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Supplementary information

Deconstruction of a spino-brain–spinal cord circuit that drives chronic pain

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Supplementary information for

Deconstruction of a spino-brain-spinal cord circuit drives chronic pain

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Supplementary Fig. 1 Canula placement in the RVM or lSuC and virus expression in the PAG, lSuC, SS, MO, and DCN for chemogenetic manipulation of the PAG→RVM, lSuC→RVM, SS→lSuC, MO→lSuC pathways, and VPL-projecting DCN neurons.

Supplemental note: Strategies for Genetic Targeting of Oprm1-Expressing Cells

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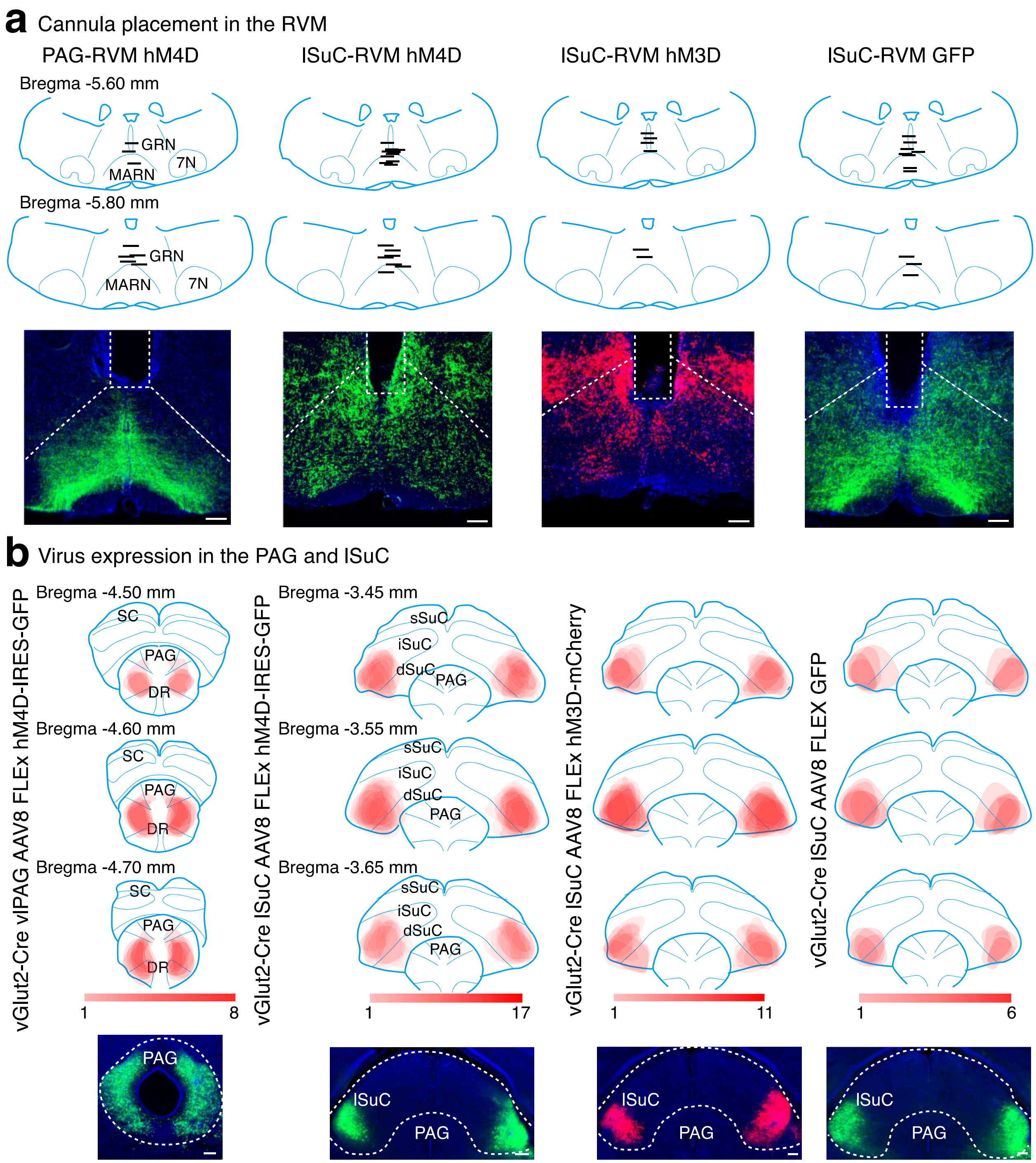
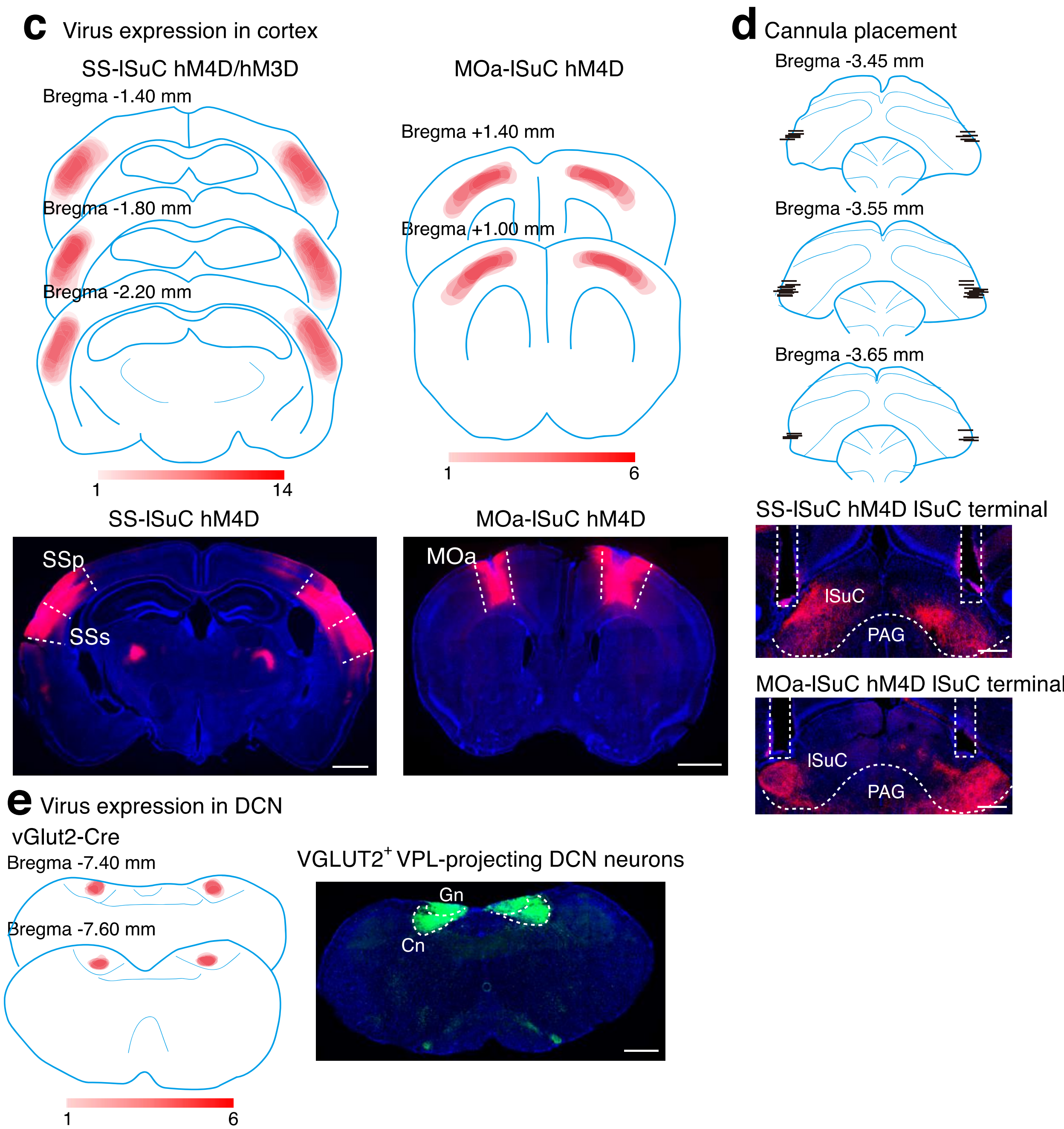
Supplementary Fig.1





