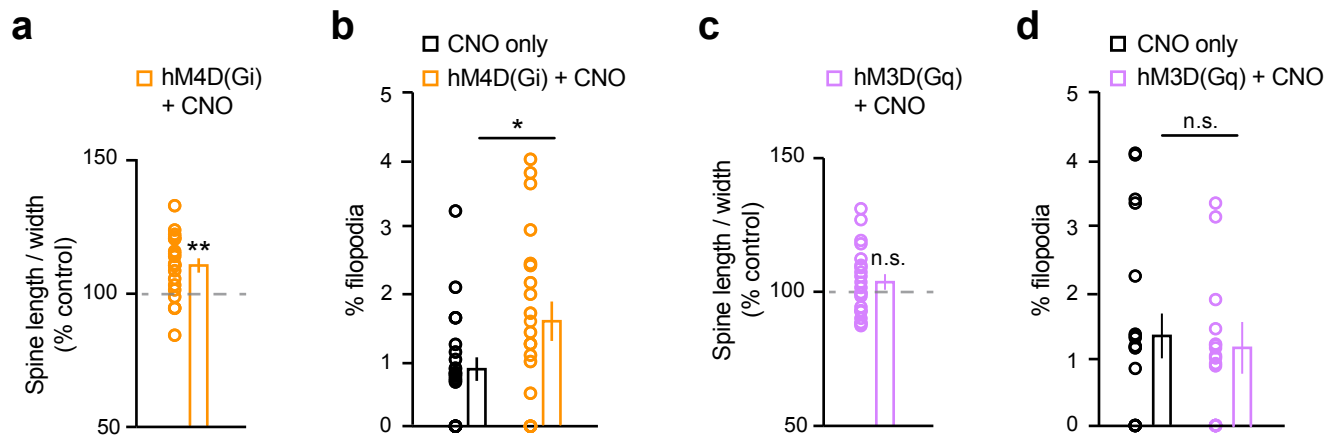
Classification: Article



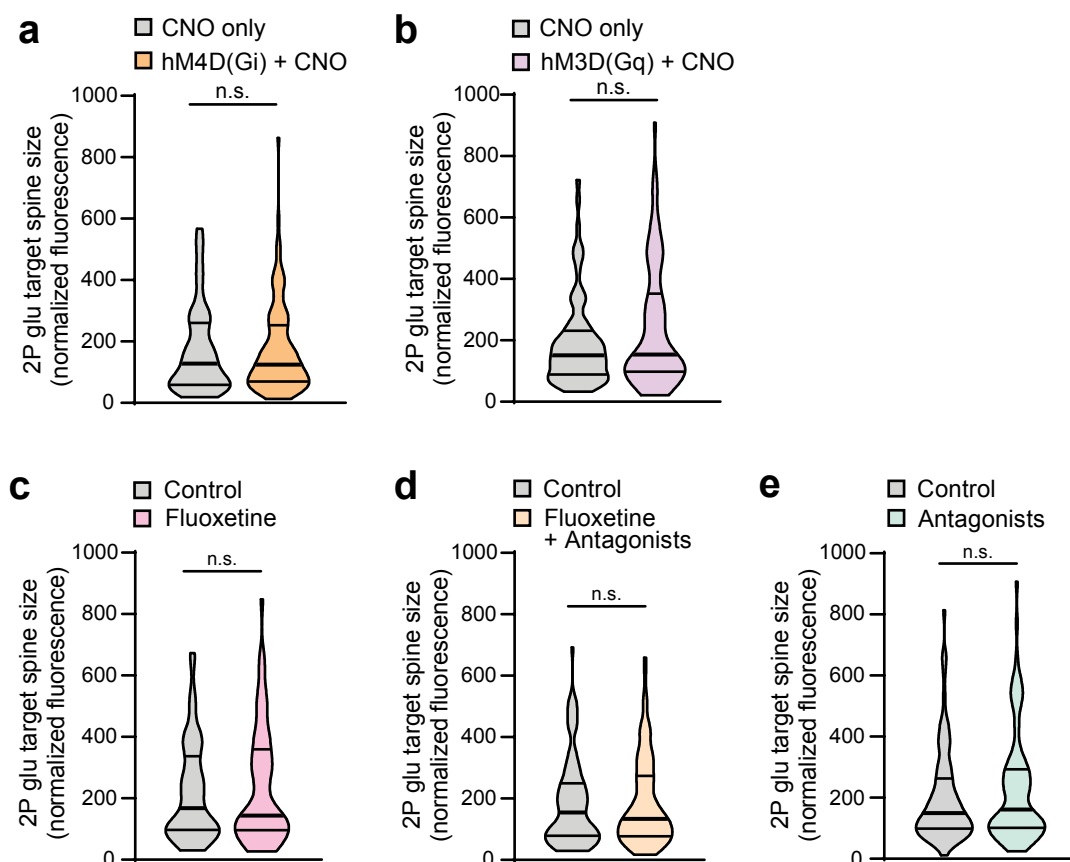
Supplementary Fig. 1. Verification of CNO on rAAV-hM4D(Gi) or hM3D(Gq) expressing 5-HT neurons in acute brainstem slices from SERT-Cre mice. **a, d, f**, Schematic of intracranial rAAV and/or retro-beads injections and experimental timeline. **b**, Diagram showing location of 2P *in vivo* and *ex vivo* imaging. **c**, Representative images of tdTomato expressing serotonergic axonal terminals in the PFC. **e**, DIC and 2P images of tdTomato and Retro-Beads expressing 5-HT neurons in the DRN. Spontaneous action potentials recorded in current-clamp mode. **g**, Example membrane potential traces evoked by current injection of 10 pA and quantitative analysis of rheobase of mCherry positive neurons after CNO bath application (30-60 min, 1 μ M) in hM4D(Gi) expressing brainstem slices (Gi: n = 10 cells, 4 mice; Gi + CNO: n = 10 cells, 4 mice). **h**, Quantitative analysis of resting membrane potential (Gi: n = 17 cells, 7 mice; Gi + CNO: n = 18 cells, 7 mice; 4 males, 3 females per group). **i**, Example traces evoked by 5 pA current injection and summary of rheobase of mCherry positive neurons after CNO bath application (30-60 min, 1 μ M) in hM3D(Gq) expressing brainstem slices (Gq: n = 11 cells, 4 mice; Gq + CNO: n = 11 cells, 4 mice). **j**, Quantitative analysis of resting membrane potential (Gq: n = 16 cells, 6 mice; Gq + CNO: n = 15 cells, 6 mice; 3 males, 3 females per group). ** p < 0.05; error bars represent SEM. Source data are provided as a Source Data file.



