Supplemental Items

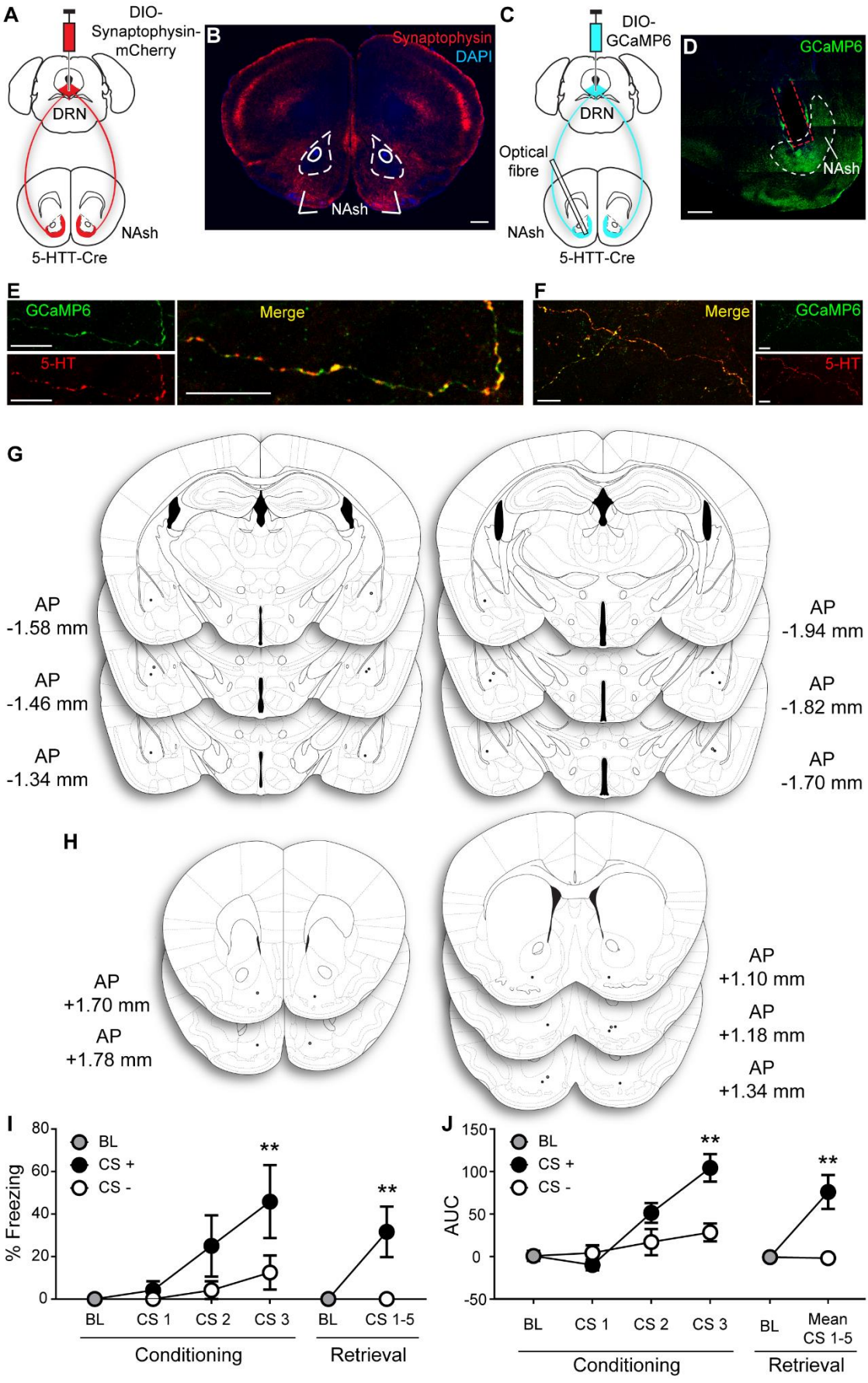

Figure S1, related to Figure 1

BA and NA placements for *in vivo* fibre photometry of DRN 5-HT projections

(A-B) Cre-dependent fluorescent synaptophysin injection into DRN of 5-HTT-Cre mice (A). Fluorescent synaptophysin expression in DRN 5-HT projections in NA (B). Scale bar=500 µm.

(C-D) Cre-dependent GCaMP6 injection into DRN and unilateral optical fibre implantation into NA of 5-HTT-Cre mice (C). GCaMP6 expression in DRN 5-HT projections in NA (D). Dashed red lines=optical fibre tract. Scale bar=500 µm.

(E-F) Overlap of GCaMP6 expression and 5-HT immunoreactivity in axons in BA (E) and NA (F). Scale bars=10 µm.

(G-H) BA (G) and NA (H) placements of optical fibre implants in GCaMP6-injected 5-HTT-Cre mice. Dots demarcate fibre tip placement; solid are from GCaMP6 and empty from YFP animals.

(I-J) Freezing behaviour (I) and DRN→BA 5-HT projection activity (J) were higher during CS+ compared to CS- presentations in a discriminative fear conditioning task. Data are mean±SEM. AUC—area under the curve, BL—baseline, NAsh—nucleus accumbens shell.