Supplementary Fig. 1 Canula placement in the RVM or lSuC and virus expression in the PAG, lSuC, SS, MO, and DCN for chemogenetic manipulation of the PAG→RVM, lSuC→RVM, SS→lSuC, MO→lSuC pathways, and VPL-projecting DCN neurons. (**a**) Canula placement in the RVM. Scale bar: 200 μm. GRN: gigantocellular reticular nucleus; MARN: magnocellular reticular nucleus, 7N: 7th nucleus. (**b**) Virus expression in the PAG and lSuC. Scale bar: 200 μm. SuC: superior colliculus; sSuC: superficial SuC; iSuC: intermediate SuC;

dSuC: deep SuC; PAG: periaqueductal gray; DR: dorsal raphe. Related to experiments in Fig. 3. (**c**) Virus expression and (**d**) cannula placement for manipulating the SS→lSuC or MO→lSuC pathway. Scale bar: 1 mm (**c**), 500 μm (**d**). SS: somatosensory cortex; MO: motor cortex. Related to experiments in Fig. 4. (**e**) Virus expression for manipulating the DCN→VPL pathway. Scale bar: 500 μm. Gn: Gracile nucleus; Cn: Cuneate nucleus. Related to experiments in Fig. 5.

Supplemental note: Strategies for Genetic Targeting of Oprm1-Expressing Cells

Several approaches have been developed to achieve precise genetic manipulation of μ-opioid receptor (MOR/Oprm1)-expressing cells.

1. The Chen lab's Oprm1Cre knock-in/knock-out line (current manuscript).

The Cre recombinase cDNA, followed by the rabbit β-globin poly-A signal, was introduced via homologous recombination immediately after the start codon in exon 1 of the mouse Oprm1 gene. This line allows Cre expression under control of endogenous Oprm1 promotor. Heterozygous mice exhibit normal phenotype, whereas homozygotes lack functional μ-opioid receptor expression and do not respond to endogenous or exogenous μ-opioid receptor ligands.