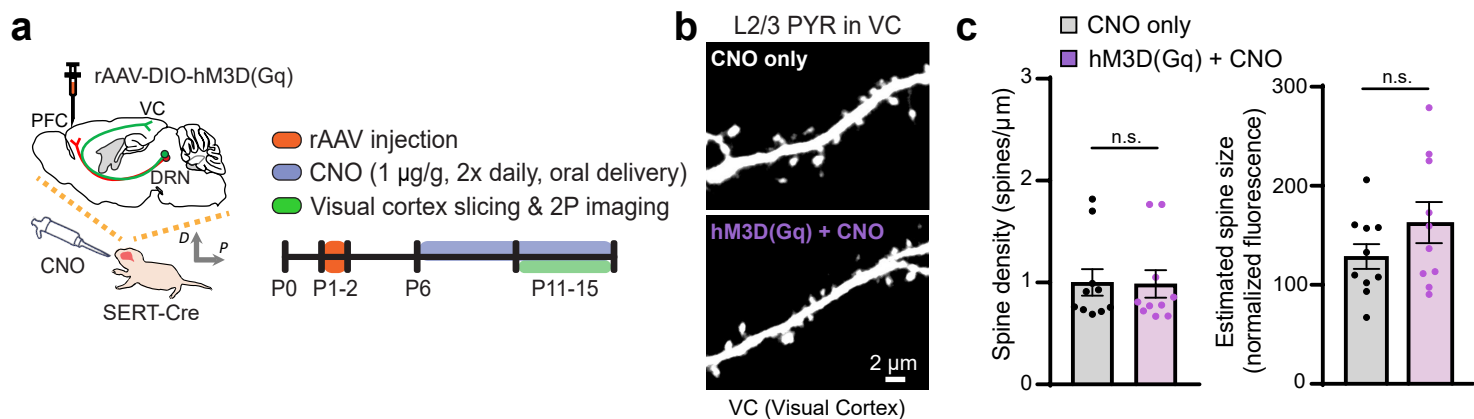
Supplementary Fig. 2. Serotonin-mediated changes in spine density of PFC layer 2/3 pyramidal neurons *in vivo*. **a**, Quantitative analysis of spine density and spine proportion by size (CNO only: $n = 62$ dendrites, 21 cells, 4 mice; hM4D(Gi) + CNO: 65 dendrites, 19 cells, 4 mice). **b**, Frequency distribution plot of all spine sizes (CNO only: $n = 2255$ spines, 21 cells, 4 mice; hM4D(Gi) + CNO: 1662 spines, 19 cells, 4 mice). **c**, Quantitative analysis of spine density and spine proportion by size (CNO only: $n = 72$ dendrites, 20 cells, 6 mice; hM3D(Gq) + CNO: 69 dendrites, 20 cells, 6 mice). **d**, Frequency distribution plot of all spine sizes (CNO only: $n = 2327$ spines, 20 cells, 6 mice; hM3D(Gq) + CNO: 2688 spines, 20 cells, 6 mice). * $p < 0.05$, ** $p < 0.01$; error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



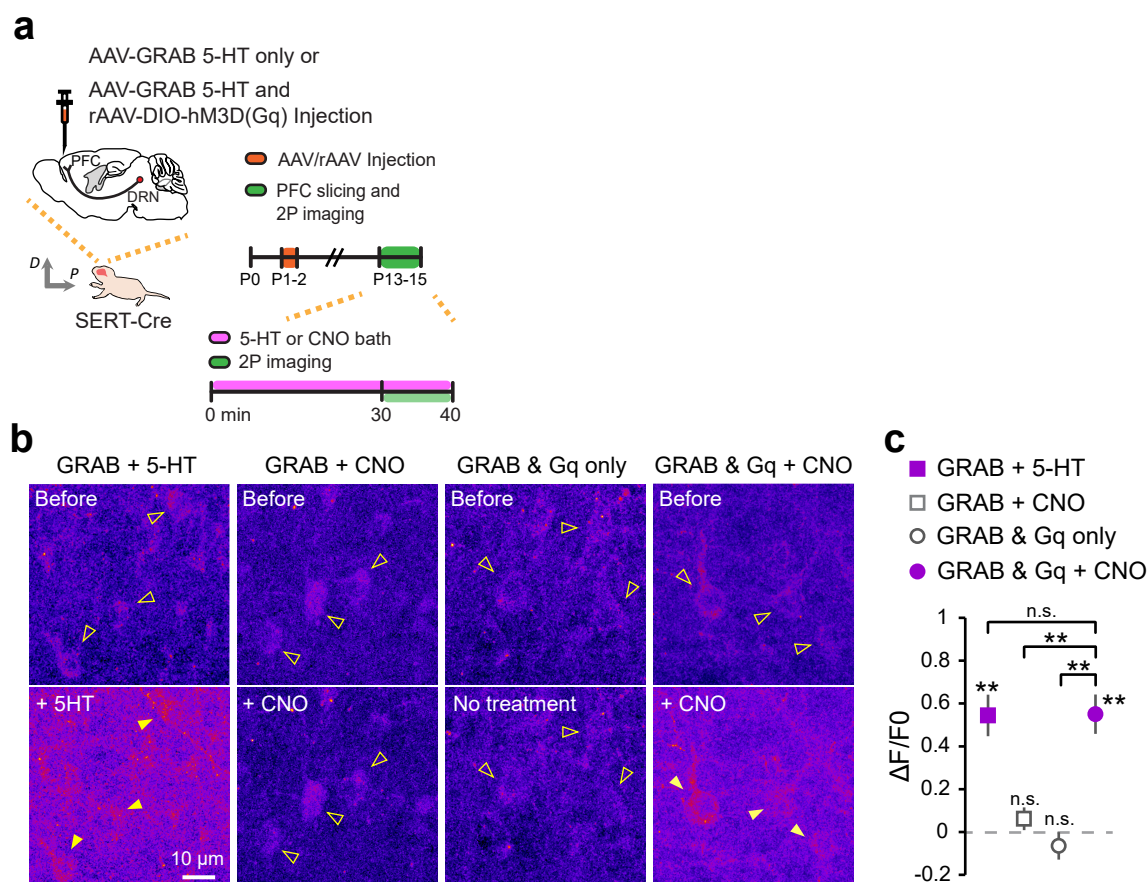
Supplementary Fig. 3. Spine morphology analysis in DREADDs experiments. Quantitative analysis of **a**, spine length / width ratio and **b**, % filopodia in hM4D(Gi) experimental group (CNO only: n = 2762 spines, 21 cells; hM4D(Gi) + CNO: n = 1675 spines, 20 cells). Quantitative analysis of **c**, spine length / width ratio and **d**, % filopodia in hM3D(Gq) experimental group (CNO only: n = 1333 spines, 18 cells; hM3D(Gq) + CNO: n = 1535 spines, 20 cells). * $p < 0.05$; error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



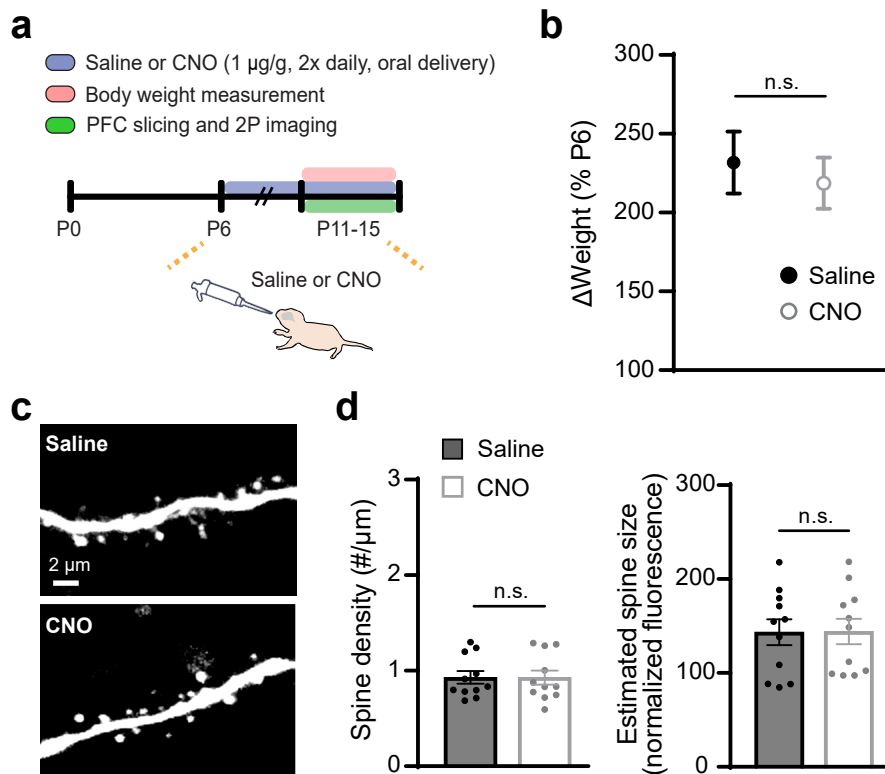
Supplementary Fig. 4. Comparable target spine sizes for two-photon uEPSC measurements from *in vivo* manipulation experiments. **a**, Violin plot distribution of spine sizes that uEPSCs were measured from in PFC slices of CNO only and hM4D(Gi) + CNO treated mice (CNO only: $n = 80$ spines, 12 cells, 4 mice; hM4D(Gi) + CNO: 115 spines, 12 cells, 4 mice). **b**, Violin plot distribution of spine sizes that uEPSCs were measured from in PFC slices of CNO only and hM3D(Gq) + CNO treated mice (CNO only: $n = 79$ spines, 13 cells, 6 mice; hM3D(Gq) + CNO: 83 spines, 14 cells, 6 mice). **c**, Violin plot distribution of spines that uEPSCs were measured from for control and FLX treated mice (Control: $n = 59$ spines, 9 cells, 3 mice; FLX: 64 spines, 11 cells, 3 mice). **d**, Violin plot distribution of spines that uEPSCs were measured from for control and FLX and antagonists treated mice (Control: $n = 95$ spines, 15 cells, 4 mice; FLX and Antagonists: 110 spines, 17 cells, 4 mice). **e**, Violin plot distribution of spines that uEPSCs were measured from for control and antagonists treated mice (Control: $n = 93$ spines, 15 cells, 4 mice; Antagonists: 93 spines, 12 cells, 4 mice). Violin plots: median $\pm$ upper and lower quartiles. n.s., not significant. Source data are provided as a Source Data file.