**p<0.01.

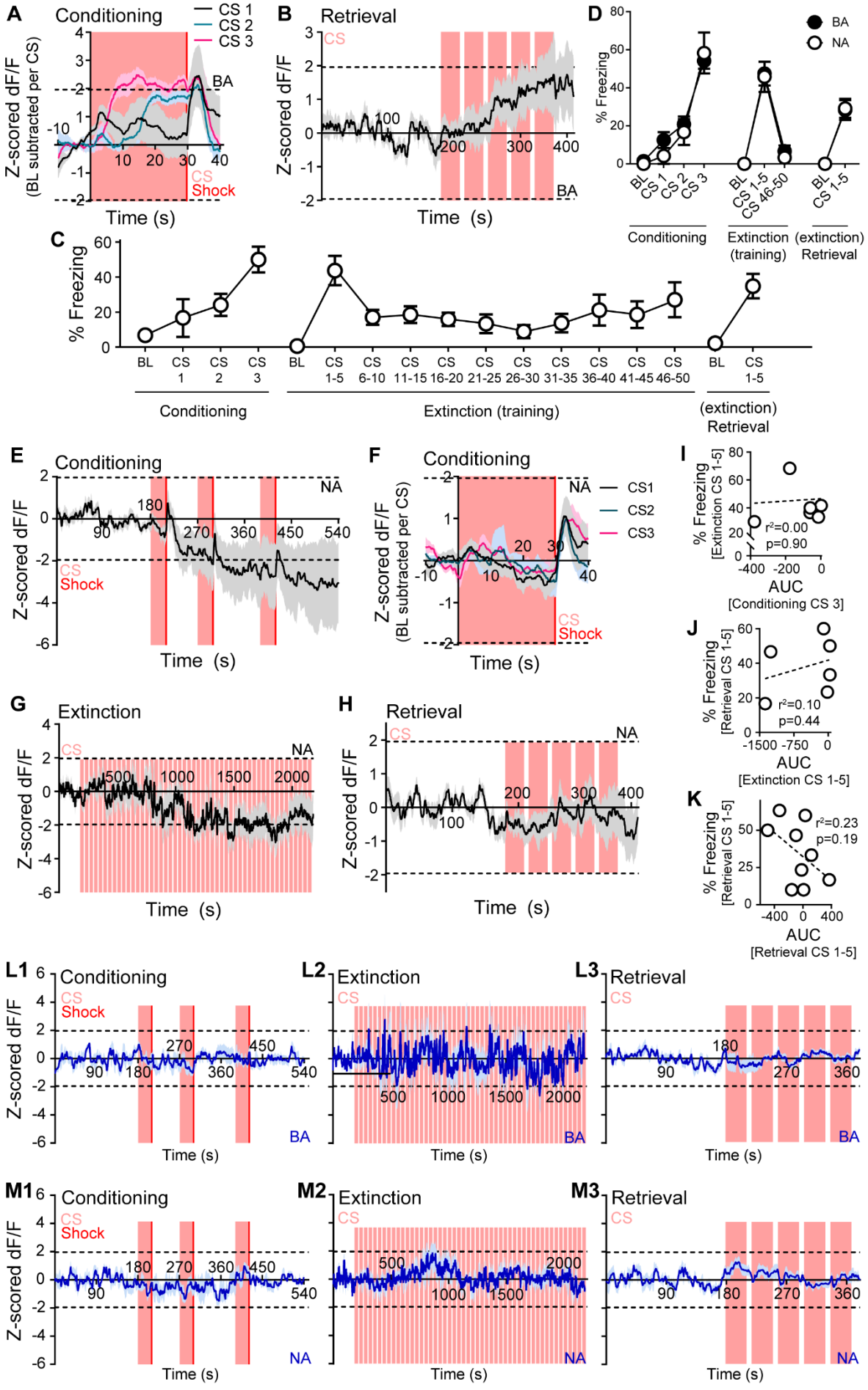
A
Conditioning
CS 1
CS 2
CS 3
Z-scored dF/F
(BL subtracted per CS)
BA
CS
Shock
Time (s)
B
Retrieval
CS
Z-scored dF/F
BA
Time (s)
D
% Freezing
BA
NA
BL
CS 1
CS 2
CS 3
CS 1-5
CS 46-50
Conditioning
Extinction
(training)
(extinction)
Retrieval
C
% Freezing
BL
CS 1
CS 2
CS 3
CS 1-5
CS 6-10
CS 11-15
CS 16-20
CS 21-25
CS 26-30
CS 31-35
CS 36-40
CS 41-45
CS 46-50
Conditioning
Extinction (training)
(extinction)
Retrieval
E
Conditioning
NA
Z-scored dF/F
CS
Shock
Time (s)
F
Conditioning
NA
CS1
CS2
CS3
Z-scored dF/F
(BL subtracted per CS)
CS
Shock
Time (s)
I
% Freezing
[Extinction CS 1-5]
r²=0.00
p=0.90
AUC
[Conditioning CS 3]
J
% Freezing
[Retrieval CS 1-5]
r²=0.10
p=0.44
AUC
[Extinction CS 1-5]
K
% Freezing
[Retrieval CS 1-5]
r²=0.23
p=0.19
AUC
[Retrieval CS 1-5]
G
Extinction
CS
NA
Z-scored dF/F
Time (s)
H
Retrieval
CS
NA
Z-scored dF/F
Time (s)
L1
Conditioning
CS
Shock
Z-scored dF/F
BA
Time (s)
L2
Extinction
CS
BA
Time (s)
L3
Retrieval
CS
BA
Time (s)
M1
Conditioning
CS
Shock
Z-scored dF/F
NA
Time (s)
M2
Extinction
CS
NA
Time (s)
M3
Retrieval
CS
NA
Time (s)

Figure S2, related to Figure 1
Distinct DRN 5-HT projection activity in BA and NA during fear learning
(A-B) Increasingly elevated DRN→BA 5-HT projection activity per CS (baseline-subtracted) during conditioning (n=7 mice) (A). DRN→BA 5-HT projection activity did not change significantly during retrieval (n=8 mice) (B). Traces show mean±SEM of z-scored dF/F GCaMP fluorescence. Dashed lines at ±1.96 demarcate statistically significant change from baseline (2-tailed, α=0.05).
(C) Freezing of DRN GCaMP6-injected and NA optical fibre-implanted 5-HTT-Cre mice during the fear learning task (n=9 mice). Data are mean±SEM.
(D) Freezing of DRN YFP-injected and BA or NA optical fibre-implanted 5-HTT-Cre mice during the fear learning task (BA n=4 mice, NA n=4 mice). Data are mean±SEM.
(E-H) DRN→NA 5-HT projection activity did not change significantly during conditioning (n=9 mice) (E), per conditioning CS (baseline-subtracted) (n=9 mice) (F), during extinction (n=6 mice) (G), or during retrieval (n=9 mice) (H). Traces show mean±SEM of z-scored dF/F GCaMP fluorescence. Dashed lines at ±1.96 demarcate statistically significant change from baseline (2-tailed, α=0.05).
(I-K) DRN→NA 5-HT projection activity during the previous conditioning session did not correlate with freezing behaviour during extinction (n=6 mice) (I). DRN→NA 5-HT projection activity during the previous extinction session did not correlate with freezing behaviour during retrieval (n=6 mice) (J). DRN→NA 5-HT projection activity did not correlate with freezing behaviour during retrieval (n=9 mice) (K).
(L1-L3) YFP fluorescence in DRN→BA 5-HT projections did not change significantly during conditioning (L1), extinction (L2), or retrieval (L3). Traces show mean±SEM of z-scored dF/F YFP fluorescence. Dashed lines at ±1.96 demarcate statistically significant change from baseline (2-tailed, α=0.05).
(M1-M3) YFP fluorescence in DRN→NA 5-HT projections did not change significantly during conditioning (M1), extinction (M2), or retrieval (M3). Traces show mean±SEM of z-scored dF/F YFP fluorescence. Dashed lines at ±1.96 demarcate statistically significant change from baseline (2-tailed, α=0.05).
AUC—area under the curve, BL—baseline.