2. The Palmiter lab's Oprm1Cre knock-in/knock-out line (Liu et al., 2021)

The cDNA of Cre recombinase and GFP fusion protein, followed by the rabbit β-globin poly-A signal, was introduced via homologous recombination immediately after the start codon in exon 1 of the mouse Oprm1 gene. This line allows expression of Cre:GFP fusion protein under control of endogenous Oprm1 promotor. Heterozygous mice exhibit normal phenotype, whereas homozygotes lack functional μ-opioid receptor expression and do not respond to endogenous or exogenous μ-opioid receptor ligands.

3. The Meletis lab's Oprm1-2A-Cre mice (Martin et al., 2019)

A T2A-Cre cassette was inserted into the fifth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of Cre recombinase under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

4. The Kieffer lab's Oprm1-2A-Cre:eGFP mice (Bailly et al., 2020)

A T2A-Cre:eGFP fusion protein cassette was inserted into the fourth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of Cre:eGFP fusion protein under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

5. The Watanabe lab's Oprm1-2A-CreER mice (Okunomiya et al., 2020)

A T2A-CreER cassette was inserted into the fourth exon of the Oprm1 gene and replacing a TAA stop codon by homologous recombination. This line allows expression of a tamoxifen-inducible Cre recombinase under control of endogenous Oprm1 promotor. The endogenous μ-opioid receptor expression has not been disrupted.

6. The Corder lab's Oprm1 targeting virus (Salimando et al., 2023)

The researcher developed a new promotor system for viral-assisted, species-specific, selective targeting of OPRM1$^+$ cells. They cloned genomic sequence spanning -1797 to -265bp upstream of the ATG site of the mouse *Oprm1* gene into plasmids encoding various reporter, then packaged these constructs into AAV1 vectors (AAV1-mMORp1-reporter). The resulting mMORp construct exhibit robust and specific expression in endogenous MOR+ neurons across the mouse central and peripheral nervous systems. This specificity was confirmed through co-localization with MOR protein, *Oprm1* mRNA, and Cre-recombinase in transgenic lines, with minimal off-target glial expression. Remarkably, the mMORp system also functioned effectively in rats, shrews, and human iPSC-derived nociceptors, but not in non-neuronal cells (like cardiomyocytes) with low receptor expression. Similarly, the human construct, hMORp1 (-1841 to -257bp upstream of the human *OPRM1*ATG site), enabled efficient and selective transduction of OPRM1^{+} neurons in rhesus macaques. This versatile toolkit facilitates precise targeting and manipulation of opioidergic cells across multiple species. It supports diverse transgenes (reporters, sensors, actuators) and is compatible with various AAV serotypes optimized for different delivery routes and tissues.

Statistical analysis table

Figure	Groups	Statistical Analysis
Fig. 1f	Baseline vs. DAMGO (6 cells from 6 mice) Baseline vs. DAMGO + CTAP (5 cells from 5 mice)	Wilcoxon matched-pairs signed-rank test Baseline vs. DAMGO: *P* = 0.0313 Baseline vs. DAMGO + CTAP: *P* = 0.4375
Fig. 1i	Pre, day2, 7, 14, and 28 post SNI (n = 8, 5 males, 3 females)	Nonparametric ANOVA (Friedman test) Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli Pre vs. D2: *P* = 0.1004 Pre vs. D7: *P* = 0.0005 Pre vs. D14: *P* = 0.0005 Pre vs. 28: *P* = 0.0038 D2 vs. D7: *P* = 0.0493 D2 vs. D14: *P* = 0.0586 D2 vs. D28: *P* = 0.1625 D7 vs. D14: *P* = 0.6427 D7 vs. D28: *P* = 0.3943 D14 vs. D28: *P* = 0.4305
Fig. 1j	Locomotion (SNI) Escape (SNI)	Pearson correlation Average Speed vs average %ΔF/F (SNI) Locomotion: Pearson r = -0.8212 95% Confidence Intervals -0.8949 to -0.7036 Locomotion: Pearson r = 0.9188 95% Confidence Intervals 0.8606 to 0.9534
Fig. 2b	hM4D Von Frey saline vs. CLZ (n = 8, 4 males, 4 females) hM3D Von Frey saline vs. CLZ (n = 10, 5 males, 5 females) hM4D Plantar Cold saline vs. CLZ (n = 9, 5 males, 4 females) hM3D Plantar Cold saline vs. CLZ (n = 9, 5 males, 4 females)	Wilcoxon matched-pairs signed rank test hM4D Von Frey saline vs. CLZ: *P* = 0.0078 hM3D Von Frey saline vs. CLZ: *P* = 0.9004 hM4D Plantar Cold saline vs. CLZ: *P* = 0.0039 hM3D Plantar Cold saline vs. CLZ: *P* = 0.4258
Fig. 2d	Control (n = 7, 3 males + 4 females) Caspase (n = 8, 4 males + 4 females)	Mann-Whitney test Pre-ablation control vs. caspase: *P* = 0.2146 Post-ablation control vs. caspase: *P* = 0.5175