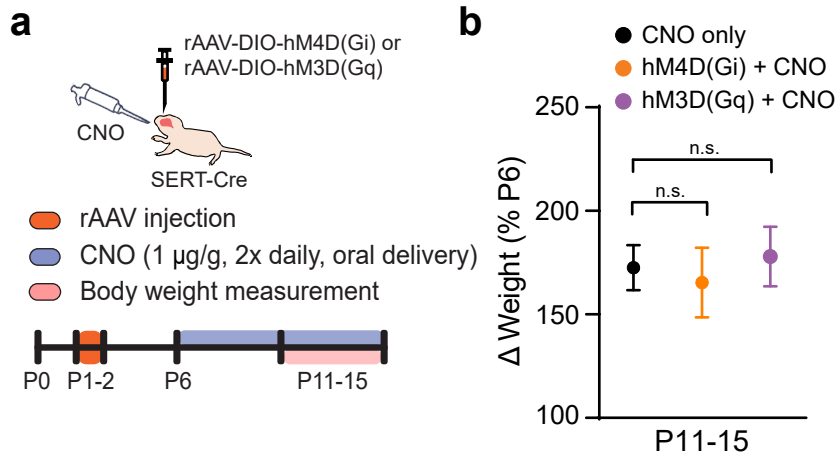
Supplementary Fig. 5. rAAV-DREADD injection in the PFC does not affect excitatory synapse development on layer 2/3 pyramidal neurons in the developing visual cortex. **a**, Schematic of rAAV injection in PFC, oral CNO delivery, and experimental timeline. **b**, Images from dendritic segments of layer 2/3 pyramidal neurons from visual cortex of CNO only and hM3D(Gq) and CNO treated mice. **c**, Quantitative analysis of spine density and size (CNO only: $n = 37$ dendrites, 10 cells, 3 mice; hM3D(Gq) + CNO: 37 dendrites, 10 cells, 3 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



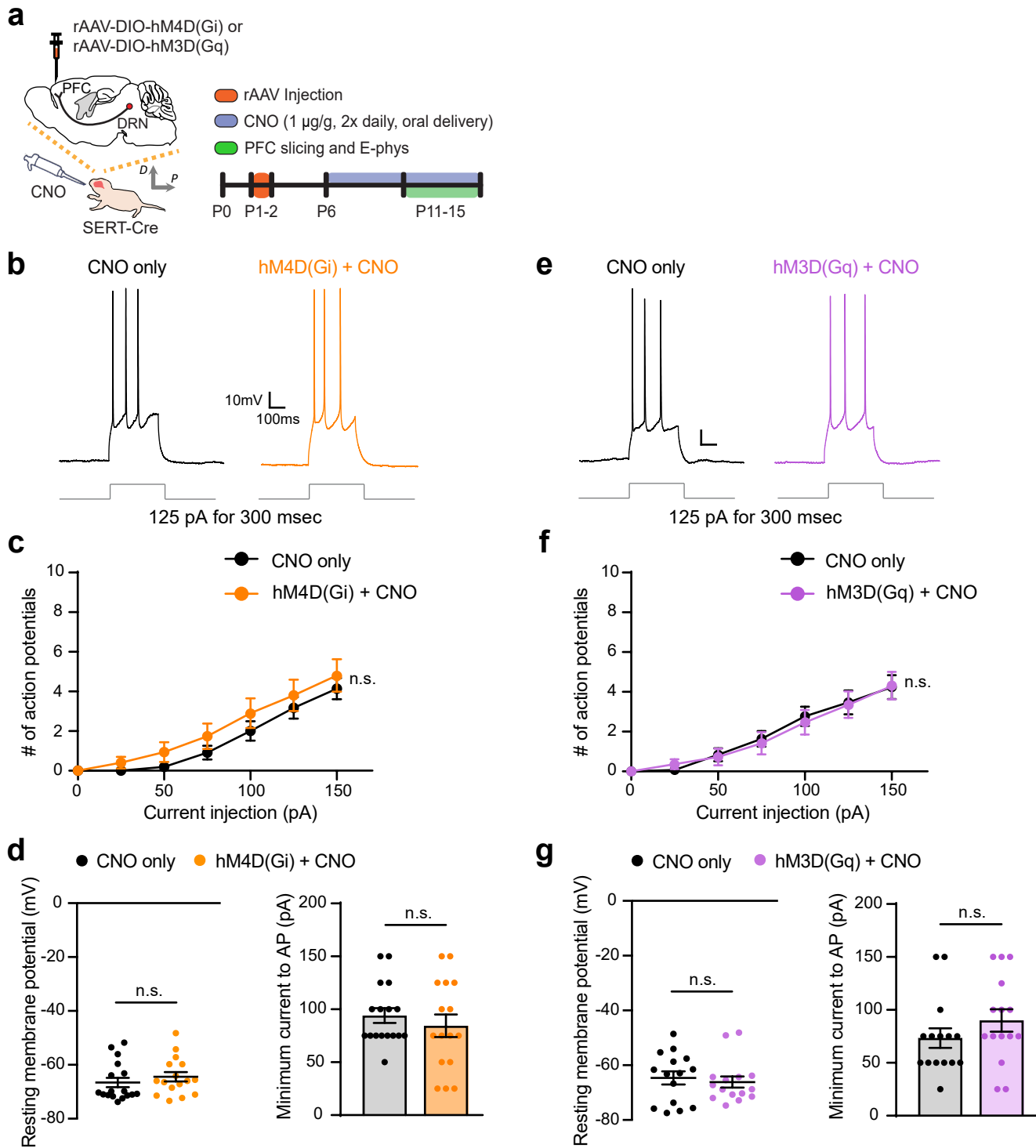
Supplementary Fig. 6. Verification of 5-HT release in the PFC of rAAV-hM3D(Gq) injected mice using GRAB 5-HT1.0. **a**, Schematic of intracranial AAV/rAAV injection and experimental timeline. **b**, Representative images of GRAB 5-HT expressing neurons from PFC layer 2/3 before and after 5-HT or CNO bath application (5-HT, 30-40 min, 10 μ M; CNO, 30-40 min, 1 μ M). **c**, Quantitative analysis of GRAB-5-HT fluorescent changes (GRAB + 5-HT: $n = 42$ cells, 2 mice; GRAB + CNO: $n = 38$ cells, 2 mice; GRAB & Gq, no treatment: $n = 24$ cells, 3 mice; GRAB & Gq + CNO: $n = 94$ cells, 3 mice). ** $p < 0.01$; error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