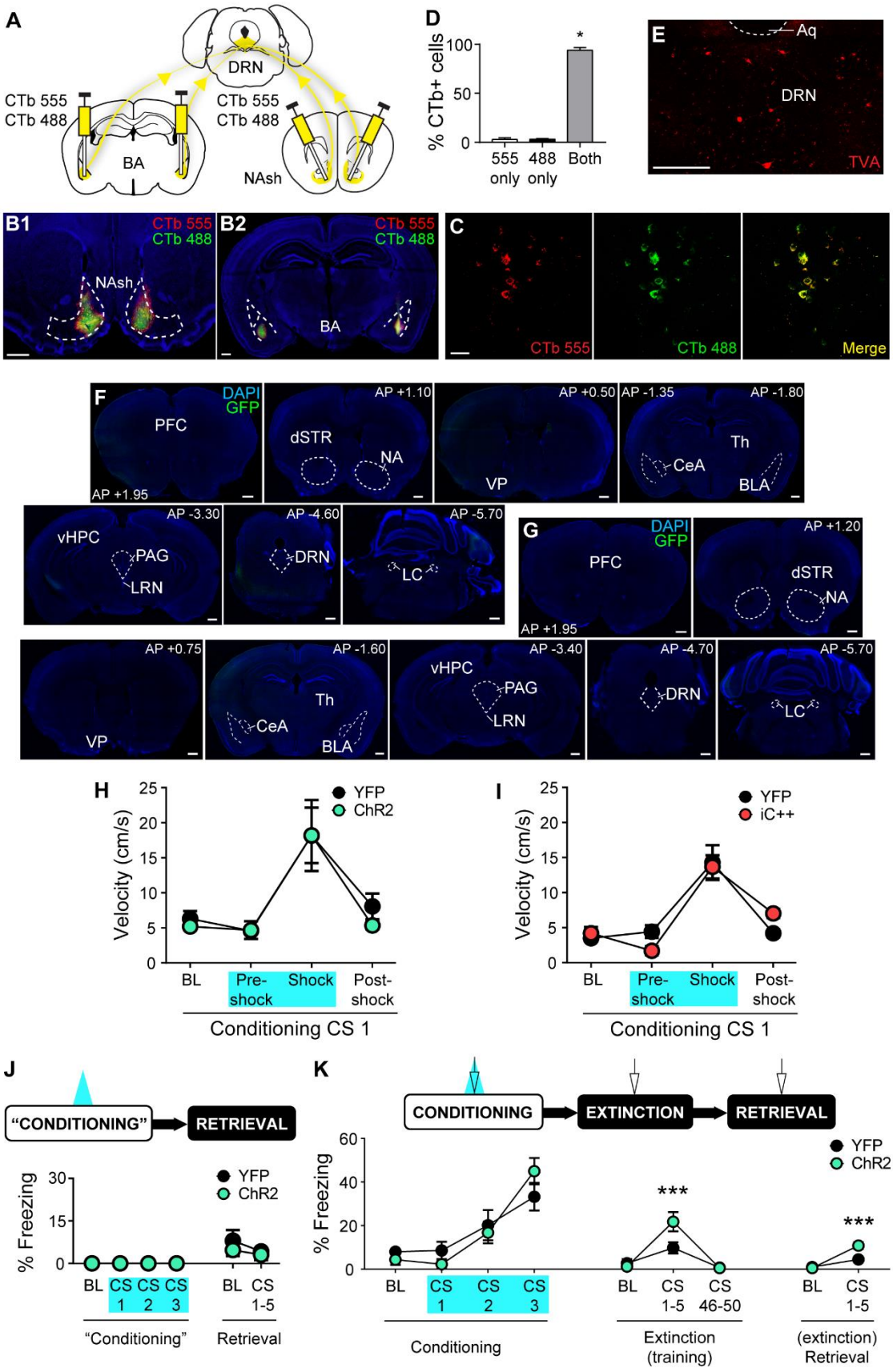
A
DRN
CTb 555
CTb 488
BA
NAsh
D
% CTb+ cells
555 only
488 only
Both
E
Aq
DRN
TVA
B1
B2
C
CTb 555
CTb 488
NAsh
BA
Merge
F
DAPI
GFP
PFC
dSTR
NA
VP
Th
CeA
BLA
vHPC
PAG
LRN
LC
G
H
I
YFP
ChR2
iC++
Velocity (cm/s)
BL
Pre-shock
Shock
Post-shock
Conditioning CS 1
J
"CONDITIONING"
RETRIEVAL
% Freezing
"Conditioning"
Retrieval
K
CONDITIONING
EXTINCTION
RETRIEVAL
Conditioning
Extinction (training)
(extinction) Retrieval

Figure S3, related to Figures 2, 3, 4, 5, 6

Control experiments for anatomical and functional investigation of DRN 5-HT projection pathways

(A-C) Bilateral dual CTb (555/488) injections into BA and NA (A). CTb (555/488) in NA (B1) and BA (B2). Scale bars=500 µm. Overlay of dual CTb-labelled cells in DRN (C). Scale bar=40 µm.

(D) CTb 555 and 488 infected the same cells (n=2 mice). Data are mean±SEM. *p<0.05 compared to theoretical zero.

(E) TVA expression enabling rabies tracing in DRN 5-HT neurons. Scale bar=250 µm.

(F-G) No GFP-expressing neurons with cTRIO tracing lacking rabies starter proteins in DRN 5-HT projection pathways to BA (F) and NA (G). Scale bars=500 µm.

(H-I) Locomotor activity during conditioning CS1 was not affected by laser stimulation in ChR2- (YFP n=8 mice, ChR2 n=8 mice) (H) or iC++-expressing (YFP n=8 mice, iC++ n=7 mice) (I) mice compared to YFP controls. Data are mean±SEM.