		2d post SNI control vs. caspase: *P* = 0.0003 7d post SNI control vs. caspase: *P* = 0.0003 14d post SNI control vs. caspase: *P* = 0.0003 28d post SNI control vs. caspase: *P* = 0.0003
Fig. 2e	Control (n = 7, 4 males + 3 females) Caspase (n = 8, 4 males + 4 females)	Mann-Whitney test Pre-ablation control vs. caspase: *P* = 0.0252 Post-ablation control vs. caspase: *P* = 0.11 1d post CFA control vs. caspase: *P* = 0.0003 2d post SNI control vs. caspase: *P* = 0.0003 3d post SNI control vs. caspase: *P* = 0.0003
Fig. 2f	CLZ-mCherry (n = 8, 4 males + 4 females) CLZ hM4D (n = 8, 4 males + 4 females)	Mann-Whitney test Pre SNI CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0103 2D post SNI CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0002 7D post SNI CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0002 14D post SNI CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0002 28D post SNI CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0002
Fig. 2g	SNI CLZ-mCherry (n = 8, 4 males + 4 females) SNI hM4D-mCherry (n = 9, 5 males + 4 females)	Mann-Whitney test SNI CLZ-mCherry vs. SNI hM4D-mCherry: *P* < 0.0001
Fig. 2h	SNI CLZ-mCherry (n = 5, 3 males + 2 females) SNI hM4D-mCherry (n = 5, 3 males + 2 females)	Mann-Whitney test SNI CLZ-mCherry vs. SNI hM4D-mCherry: *P* = 0.0159
Fig. 2i	mCherry (n = 9, 5 males + 4 females) hM3D (n = 5, 3 males + 2 females)	Mann-Whitney test Pre CLZ-mCherry vs. hM3D: *P* = 0.5884 1D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.3452 2D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.001 3D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.001 5D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.001 7D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0005 7D post CLZ-mCherry vs. CLZ-hM3D: *P*= 0.001 14D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.001 21D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.001 Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test hM3D Pre vs. D1 on CLZ: *P* > 0.9999

		hM3D Pre vs. D2 on CLZ: $P > 0.9999$ hM3D Pre vs. D3 on CLZ: $P = 0.5174$ hM3D Pre vs. D5 on CLZ: $P = 0.1225$ hM3D Pre vs. D7 on CLZ: $P = 0.0146$ hM3D Pre vs. D7 post CLZ: $P = 0.0177$ hM3D Pre vs. D14 post CLZ: $P = 0.0374$ hM3D Pre vs. D21 post CLZ: $P = 0.0311$
Fig. 3b	Saline vs. CLZ (n = 8, 4 males + 4 females)	Wilcoxon matched-pairs signed rank test Pre SNI saline vs. CLZ: $P = 0.5884$ 2d post SNI saline vs. CLZ: $P > 0.9999$ 14d post SNI saline vs. CLZ: $P = 0.7422$
Fig. 3g	Saline vs. CLZ (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test Pre SNI saline vs. CLZ: $P = 0.5625$ 2d post SNI saline vs. CLZ: $P = 0.0313$ 14d post SNI saline vs. CLZ: $P = 0.0313$
Fig. 3h	hM3D (n = 6, 4 males + 2 females)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test hM3D Pre vs. D1 on CLZ: $P > 0.9999$ hM3D Pre vs. D2 on CLZ: $P > 0.9999$ hM3D Pre vs. D3 on CLZ: $P = 0.0300$ hM3D Pre vs. D5 on CLZ: $P = 0.0059$ hM3D Pre vs. D7 on CLZ: $P = 0.0033$ hM3D Pre vs. D7 post CLZ: $P = 0.0022$ hM3D Pre vs. D14 post CLZ: $P = 0.0059$ hM3D Pre vs. D21 post CLZ: $P = 0.0150$
Fig. 3i	CLZ-GFP (n = 5, 3 males + 2 females) CLZ-hM4D (n = 5, 3 males + 2 females)	Mann-Whitney test CLZ-GFP vs. CLZ-hM4D phase I: $P > 0.9999$ CLZ-GFP vs. CLZ-hM4D phase II: $P = 0.8413$
Fig. 4c	Saline vs. CLZ (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test Pre SNI saline vs. CLZ : $P > 0.9999$ 2d post SNI saline vs. CLZ : $P = 0.25$ 14d post SNI saline vs. CLZ : $P = 0.5$
Fig. 4d	Saline vs. CLZ (n = 7, 4 males + 3 females)	Wilcoxon matched-pairs signed rank test Pre SNI saline vs. CLZ: $P = 0.8438$ 2d post SNI saline vs. CLZ: $P = 0.0156$ 14d post SNI saline vs. CLZ: $P = 0.0156$
Fig. 4e	CLZ-mCherry (n = 6, 3 males + 3 females) CLZ-hM4D (n = 7, 4 males + 3 females)	Mann-Whitney test CLZ-mCherry vs. CLZ-hM4D: $P = 0.0012$