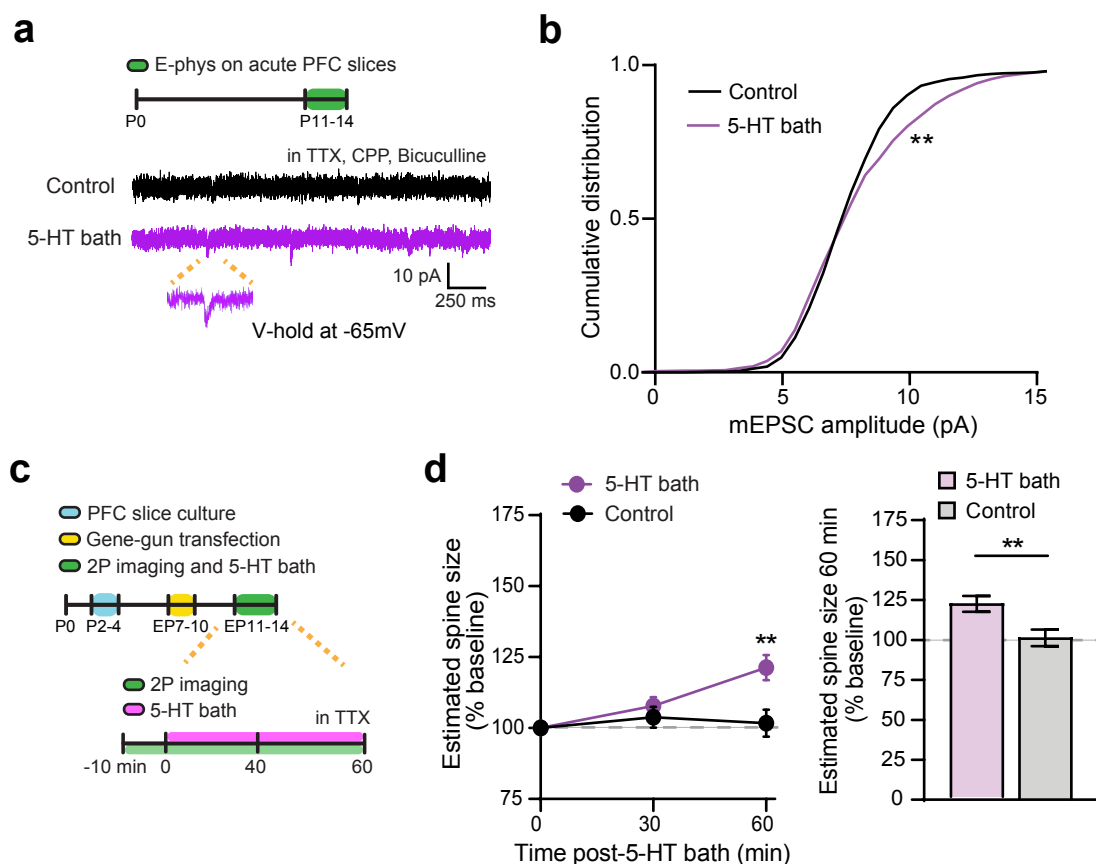
Supplementary Fig. 7. *In vivo* CNO administration alone does not affect mouse health or dendritic spine development on layer 2/3 pyramidal neurons in the PFC. **a**, Schematic of oral delivery of saline or CNO and timeline for pup weight measurements, acute PFC slicing, and 2-photon imaging, **b**, Quantative analysis of pup weight changes from start of saline or CNO delivery to time of experiments at P11-15 (saline: $n = 4$ mice; CNO: $n = 4$ mice). **c**, Representative 2-photon images of dendritic segments in saline and CNO conditions. **d**, Summary of spine density and size in saline and CNO treated mice (saline: $n = 11$ cells, 4 mice; CNO: $n = 11$ cells, 4 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



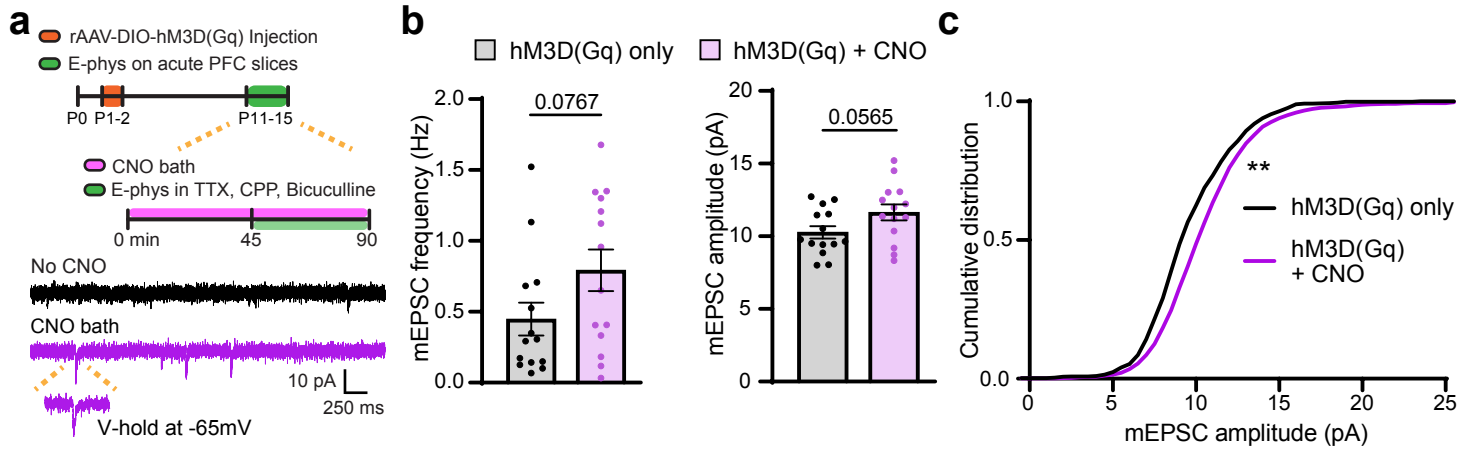
Supplementary Fig. 8. rAAV-hM4D(Gi) or hM3D(Gq) injected mice show normal growth rates. **a**, Schematic of rAAV injection, CNO oral delivery, and timeline for pup weight measurements. **b**, Summary of pup weight changes from start of CNO treatment to time of experiments at P11-15 (CNO only: $n = 14$ mice; hM4D(Gi) + CNO: 4 mice; hM3D(Gq) + CNO: 6 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