(J) Pseudo fear conditioning with 20 Hz laser stimulation but without shocks did not elevate freezing (YFP n=9 mice, ChR2 n=9 mice). Data are mean±SEM.

(K) *Top*: 3-day fear learning task with *in vivo* electrophysiological recordings. 20 Hz laser stimulation was delivered at conditioning during CS presentations. *Bottom*: ChR2-expressing mice froze more compared to YFP controls during extinction and retrieval (YFP n=9 mice, ChR2 n=9 mice). Data are mean±SEM.

Aq—aqueduct, BL—baseline, BLA—basolateral amygdala, CeA—central amygdala, dSTR—dorsal striatum, LC—locus coeruleus, LRN—linear raphe nucleus, NAsh—nucleus accumbens shell, PAG—periaqueductal grey, PFC—prefrontal cortex, Th—thalamus, vHPC—ventral hippocampus, VP—ventral pallidum.

*p<0.05, ***p<0.001.

Table S1, related to Figure 2
Distribution of identified inputs onto DRN 5-HT pathways to BA and NA

Brain region	% Labelled input cells to DRN 5-HT pathway			
	BA n=5 mice		NA n=5 mice	
	Mean	SEM	Mean	SEM
Anterior amygdaloid area	0.00	0.00	0.19	0.19
Anterior hypothalamic area	0.00	0.00	0.58	0.58
Basal amygdala	0.01	0.01	0.00	0.00
Bed nucleus of the stria terminalis	2.61	2.61	0.03	0.03
Caudate putamen	0.00	0.00	0.02	0.02
Central amygdala	0.87	0.87	0.03	0.02
Central grey of the pons	0.00	0.00	0.48	0.48
Cerebellar peduncle	0.00	0.00	0.29	0.18
Cerebellum	1.56	1.08	0.16	0.10
Cingulate cortex	0.47	0.47	0.00	0.00
Deep mesencephalic nucleus	2.53	1.10	1.02	0.65
Dorsal raphe nucleus	16.35	5.16	30.39	10.24
Dorsal tenia tecta	0.00	0.00	0.02	0.02
Dorsomedial tegmental area	0.00	0.00	0.85	0.75
Epimicrocellular nucleus	0.00	0.00	0.02	0.02
Gigantocellular reticular nucleus	2.11	1.18	0.40	0.38
Granular insular cortex	0.00	0.00	1.19	1.19
Inferior colliculus	0.00	0.00	0.02	0.02
Infralimbic prefrontal cortex	0.00	0.00	0.02	0.02
Intermediate reticular nucleus	0.00	0.00	0.02	0.02
Interpeduncular nucleus	0.00	0.00	0.97	0.96
Interstitial nucleus of Cajal	0.05	0.05	0.00	0.00
Lateral habenula	0.00	0.00	0.37	0.17
Lateral hypothalamus	0.01	0.01	2.96	1.52
Lateral lemniscus	0.43	0.43	0.00	0.00
Lateral parabrachial nucleus	0.00	0.00	0.24	0.24
Lateral septal nucleus	0.43	0.43	0.05	0.04
Linear raphe nucleus	11.46	4.69	12.38	4.64
Locus coeruleus	7.55	3.17	2.66	1.15
Magnocellular preoptic nucleus	0.43	0.43	0.00	0.00
Mammillary nucleus	0.00	0.00	0.02	0.02
Medial forebrain bundle	2.62	2.61	0.00	0.00
Medial globus pallidus	0.00	0.00	0.01	0.01
Medial lemniscus	0.00	0.00	0.38	0.38
Median raphe nucleus	0.00	0.00	1.19	0.69
Mesencephalic trigeminal nucleus	0.42	0.42	0.00	0.00
Motor root of the trigeminal nerve	0.00	0.00	0.02	0.02
Nucleus accumbens core	1.30	1.30	0.00	0.00
Nucleus accumbens shell	2.51	1.72	4.67	4.19

Nucleus of origin of efferents of the vestibular nerve	0.00	0.00	0.24	0.24
Nucleus of the horizontal limb of the diagonal band	0.00	0.00	0.48	0.48
Nucleus of the lateral lemniscus	0.00	0.00	0.19	0.19
Oculomotor nucleus	1.11	1.11	0.00	0.00
Orbital cortex	0.00	0.00	0.31	0.17
Paracochlear glial substance	0.00	0.00	0.38	0.38
Paralemniscal nucleus	0.00	0.00	0.02	0.02
Parvicellular reticular nucleus	0.00	0.00	0.26	0.23
Pedunculopontine tegmental nucleus	0.87	0.00	0.47	0.19
Periaqueductal grey	25.03	9.13	11.52	4.49
Pontine nucleus	0.87	0.87	0.00	0.00
Pontine reticular nucleus	0.02	0.02	2.27	0.99
Posterior hypothalamic area	0.00	0.00	0.01	0.01
Posterior thalamic nuclear group	0.00	0.00	0.09	0.07
Prelimbic cortex	0.43	0.43	0.00	0.00
Preoptic area	0.00	0.00	0.32	0.17
Primary motor cortex	0.43	0.43	0.24	0.24
Primary somatosensory cortex	1.21	1.21	0.00	0.00
Principal sensory trigeminal nucleus	0.00	0.00	0.03	0.02
Raphe magnus nucleus	0.00	0.00	0.10	0.07
Red nucleus	0.00	0.00	0.01	0.01
Reticulotegmental nucleus of the pons	0.43	0.43	0.25	0.19
Retrosplenial agranular cortex	0.00	0.00	0.01	0.01
Secondary motor cortex	0.00	0.00	0.23	0.18
Substantia nigra pars reticulata	0.43	0.43	4.41	3.11
Superior cerebellar peduncle	2.87	2.10	0.00	0.00
Superior colliculus	9.34	6.20	6.62	3.06
Superior vestibular nucleus	0.00	0.00	0.99	0.94
Tuber cinereum area	0.01	0.01	0.00	0.00
Ventral anterior thalamic nucleus	0.00	0.00	0.02	0.02
Ventral pallidum	0.02	0.02	6.93	2.70
Ventral tegmental area	2.01	1.40	1.01	0.49
Ventromedial hypothalamic nucleus	0.00	0.00	0.24	0.18
Ventromedial thalamic nucleus	0.03	0.03	0.00	0.00
Visual tegmental relay zone	1.11	1.11	0.00	0.00
Zona incerta	0.00	0.00	0.71	0.71