Fig. 4f	Control (4 male + 3 female) Ablation (5 male + 3 female)	Mann-Whitney test Pre CLZ-control vs. Pre CLZ-ablation: *P* = 0.0609 1D on CLZ-control vs. CLZ-ablation: *P* = 0.0031 2D on CLZ-control vs. CLZ-ablation: *P* = 0.0003 3D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0003 5D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0003 7D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0003 7D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0003 14D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0002 21D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0003 Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test control pre vs. D1 on CLZ: *P* > 0.9999 control Pre vs. D2 on CLZ: *P* = 0.2544 control Pre vs. D3 on CLZ: *P* = 0.0121 control Pre vs. D5 on CLZ: *P* = 0.0035 control Pre vs. D7 on CLZ: *P* = 0.0121 control Pre vs. D7 post CLZ: *P* = 0.0005 control Pre vs. D14 post CLZ: *P* = 0.0051 control Pre vs. D21 post CLZ: *P* = 0.0043
Fig. 4g	CLZ-mCherry (n = 6, 3 males + 3 females) CLZ-hM3D (n = 7, 4 males + 3 females)	Mann-Whitney test CLZ-mCherry vs. CLZ-hM3D: P = 0.0012
Fig. 5c	SC-VPL Saline vs. CLZ (n = 7, 4 males + 3 females) SC-PO Saline vs. CLZ (n = 5, 3 males + 2 females)	Wilcoxon matched-pairs signed rank test VPL Pre SNI saline vs. CLZ: *P* = 0.6250 2d post SNI saline vs. CLZ: *P* = 0.0625 14d post SNI saline vs. CLZ: *P* = 0.0625 Po Pre SNI saline vs. CLZ: *P* > 0.9999 2d post SNI saline vs. CLZ: *P* > 0.9999 14d post SNI saline vs. CLZ: *P* > 0.9999
Fig. 5d	SC-VPL+Po Saline vs.CLZ (n = 7, 4 males + 3 females)	Pre SNI saline vs. CLZ: *P* = 0.8125 2d post SNI saline vs. CLZ: *P* = 0.0156 14d post SNI saline vs. CLZ: *P* = 0.0156
Fig. 5e	VPL+PO hM4D (n = 5, 3 males + 2 females) VPL hM4D (n = 6, 3 males + 3 females) Po hM4D (n = 5, 3 males + 2 females)	One-way ANOVA, followed by Tukey's multiple comparisons test VPL+Po hM4D vs. SpC to VPL hM4D: *P* < 0.0001 VPL+Po hM4D vs. SpC to PO hM4D: *P* = 0.0003 VPL hM4D SNI vs. Po hM4D SNI: *P* = 0.0471

Fig. 5f	Po hM3D (n = 5, 3 males + 2 females) VPL hM3D (n = 5, 3 males + 2 females)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test Po hM3D Pre vs. D1 on CLZ: *P* > 0.9999 hM3D Pre vs. D2 on CLZ: *P* > 0.9999 hM3D Pre vs. D3 on CLZ: *P* =0.3972 hM3D Pre vs. D5 on CLZ: *P* = 0.0146 CLZ-hM3D Pre vs. D7 on CLZ: *P* = 0.0028 hM3D Pre vs. D7 post CLZ: *P* = 0.0022 hM3D Pre vs. D14 post CLZ: *P* = 0.0007 hM3D Pre vs. D21 post CLZ: *P* = 0.0374 VPL hM3D Pre vs. D1 on CLZ: *P* > 0.9999 hM3D Pre vs. D2 on CLZ: *P* > 0.9999 hM3D Pre vs. D3 on CLZ: *P* = 0.0750 hM3D Pre vs. D5 on CLZ: *P* = 0.0018 CLZ-hM3D Pre vs. D7 on CLZ: *P* = 0.0080 hM3D Pre vs. D7 post CLZ: *P* = 0.0034 hM3D Pre vs. D14 post CLZ: *P* = 0.0146 hM3D Pre vs. D21 post CLZ: *P* = 0.0374
Fig. 5g	Control (n = 5, 3 males + 2 females) VPL hM4D (n = 5, 3 males + 2 females) PO hM4D (n = 5, 3 males + 2 females)	Nonparametric ANOVA (Kruskal-Wallis test), Dunn's multiple comparison test Cntl vs. VPL hM3D: *P* = 0.0265 Cntl vs. Po hM3D: *P* = 0.0215 VPL hM3D vs. Po hM3D: *P* > 0.9999
Fig. 5h	VPL+Po hM4D (n = 5, 3 males + 2 females) DCN-VPL hM4D (n = 6, 3 males + 3 females) WT (n = 5, 3 males + 2 females)	Wilcoxon matched-pairs signed rank test VPL+Po hM4D saline vs. CLZ: *P* = 0.0625 DCN-VPL hM4D saline vs. CLZ: *P* = 0.0313 WT saline vs. CLZ: *P* = 0.1250
Extended Data Fig. 1b	WT saline vs. 2.5 mg/Kg morphine vs. 5 mg/kg morphine (n = 5, 3 males, 2 females)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test WT Saline vs. 2.5 mg/kg morphine: *P* = 0.1733 WT Saline vs. 5 mg/kg morphine: *P* = 0.0133