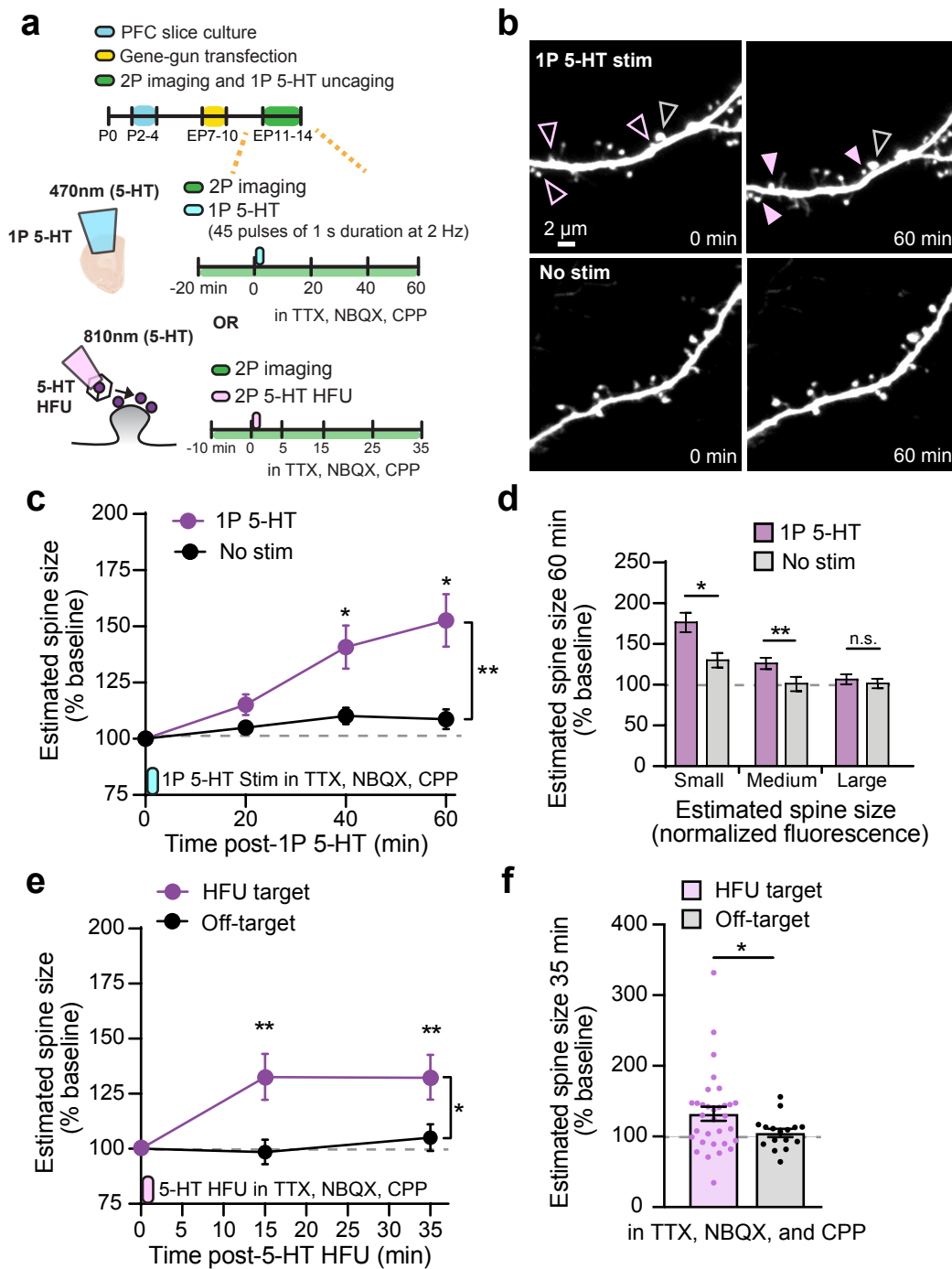
Supplementary Fig. 9. Chemogenetic manipulation of PFC-projecting 5-HTergic neurons does not alter intrinsic properties of PFC layer 2/3 pyramidal neurons. **a**, Schematic of rAAV injection, oral CNO delivery, and experimental timeline. **b**, Representative whole-cell traces of action potential firing evoked by current injection of 125 pA from CNO only and hM4D(Gi) and CNO treated mice. **c**, Quantative analysis of number of action potentials fired at increasing current injection steps. **d**, Quantative analysis of resting membrane potential and minimum current to elicit an action potential (CNO only: n = 17 cells, 3 mice; hM4D(Gi) + CNO: n = 16 cells, 3 mice). **e**, Representative traces of action potential firing evoked by current injection of 125 pA from CNO only and hM3D(Gq) and CNO treated mice. **f**, Quantative analysis of number of action potentials fired at increasing current injection steps. **g**, Summary of resting membrane potential and minimum current to elicit an action potential (CNO only: n = 15 cells, 3 mice; hM3D(Gq) + CNO: n = 15 cells, 3 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



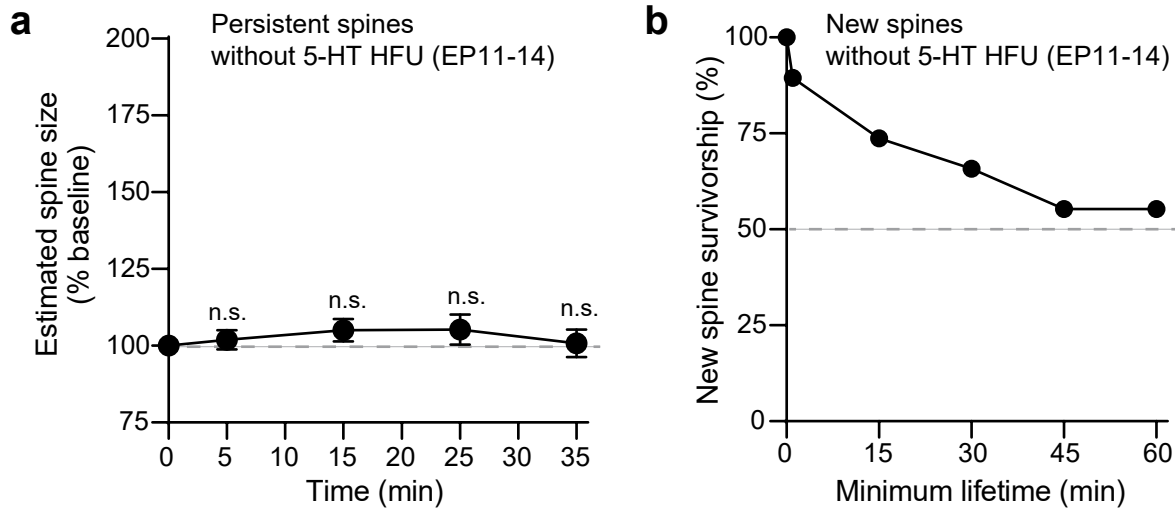
Supplementary Fig. 10. Serotonin bath application induces cell-wide excitatory synaptic potentiation in the PFC. **a**, Schematic of experimental timeline and representative mEPSC traces by whole-cell electrophysiological recordings. **b**, Frequency distribution plot of mEPSC amplitudes after 1 hr 5-HT bath application (Control: $n = 11$ cells; 5-HT: 9 cells, 10 μM). **c**, Schematic of experimental timeline. **d**, Time courses of spine size changes and quantitative analysis at 1 hr post 5-HT bath application (Control: $n = 26$ dendrites, 6 cells; 5-HT: 28 dendrites, 8 cells, 50 μM). ** $p < 0.01$; error bars represent SEM. Source data are provided as a Source Data file.