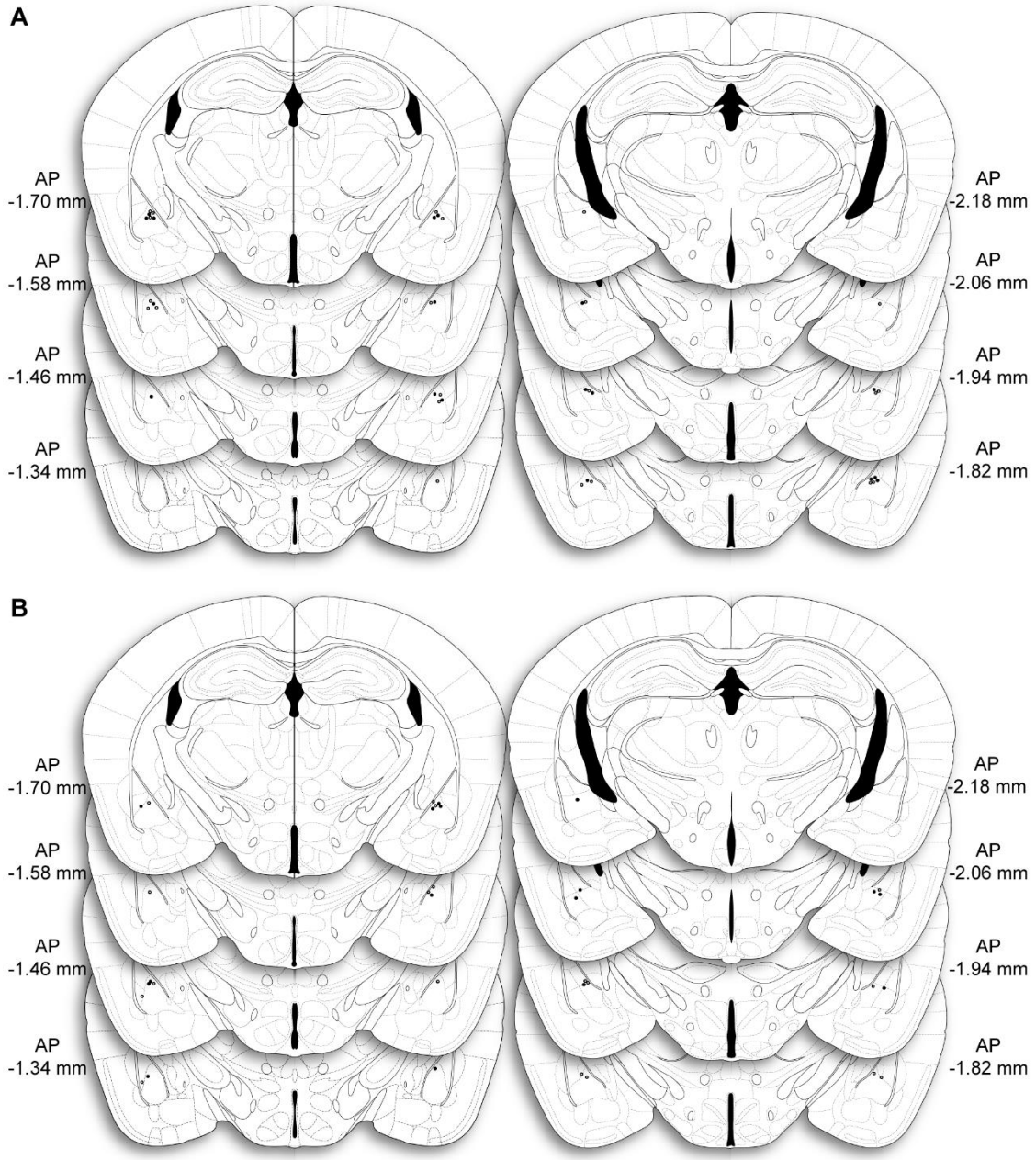

Figure S4, related to Figure 3

BA placements for *in vivo* optogenetics in DRN 5-HT projections during fear conditioning

(A-B) BA placements of optical fibre implants in ChR2- (A) and iC++-injected (B) 5-HTT-Cre mice. Dots demarcate fibre tip placement; solid are from opsin and empty from YFP animals.