	Oprm1Cre/+ saline vs. 2.5 mg/Kg morphine vs. 5 mg/kg morphine (n = 5, 3 males, 2 females) Oprm1Cre/Cre saline vs. 2.5 mg/Kg morphine vs. 5 mg/kg morphine (n = 5, 3 males, 2 females)	WT 2.5 mg/kg morphine vs. 5 mg/kg morphine: $P > 0.9999$ Oprm1Cre/+ Saline vs. 2.5 mg/kg morphine: $P = 0.6177$ Oprm1Cre/+ Saline vs. 5 mg/kg morphine: $P = 0.0080$ Oprm1Cre/+ 2.5 mg/kg morphine vs. 5 mg/kg morphine: $P = 0.2460$ Oprm1Cre/Cre Saline vs. 2.5 mg/kg morphine: $P > 0.9999$ Oprm1Cre/Cre Saline vs. 5 mg/kg morphine: $P > 0.9999$ Oprm1Cre/Cre 2.5 mg/kg morphine vs. 5 mg/kg morphine: $P > 0.9999$
Extended Data Fig. 1c	WT saline vs. 15 mg/Kg morphine (n = 6, 3 males, 3 females) Oprm1Cre/+ saline vs. 15 mg/Kg morphine (n = 6, 3 males, 3 females) Oprm1Cre/Cre saline vs. 15 mg/Kg morphine (n = 5, 3 males, 2 females)	Wilcoxon matched-pairs signed rank test WT saline vs. 15 mg/Kg morphine: $P = 0.0313$ Oprm1Cre/+ saline vs. 15 mg/Kg morphine: $P = 0.0313$ Oprm1Cre/Cre saline vs. 15 mg/Kg morphine: $P = 0.0625$
Extended Data Fig. 4b	Normal nonwithdrawal vs. withdrawal (n = 6, 4 males, 2 females) SNI nonwithdrawal vs. withdrawal: (n = 6, 3 males, 3 females)	Paired t test Normal nonwithdrawal vs. withdrawal: $P = 0.006$ SNI nonwithdrawal vs. withdrawal: $P = 0.0017$
Extended Data Fig. 4c	Von Frey normal (n = 7, 4 males, 3 females) vs. SNI (n = 8, 5 males, 3 females) Hargreaves normal (n = 7, 4 males, 3 females) vs. SNI (n = 7, 4 males, 3 females) Plantar cold normal (n = 7, 4 males, 3 females) vs. SNI (n = 7, 4 males, 3 females)	Mann-Whitney test Von Frey normal vs. SNI: $P = 0.0003$ Hargreaves normal vs. SNI: $P = 0.62$ Plantar cold normal vs. SNI: $P = 0.0041$

Extended Data Fig. 4e	Von Frey Pre, day2, 7, 21 post CFA Hargreaves Pre, day2, 7, 21 post CFA (n = 6, 3 males, 3 females for all groups)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test Von Frey Pre vs. 2D post CFA: $P = 0.0048$ Pre vs. 7D post CFA: $P > 0.9999$ Pre vs. 21D post CFA: $P > 0.9999$ 2D post CFA vs. 7D post CFA: $P = 0.1521$ 2D post CFA vs. 21D post CFA: $P = 0.0834$ 7D post CFA vs. 21D post CFA: $P > 0.9999$ Hargreaves Pre vs. 2D post CFA: $P = 0.4418$ Pre vs. 7D post CFA: $P > 0.9999$ Pre vs. 21D post CFA: $P > 0.9999$ 2D post CFA vs. 7D post CFA: $P = 0.7051$ 2D post CFA vs. 21D post CFA: $P = 0.2650$ 7D post CFA vs. 21D post CFA: $P > 0.9999$
Extended Data Fig. 4f	Locomotion (normal) Escape (normal)	Pearson correlation Average Speed vs average %ΔF/F (SNI) Locomotion: Pearson r = -0.1453 95% Confidence Intervals -0.4072 to 0.1386 Locomotion: Pearson r = 0.9176 95% Confidence Intervals 0.8585 to 0.9526
Extended Data Fig. 4g	Loconotion BL Escape BL Locomotion SNI Escape SNI (n = 6, 3 males, 3 females for all groups)	One-way ANOVA, followed by Tukey's multiple comparisons test Locomotion BL vs. escape BL: $P = 0.0169$ Locomotion BL vs. Locomotion SNI: $P = 0.9686$ Locomotion BL vs. escape SNI: $P = 0.0083$ Escape BL vs. Locomotion SNI: $P = 0.0026$ Escape BL vs. Escape SNI: $P = 0.0351$ Locomotion SNI vs. Escape SNI: $P = 0.0039$
Extended Data Fig. 4h	Evoked Spontaneous (n = 7, 4 males, 3 females for all groups)	Paired t test $P = 0.1553$
Extended Data Fig. 4k	Pre-SNI Post-SNI (n = 6, 3 males, 3 females for all groups)	Paired t test $P = 0.0069$