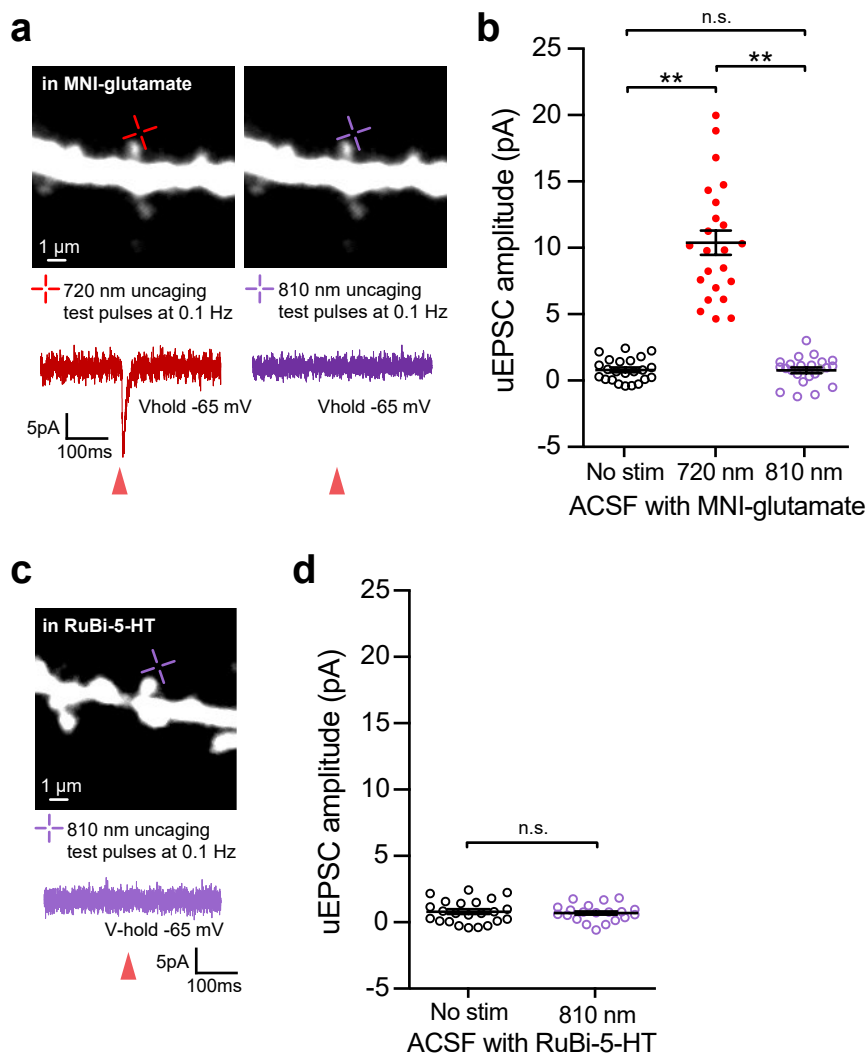
Supplementary Fig. 11. hM3D(Gq)-mediated 5-HT release induces cell-wide excitatory synaptic potentiation in the PFC. a, Schematic of rAAV injection, experimental timeline, and representative mEPSC traces. **b**, Quantitative analysis of mEPSC frequency and amplitude recorded at -65 mV 45-90 min after CNO bath application (1 μ M) (Gq only: n = 13 cells, 3 mice; Gq + CNO: 13 cells, 3 mice). **c**, Frequency distribution plot of mEPSC amplitudes. ** $p < 0.01$; error bars represent SEM. Source data are provided as a Source Data file.



Supplementary Fig. 12. 5-HT drives structural potentiation of dendritic spines independently of glutamatergic signaling. **a**, Schematic of slice culture and experimental timeline for structural LTP after 1-photon (top) and 2-photon (bottom) 5-HT uncaging in TTX (1 μM), NBQX (10 μM), and CPP (10 μM). **b**, Two-photon images of dendrites with and without 1-photon 5-HT uncaging stimulation. Purple arrows indicate small and medium spines that underwent sLTP. Gray arrow shows a large spine that was unchanged. **c**, Time courses for spine size changes post 1-photon 5-HT uncaging. Blue indicator represents 1-photon 5-HT time point (Control: n = 20 dendrites, 5 cells; 1P 5-HT: 26 dendrites, 6 cells). **d**, Quantitative analysis of spine size change at 60 min, subdivided by spine size. **e**, Time courses for spine size changes following 5-HT HFU and **f**, summary of spine size at 35 min post 5-HT HFU. Purple indicator shows 2P 5-HT HFU time point (HFU target: n = 32 spines, 16 cells; Off-target: 16 ROIs, 16 cells). * $p < 0.05$, ** $p < 0.01$; error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.

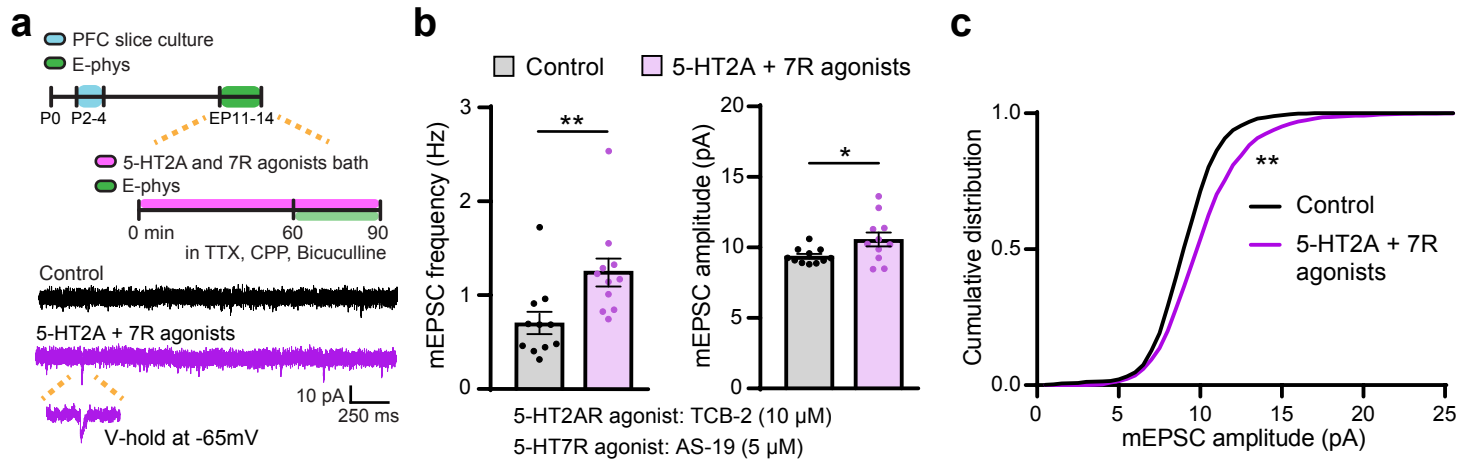


Supplementary Fig. 13. Dynamics of dendritic spines of PFC layer 2/3 pyramidal neurons in RuBi-5-HT and TTX containing ACSF. a, Time course for spine size changes of no stim control spines (n = 20 dendrites, 10 cells). **b,** Time course for new spine survivorship of no stim control spines (n = 38 spines, 7 cells). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.