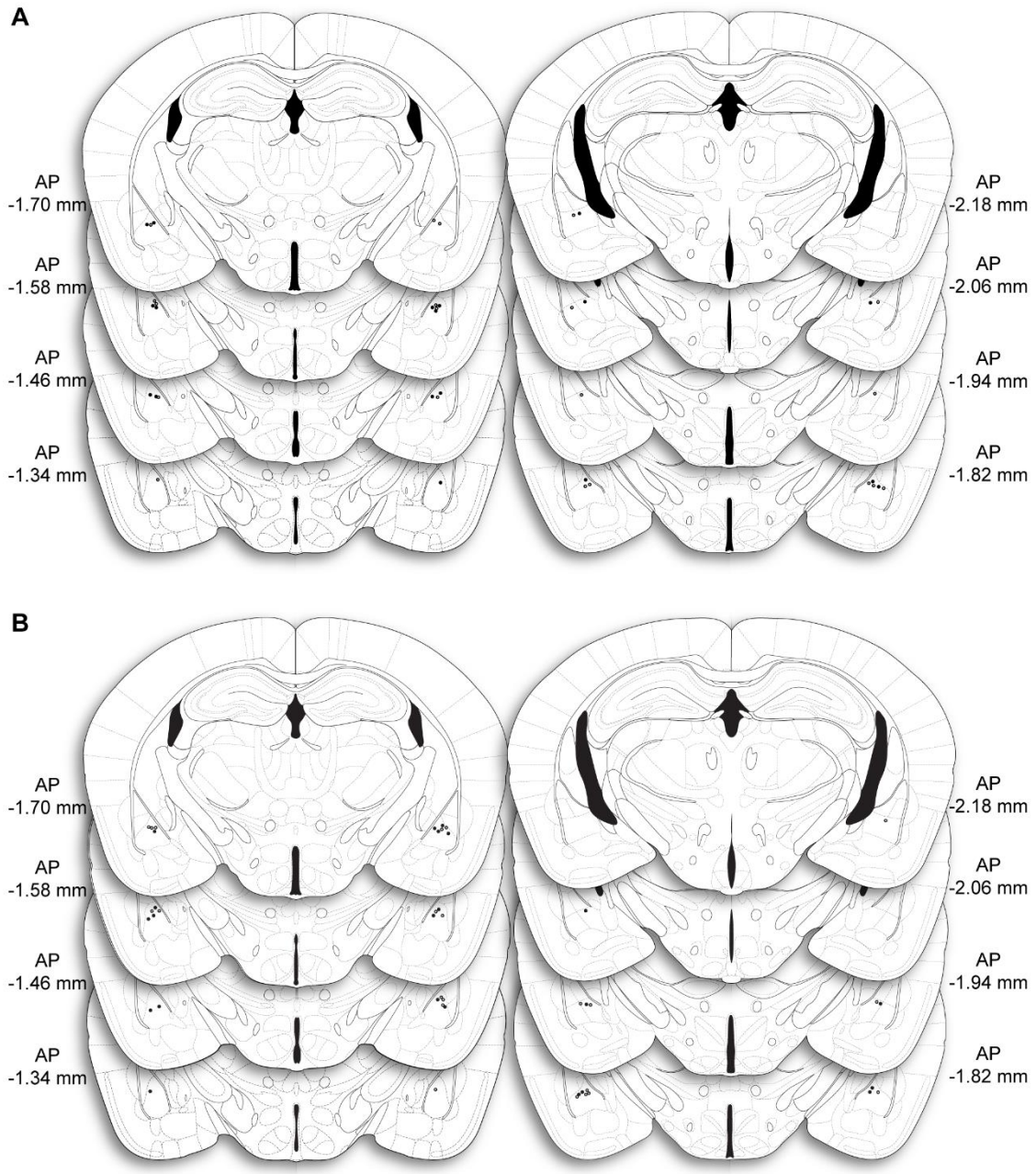

Figure S5, related to Figure 3
BA placements for *in vivo* optogenetics in DRN 5-HT projections during fear extinction
(A-B) BA placements of optical fibre implants in ChR2- (A) and iC++-injected (B) 5-HTT-Cre mice. Dots demarcate fibre tip placement; solid are from opsin and empty from YFP animals.

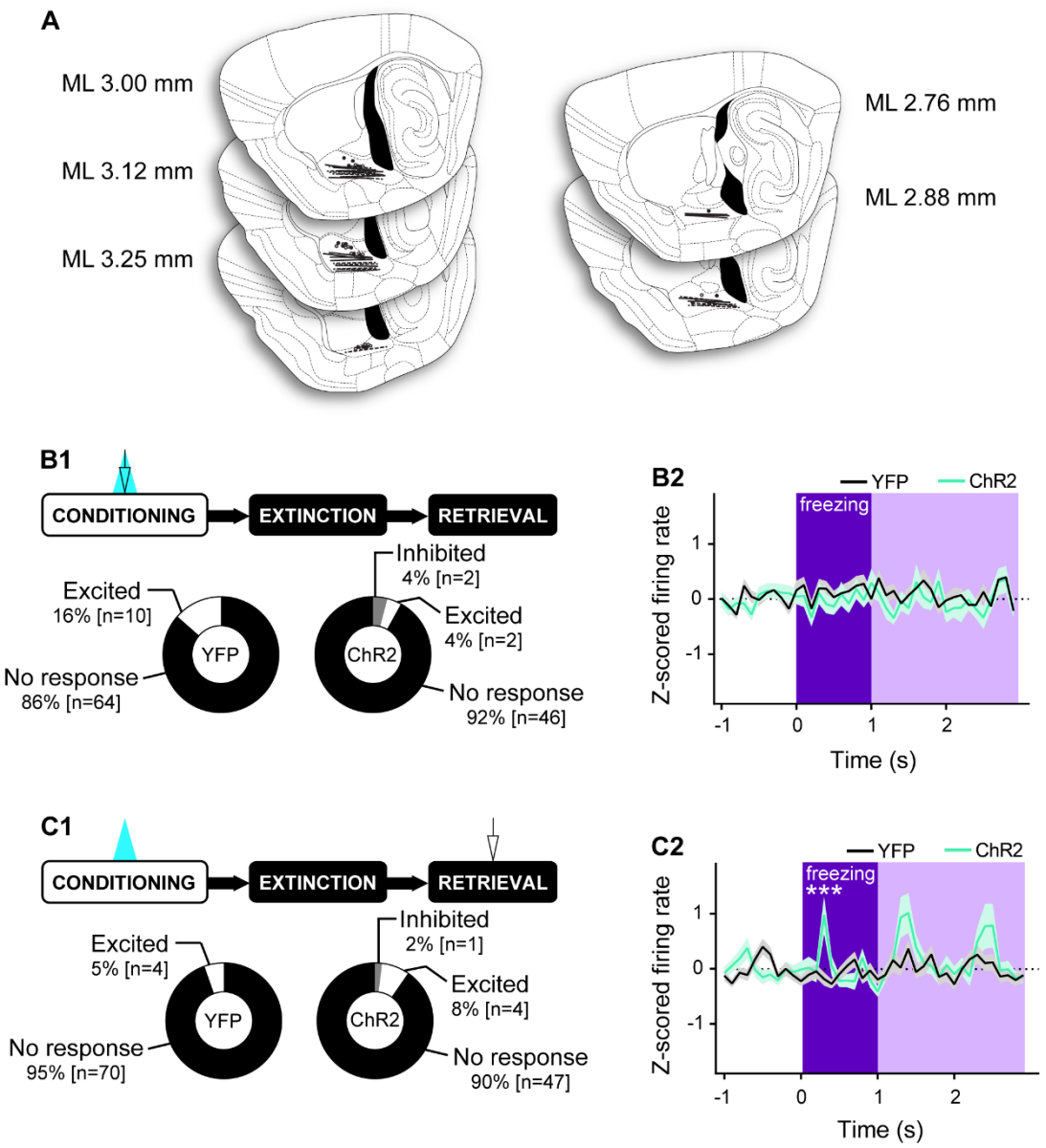
A
ML 3.00 mm
ML 3.12 mm
ML 3.25 mm
ML 2.76 mm
ML 2.88 mm
B1
CONDITIONING
EXTINCTION
RETRIEVAL
Excited
16% [n=10]
No response
86% [n=64]
YFP
Inhibited
4% [n=2]
Excited
4% [n=2]
No response
92% [n=46]
ChR2
B2
YFP
ChR2
freezing
Z-scored firing rate
Time (s)
C1
CONDITIONING
EXTINCTION
RETRIEVAL
Excited
5% [n=4]
No response
95% [n=70]
YFP
Inhibited
2% [n=1]
Excited
8% [n=4]
No response
90% [n=47]
ChR2
C2
YFP
ChR2
freezing
***
Z-scored firing rate
Time (s)

Figure S6, related to Figures 4, 5, 6

DRN→BA 5-HT pathway alters BA neuronal activity during fear behaviour

(A) Sagittal BA placements of optrode array implants in ChR2-injected 5-HTT-Cre mice. Dots demarcate fibre tip placement; solid are from ChR2 and empty from YFP animals. Lines depict electrode array placement; solid are from ChR2 and dashed from YFP animals.