Extended Data Fig. 5b	hM4D saline vs. clozapine (n = 5, 3 males, 2 females) hM3D saline vs. clozapine (n = 5, 3 males, 2 females)	Wilcoxon matched-pairs signed rank test hM4D saline vs. clozapine: *P* = 0.8125 hM3D saline vs. clozapine: *P* = 0.3125
Extended Data Fig. 5c	saline vs. CLZ (n = 6, 3 males, 3 females)	Two-way ANOVA, followed by Šídák's multiple comparisons test Saline vs. CLZ: *P* > 0.9 for all time points compared
Extended Data Fig. 5f	Baseline vs. after CNO (6 cells from 3 mice)	Wilcoxon matched-pairs signed rank test Baseline vs. after CNO: *P* = 0.0313
Extended Data Fig. 5g	hM4D saline vs. clozapine (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test hM4D saline vs. clozapine: *P* = 0.8438
Extended Data Fig. 5i	Control no light vs. 20 Hz light (n = 6, 3 males + 3 females) ChR2 no light vs. 20 Hz light (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test Control no light vs. 20 Hz light: *P* = 0.2188 ChR2 no light vs. 20 Hz light: *P* = 0.6875
Extended Data Fig. 5j	control no light vs. 20 Hz light (n = 6, 3 males + 3 females) ChR2 no light vs. 20 Hz light (n = 5, 3 males + 2 females)	Wilcoxon matched-pairs signed rank test Control no light vs. 20 Hz light: *P* = 0.0938 ChR2 no light vs. 20 Hz light: *P* = 0.8125
Extended Data Fig. 5k	Control no light vs. 20 Hz light (n = 6, 3 males + 3 females) ChR2 no light vs. 20 Hz light (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test Control no light vs. 20 Hz light: *P* = 0.0625 ChR2 no light vs. 20 Hz light: *P* = 0.1563
Extended Data Fig. 6a	hM4D Von Frey saline vs. CLZ (n = 8, 4 males, 4 females) hM3D Von Frey saline vs. CLZ (n = 10, 5 males, 5 females)	Wilcoxon matched-pairs signed rank test hM4D Von Frey saline vs. CLZ: *P* = 0.8125 hM3D Von Frey saline vs. CLZ: *P* = 0.9004

Extended Data Fig. 6b	pre vs. post clozapine (n = 10, 5 males, 5 females)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test pre vs. post clozapine: $P > 0.9999$ for all pairs tested
Extended Data Fig. 6f	CLZ-mCherry (n = 6, 3 males, 3 females) CLZ-hM3D (n = 10, 5 males, 5 females)	Mann Whitney test CLZ-mCherry vs. CLZ-hM3D: $P < 0.0119$
Extended Data Fig. 6g	pre vs. post clozapine (n = 5, 3 males, 2 females)	Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test pre vs. post clozapine: $P > 0.9999$ for all pairs tested
Extended Data Fig. 6h	CLZ-mCherry (n = 8, 4 males, 4 females) CLZ-hM4D (n = 8, 4 males, 4 females)	Mann Whitney test CLZ-mCherry vs. CLZ-hM4D pre CFA: $P = 0.7389$ CLZ-mCherry vs. CLZ-hM4D 1d post CFA: $P = 0.0002$ CLZ-mCherry vs. CLZ-hM4D 2d post CFA: $P = 0.0002$ CLZ-mCherry vs. CLZ-hM4D 3d post CFA: $P = 0.0002$
Extended Data Fig. 6i	CLZ-mCherry (n = 8, 4 males, 4 females) CLZ-hM4D (n = 8, 4 males, 4 females)	Mann Whitney test CLZ-mCherry vs. CLZ-hM4D pre CFA: $P = 0.7002$ CLZ-mCherry vs. CLZ-hM4D 1d post CFA: $P = 0.7002$ CLZ-mCherry vs. CLZ-hM4D 2d post CFA: $P = 0.2047$ CLZ-mCherry vs. CLZ-hM4D 3d post CFA: $P = 0.0985$
Extended Data Fig. 7b	Control (n = 5, 3 males, 2 females) Caspase (n = 5, 3 males, 2 females)	Mann Whitney test Control vs. caspase: $P = 0.0079$
Extended Data Fig. 7c	Control (n = 3, 3 males) Caspase (n = 3, 3 males)	Mann Whitney test Control vs. caspase: $P > 0.9999$
Extended Data Fig. 7d	Control (n = 4, 2 males + 2 females) Caspase (n = 4, 2 males + 2 females)	Mann Whitney test Control vs. caspase: $P = 0.0286$
Extended Data Fig. 7e	Control (n = 7, 4 males + 3 females) Caspase (n = 8, 4 males + 4 females)	Mann-Whitney test Pre-ablation control vs. caspase: $P = 0.5358$ Post-ablation control vs. caspase: $P = 0.5158$ 1d post CFA control vs. caspase: $P = 0.0025$ 2d post SNI control vs. caspase: $P = 0.0101$ 3d post SNI control vs. caspase: $P = 0.0005$