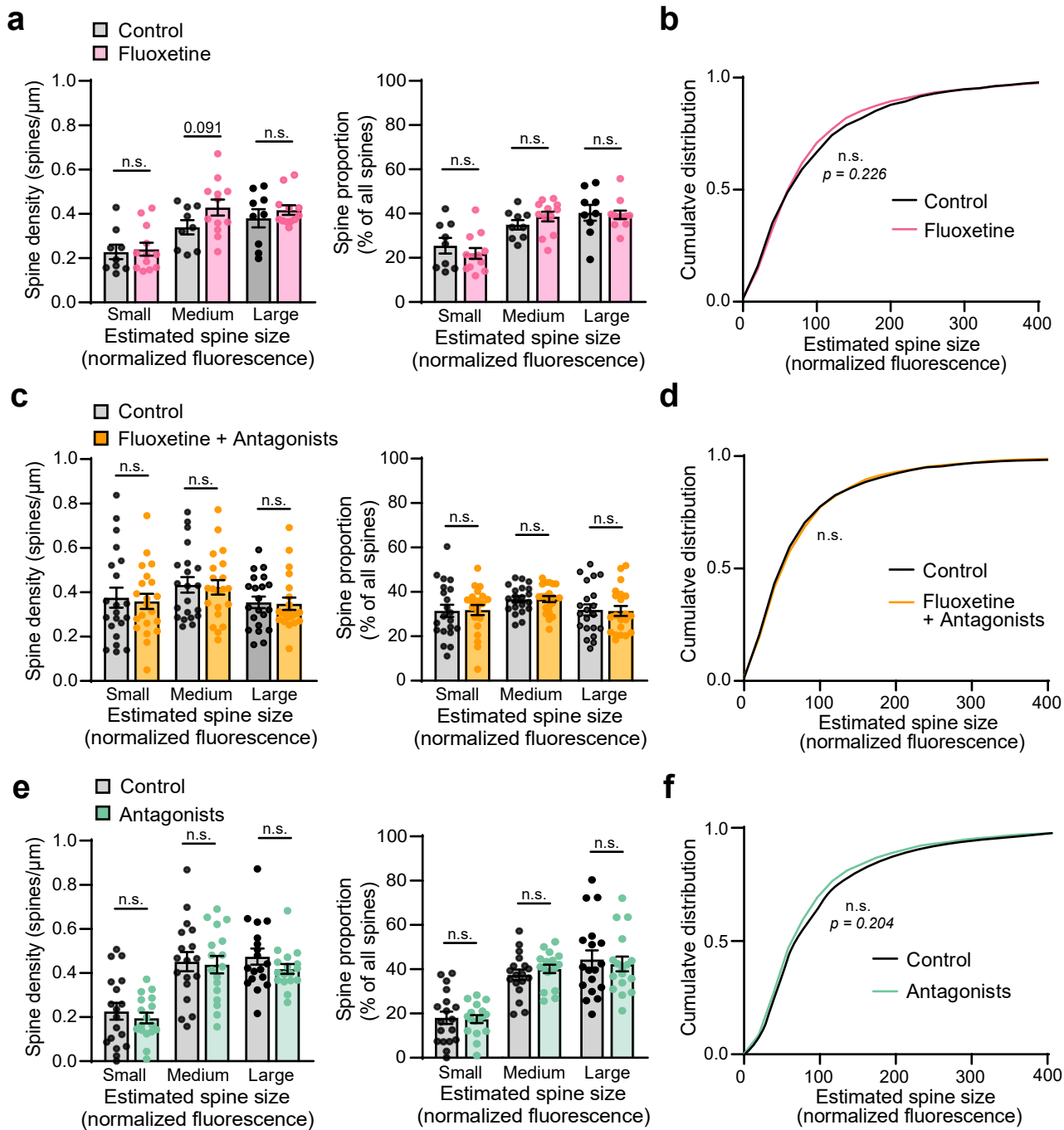


Supplementary Fig. 14. Two-photon laser wavelength specific uncaging.

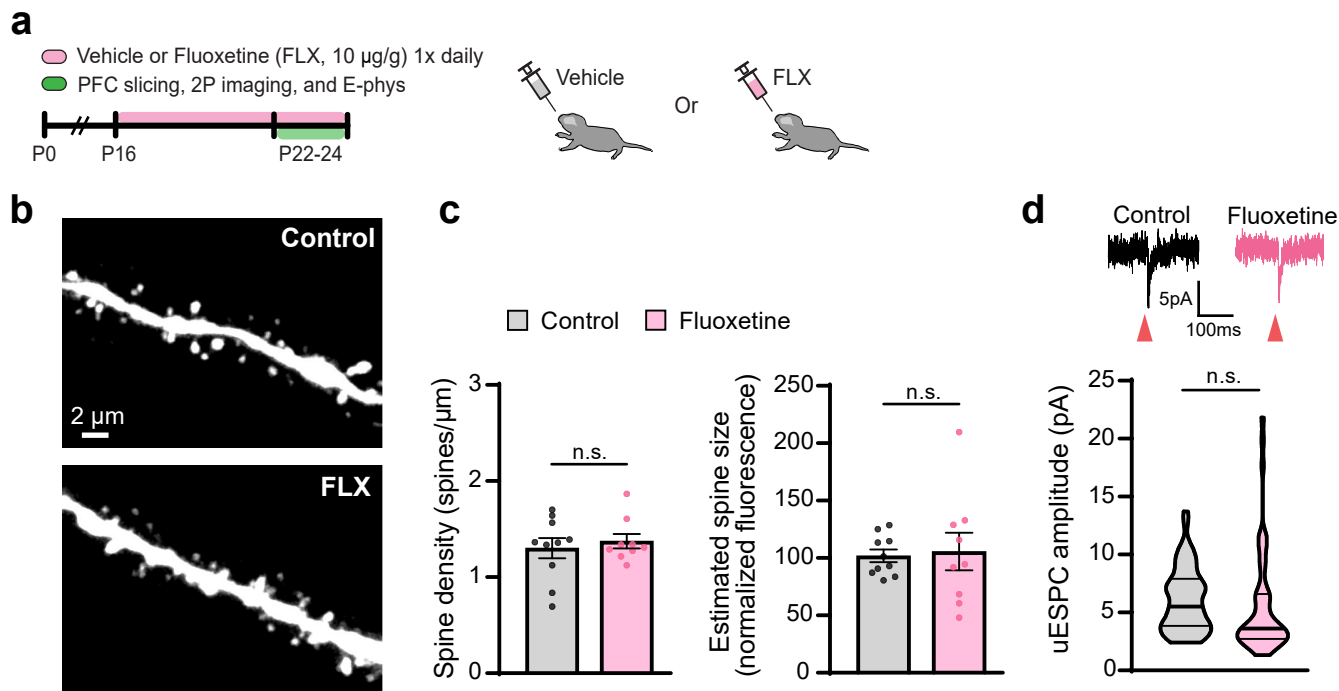
a, Representative 2-photon images and uEPSC traces from the same spine evoked by 720 nm (left) or 810 nm (right) in MNI-glutamate (2.5 mM) containing ACSF (red cross represents 720 nm uncaging point, purple cross represents 810 nm uncaging point; red arrows indicate uncaging time point). **b**, Summary of uEPSCs (No stim: $n = 23$ off-stim responses, 5 cells; 720 nm: $n = 23$ spines, 5 cells; 810 nm: $n = 23$ spines, 5 cells). **c**, A representative image and an uncaging response recorded at -65 mV by 810 nm laser in RuBi-5-HT (0.1 mM) containing ACSF (purple cross represents 810 nm uncaging point; red arrows indicate uncaging time point). **d**, Summary data (No stim: $n = 23$ off-stim responses, 5 cells; 810 nm: $n = 20$ spines, 2 cells). $**p < 0.01$; error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



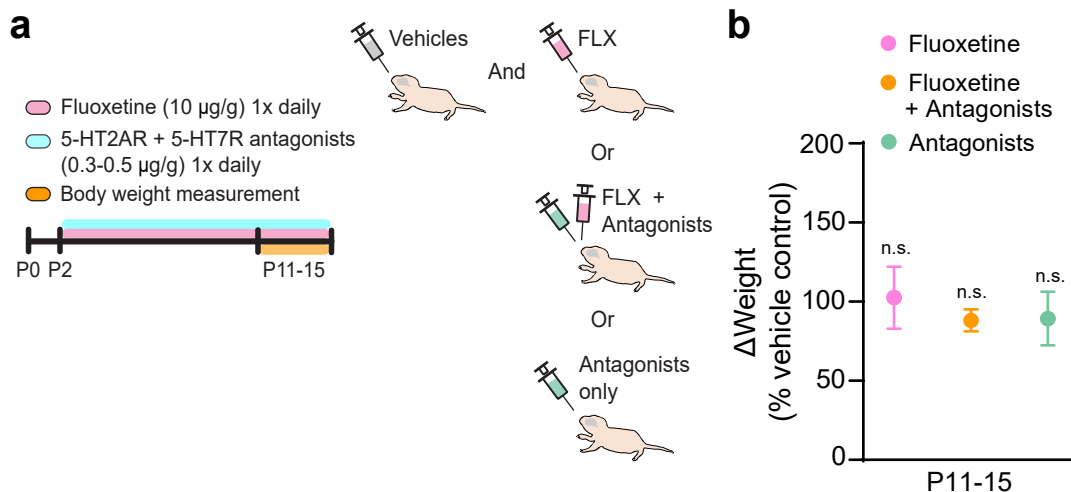
Supplementary Fig. 15. 5-HT_{2A} and 5-HT₇ receptor agonist bath application increases mEPSC frequency and amplitude on PFC layer 2/3 pyramidal neurons. **a**, Schematic of experimental timeline and representative mEPSC traces by whole-cell electrophysiological recordings after bath application of 5-HT_{2A} agonist (TCB-2, 10 μ M) and 5-HT₇R agonist (AS-19, 5 μ M). **b**, Quantative analysis of mEPSC frequency and amplitude recorded 60-90 min after bath application of agonists (Control: n = 11 cells; TCB-2 + AS-19: n = 11 cells). **c**, Frequency distribution plot of mEPSC amplitudes. * p < 0.05, ** p < 0.01; error bars represent SEM. Source data are provided as a Source Data file.