(B1) *Top*: 3-day fear learning task. 20 Hz laser stimulation was delivered at conditioning during CS presentations. *In vivo* electrophysiology data from conditioning. *Bottom*: No difference in proportion of BA single unit firing during freezing onset (YFP n=74 units, 9 mice; ChR2 n=50 units, 8 mice).

(B2) No difference between firing rates of BA single units in ChR2 vs. YFP mice during freezing onset (YFP n=74 units, 9 mice; ChR2 n=50 units, 8 mice). Traces show mean±SEM of z-scored firing rate. ±1.96 represents statistically significant change from baseline (2-tailed, $\alpha=0.05$).

(C1) *Top*: 3-day fear learning task. 20 Hz laser stimulation was delivered at conditioning during CS presentations. *In vivo* electrophysiology data from retrieval. *Bottom*: No difference in proportion of BA single unit firing during freezing onset (YFP n=74 units, 8 mice; ChR2 n=52 units, 9 mice).

(C2) Firing rates of BA single units in ChR2-expressing mice was higher than that of YFP controls during freezing onset (YFP n=74 units, 8 mice; ChR2 n=50 units, 8 mice). Traces show mean±SEM of z-scored firing rate. ±1.96 represents statistically significant change from baseline (2-tailed, $\alpha=0.05$).

***$p<0.001$.

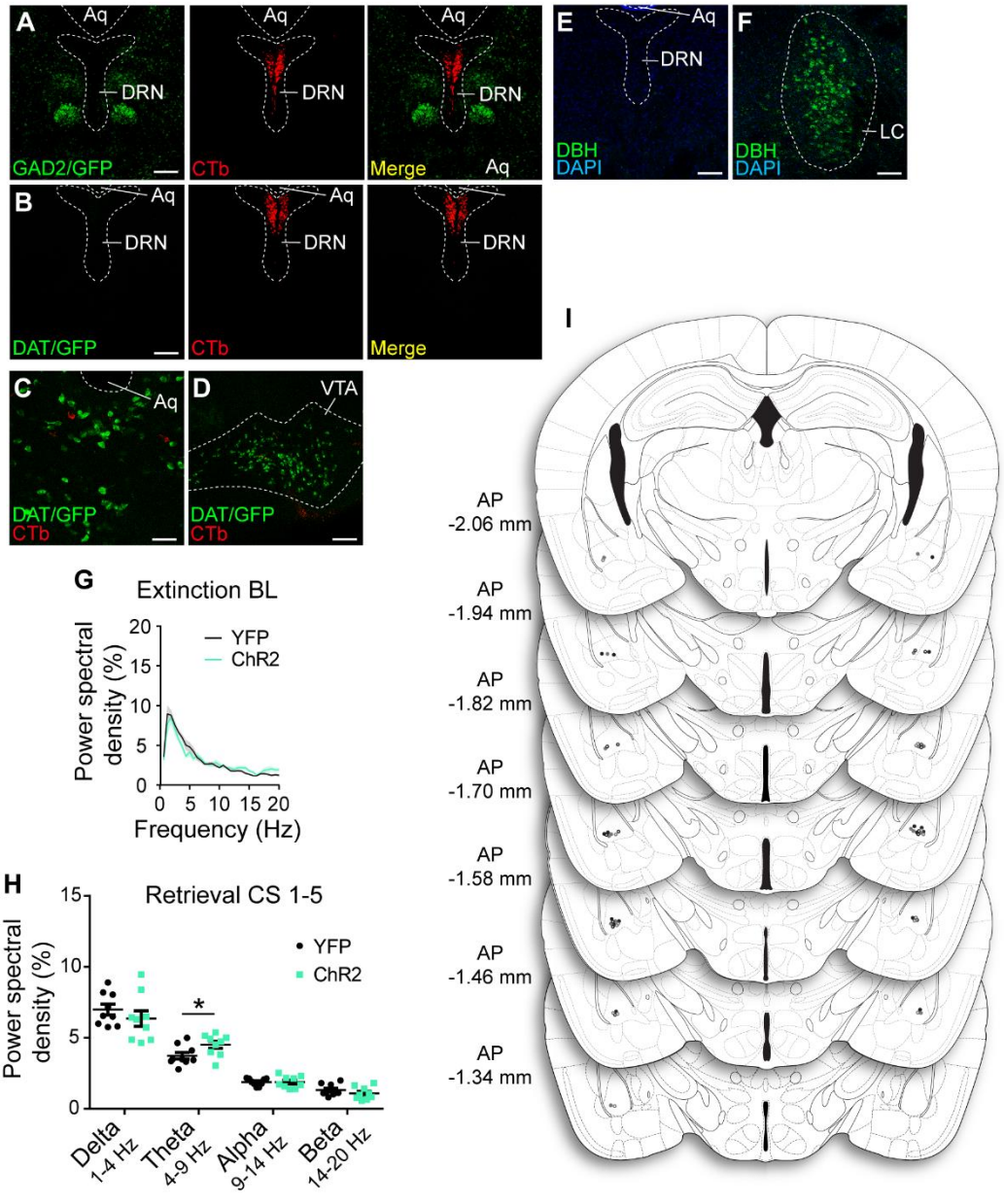
A
Aq
DRN
GAD2/GFP
CTb
Merge
B
DAT/GFP
C
D
VTA
E
DBH
DAPI
F
LC
G
Extinction BL
YFP
ChR2
Power spectral density (%)
Frequency (Hz)
H
Retrieval CS 1-5
Delta 1-4 Hz
Theta 4-9 Hz
Alpha 9-14 Hz
Beta 14-20 Hz
I
AP -2.06 mm
AP -1.94 mm
AP -1.82 mm
AP -1.70 mm
AP -1.58 mm
AP -1.46 mm
AP -1.34 mm

Figure S7, related to Figures 5, 6, 7

Anatomical and functional investigation of DRN→BA 5-HT pathway

(A-B) Overlay of CTb+ and GAD2- (A) or DAT-expressing (B) cells in middle/caudal DRN. Scale bars=150 µm.