Supplementary Fig. 16. Spine density changes by *in vivo* treatment of fluoxetine, fluoxetine and 5-HT2AR and 5-HT7R antagonists, or 5-HT2AR and 5-HT7R antagonists only. **a**, Quantitative analysis of spine density and spine proportion by size after oral FLX exposure (Control: $n = 50$ dendrites, 13 cells, 5 mice; FLX: 69 dendrites, 18 cells, 5 mice). **b**, Frequency distribution plot of all spine sizes (Control: $n = 792$ spines, 13 cells, 5 mice; FLX: 1417 spines, 18 cells, 5 mice). **c**, Summary of spine density and spine proportion by size after FLX and 5-HT2AR and 5-HT7R antagonists treatment (Control: $n = 64$ dendrites, 21 cells, 4 mice; FLX + Antagonists: 67 dendrites, 21 cells, 4 mice). **d**, Frequency distribution plot of all spine sizes (Control: $n = 2131$ spines, 21 cells, 4 mice; FLX + Antagonists: 2180 spines, 21 cells, 4 mice). **e**, Quantitative analysis of spine density and spine proportion by size after oral 5-HT2AR and 5-HT7R antagonists treatment (Control: $n = 60$ dendrites, 18 cells, 4 mice; Antagonists: 60 dendrites, 17 cells, 4 mice). **f**, Frequency distribution plot of all spine sizes (Control: $n = 2188$ spines, 18 cells, 4 mice; Antagonists: 1797 spines, 17 cells, 4 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



Supplementary Fig. 17. Fluoxetine administration to old pups aged P16-24 does not alter excitatory synapses on layer 2/3 pyramidal neurons in the PFC. **a**, Schematic of experimental timeline and oral FLX administration. **b**, 2-photon images of dendrites from PFC layer 2/3 pyramidal neurons from vehicle control and FLX treated mice. Quantitative analysis of **c**, spine density and size (Control: $n = 31$ dendrites, 10 cells, 3 mice; FLX: 27 dendrites, 9 cells, 3 mice) and **d**, uEPSCs recorded at -65 mV (Control: $n = 59$ spines, 10 cells, 3 mice; FLX: 50 spines, 9 cells, 3 mice; red arrows indicate 2P uncaging time point). Violin plots: median $\pm$ upper and lower quartiles. Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.



Supplementary Fig. 18. Fluoxetine only, fluoxetine and 5-HT2AR and 5-HT7R antagonists, or 5-HT2AR and 5-HT7R antagonists only exposed pups show normal growth rates. **a**, Schematic of FLX, FLX and antagonists, or antagonists only oral delivery and timeline for pup weight measurements. **b**, Pup weight changes compared to age, litter, and gender-matched control pups from start of FLX, FLX and antagonists, or antagonists only exposure to time of experiments (Control: n = 9 mice, FLX: 5 mice, FLX + Antagonists: 4 mice, Antagonists only: 4 mice). Error bars represent SEM. n.s., not significant. Source data are provided as a Source Data file.

Treatment	Total # of spines	# of spines that underwent enlargement (sLTP)	% of spines that underwent enlargement (sLTP)	Spine size changes (% baseline)
No stim (EP11-14)	337	137/337	40.7 %	100.76 % $\pm$ 4.50
HFU target (EP11-14)	30	21/30	70.0 %	140.13 % $\pm$ 11.42
Off-target (EP11-14)	151	61/151	40.7 %	103.86 % $\pm$ 3.60
HFU target (EP15-20)	22	11/22	50.0 %	112.65 % $\pm$ 6.21
5-HT HFU (shift)	22	9/22	40.9 %	102.04 % $\pm$ 15.85
LFU target (0.1Hz)	23	12/23	52.2 %	110.38 % $\pm$ 5.43
5-HT HFU (Layer V)	28	14/28	50.0 %	113.78 % $\pm$ 11.43
HFU target + MDL	18	7/18	38.9 %	96.95 % $\pm$ 7.67
Off-target in MDL	52	19/52	36.5 %	100.76 % $\pm$ 3.35
HFU target + Gö	15	8/15	53.3 %	119.11 % $\pm$ 16.38
Off-target in Gö	187	81/187	43.3 %	104.04 % $\pm$ 5.11
HFU target + SB	18	9/18	50.0 %	117.78 % $\pm$ 12.19
Off-target in SB	102	45/102	44.1 %	104.54 % $\pm$ 7.25
HFU target 0mM Ca	20	10/20	50.0 %	99.92 % $\pm$ 7.22
Off-target 0mM Ca	98	43/98	43.8 %	101.75 % $\pm$ 4.99
HFU target in MDL + DR	20	9/20	45.0 %	114.10 % $\pm$ 10.41
Off-target in MDL + DR	94	34/94	36.2 %	106.94 % $\pm$ 6.75
HFU target in NBQX/CPP	32	22/32	68.8 %	132.21 % $\pm$ 9.98
Off-target in NBQX/CPP	140	77/140	55.0 %	105.08 % $\pm$ 5.75

Supplementary Table 1. Two-photon 5-HT HFU increases the proportion of spines that undergo sLTP. Summary of the number and percentage of spines that underwent sLTP at 35 min post-stim for 2-photon 5-HT HFU sLTP data. Spine size changes in each of experimental conditions are also shown for reference. Source data are provided as a Source Data file.

Treatment	Estimated initial spine size (+/- SEM)	
HFU target (EP11-14)	124.55 ± 12.54	n.s.
5-HT HFU (shift)	127.75 ± 21.99	
HFU target (EP15-20)	89.02 ± 11.82	
LFU target (0.1Hz)	115.53 ± 14.23	
5-HT HFU (Layer V)	84.78 ± 10.38	
HFU target + MDL	121.78 ± 7.79	
HFU target + Gö	86.92 ± 10.56	
HFU target + SB	115.57 ± 13.95	
HFU target 0mM Ca	123.72 ± 20.31	
HFU target in MDL + DR	89.14 ± 10.91	
HFU target in NBQX/CPP	115.34 ± 13.65	

Supplementary Table 2. Initial spine sizes are comparable for all two-photon 5-HT uncaging sLTP target spines. Summary of initial spine sizes for all stimulated target spines for all 5-HT HFU sLTP experimental conditions. One-way ANOVA; n.s., not significant. Source data are provided as a Source Data file.

Groups	Initial SEP <u>GluA2</u> Expression (+/- SEM)
Persistent Spines (no drug)	0.588 ± 0.059
Persistent Spines (MDL + SB)	0.527 ± 0.061
Newly formed spines	0.335 ± 0.043

n.s.

**

Supplementary Table 3. Initial SEP-GluA2 expression of 5-HT HFU target spines. Summary of initial SEP-GluA2 for all stimulated target spines for 5-HT HFU experimental conditions. ***p* < 0.01; n.s., not significant. Source data are provided as a Source Data file.