(C-D) CTb+ and DAT-expressing cells in rostral DRN (C) and VTA (D). Scale bars=50 µm (C) and 150 µm (D).

(E-F) DBH+ (noradrenergic) cells in DRN (E) and locus coeruleus (F). Scale bars=150 µm (E) and 50 µm (F).

(G) Power spectral densities of BA local field potentials during extinction baseline (YFP n=9 mice, ChR2 n=8 mice). Data are mean±SEM.

(H) Mean power spectral densities of BA local field potentials in delta, theta, alpha, and beta oscillation ranges during retrieval CS presentations (YFP n=9 mice, ChR2 n=9 mice). Data are mean±SEM.

(I) BA placements of opto-fluid cannula implants in ChR2-injected 5-HTT-Cre mice. Dots demarcate infusion site; solid are from ChR2 and empty from YFP animals, black are from drug-treated and grey from vehicle-treated animals.

Aq—aqueduct, LC—locus coeruleus, VTA—ventral tegmental area.

*p<0.05.

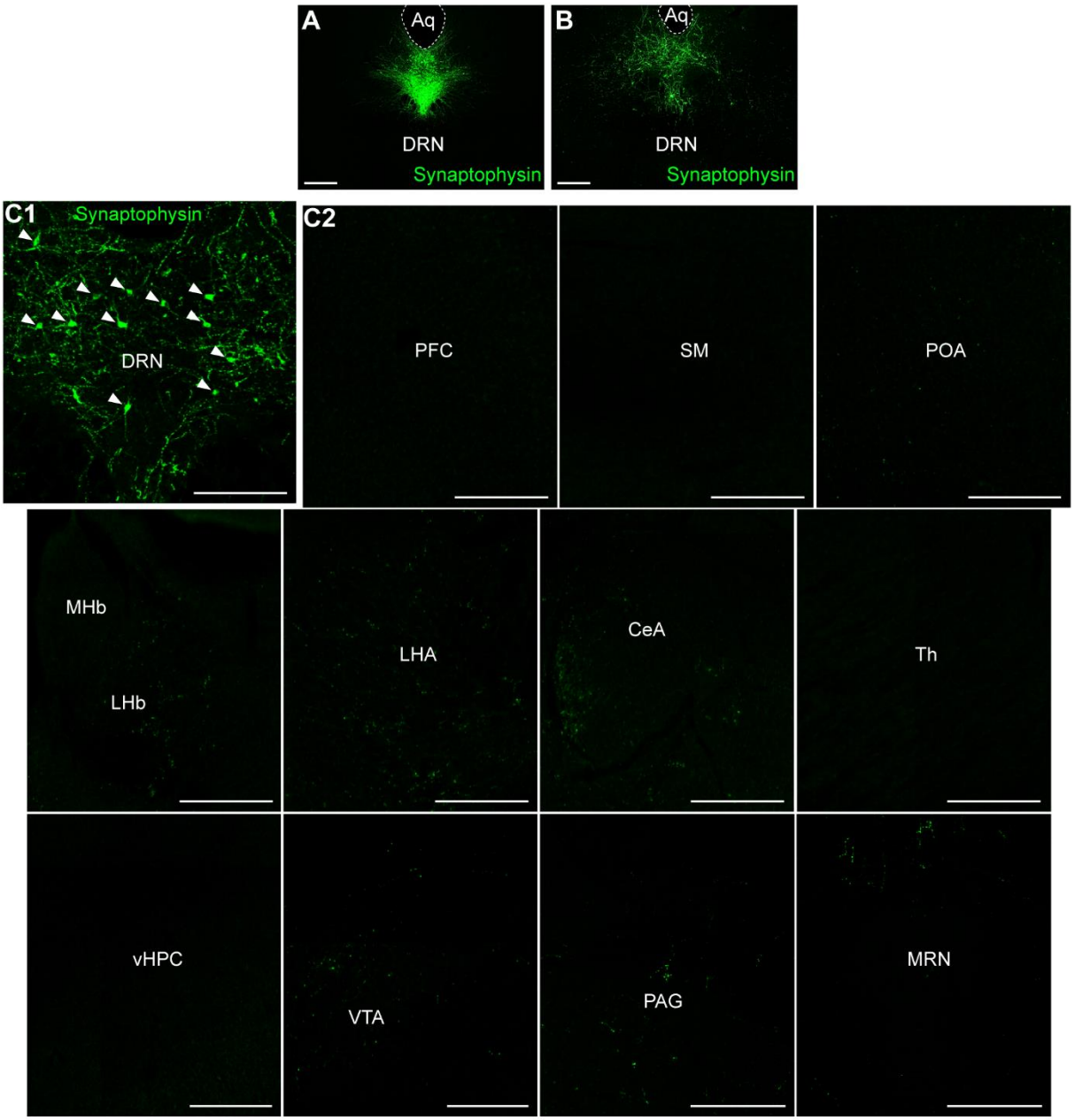

Figure S8, related to Figure 8
Anatomical specificity of DRN→BA 5-HT pathway
(A-B) Cre-dependent fluorescent synaptophysin expression in DRN of 5-HTT-Cre mice (A) or wild-type mice injected with retro Cre in BA (B). Scale bars=250 µm.
(C) In wild-type mice injected with retro Cre in BA and Cre-dependent fluorescent synaptophysin in DRN, cell bodies were transfected in DRN (C1), but not in other brain regions (C2). Scale bars=250 µm.
Aq—aqueduct, CeA—central amygdala, LC—locus coeruleus, LHA—lateral hypothalamus, LHb—lateral habenula, MHb—medial habenula, MRN—median raphe nucleus, PAG—periaqueductal grey, PFC—prefrontal cortex, POA—preoptic area, SM—sensory/motor cortices, Th—thalamus, vHPC—ventral hippocampus, VTA—ventral tegmental area.