Extended Data Fig. 8a	eGFP+7DCLZ (n= 5, 3 males+ 2 females) hM3D+7DCLZ (n = 6, 3 males + 3 females) SNI_eGFP+CLZ (n = 6, 3 males + 3 females) SNI_hM4D+CLZ (n = 5, 3 males + 2 females)	One-way ANOVA, followed by Tukey's multiple comparisons test 0.007g eGFP+7DCLZ vs. hM3D+7DCLZ: *P* < 0.0001 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* < 0.0001 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0265 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9424 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0001 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* < 0.0001 0.4g eGFP+7DCLZ vs. hM3D+7DCLZ: *P* < 0.0001 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* < 0.0001 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.3070 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9579 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0004 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.0004 2g eGFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.0136 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0111 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.9975 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9996 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0088 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.0072
Extended Data Fig. 8b	eGFP+7DCLZ (n = 9, 5 males+ 4 females) hM3D+7DCLZ (n = 10, 5 males + 5 females) SNI_eGFP+CLZ (n = 8, 4 males + 4 females) SNI_hM4D+CLZ (n = 10, 5 males + 5 females)	One-way ANOVA, followed by Tukey's multiple comparisons test eGFP+7DCLZ vs. hM3D+7DCLZ: *P* =0.7413 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0024 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* > 0.9999 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0001 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* =0.7338 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.0018
Extended Data Fig. 8c	eGFP+7DCLZ (n = 8, 4 males + 4 females) hM3D+7DCLZ (n = 8, 4 males + 4 females)	Nonparametric ANOVA (Kruskal-Wallis test), Dunn's multiple comparison test eGFP+7DCLZ vs. hM3D+7DCLZ: *P* > 0.9999 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0147 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* > 0.9999 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0023

	SNI_eGFP+CLZ (n = 10, 5 males + 5 females) SNI_hM4D+CLZ (n = 8, 4 males + 4 females)	hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* >0.9999 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.0019
Extended Data Fig. 8d	eGFP+7DCLZ (n = 6, 3 males + 3 females) hM3D+7DCLZ (n = 5, 3 males + 2 females) SNI_eGFP+CLZ (n = 5, 3 males + 2 females) SNI_hM4D+CLZ (n = 5, 3 males + 2 females)	One-way ANOVA, followed by Tukey's multiple comparisons test eGFP+7DCLZ vs. hM3D+7DCLZ: *P* =0.7645 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9834 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.9992 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.5871 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.8476 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.9652
Extended Data Fig. 8e	eGFP+7DCLZ (n = 9, 5 males+ 4 females) hM3D+7DCLZ (n = 10, 5 males + 5 females) SNI_eGFP+CLZ (n = 9, 5 males + 4 females) SNI_hM4D+CLZ(n = 7, 4 males + 3 females)	Nonparametric ANOVA (Kruskal-Wallis test), Dunn's multiple comparison test Total distance eGFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.2431 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.3374 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.1490 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* > 0.9999 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* > 0.9999 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* > 0.9999 One-way ANOVA, followed by Tukey's multiple comparisons test % time in center eGFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.0179 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.009 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0023 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9832 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.6992 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.8834
Extended Data Fig. 8f	eGFP+7DCLZ (n = 7, 4 males+ 3 females) hM3D+7DCLZ (n = 10, 5 males + 5 females)	One-way ANOVA, followed by Tukey's multiple comparisons test eGFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.023 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.0042 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0002

	SNI_eGFP+CLZ (n = 9, 5 males + 4 females) SNI_hM4D+CLZ (n = 9, 5 males + 4 females)	hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.9987 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.7408 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* = 0.6675
Extended Data Fig. 8g	eGFP+7DCLZ (n = 6, 3 males+ 3 females) hM3D+7DCLZ (n = 8, 4 males + 4 females) SNI_eGFP+CLZ (n = 8, 4 males + 4 females) SNI_hM4D+CLZ (n = 9, 5 males + 4 females)	Nonparametric ANOVA (Kruskal-Wallis test), Dunn's multiple comparison test eGFP+7DCLZ vs. hM3D+7DCLZ: *P* > 0.9999 eGFP+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.4322 eGFP+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0086 hM3D+7DCLZ vs. SNI_eGFP+CLZ: *P* = 0.3926 hM3D+7DCLZ vs. SNI_hM4D+CLZ: *P* = 0.0048 SNI_eGFP+CLZ vs. SNI_hM4D+CLZ: *P* > 0.9999
Extended Data Fig. 9a	CLZ-mCherry (n = 6, 3 males + 3 females) CLZ-hM4D (n = 6, 3 males+ 3 females)	Mann-Whitney test CLZ-mCherry vs. CLZ-hM4D: *P* = 0.0022
Extended Data Fig. 9b	CLZ-mCherry (n = 6, 3 males + 3 females) CLZ-hM3D (n = 5, 3 males+ 2 females)	Mann-Whitney test CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022
Extended Data Fig. 10b	Control (n = 6, 3 males + 3 females) Ablation (n = 6, 3 males + 3 females)	Mann-Whitney test Pre CLZ-control vs. Pre CLZ-ablation: *P* = 0.05779 1D on CLZ-control vs. CLZ-ablation: *P* = 0.0065 2D on CLZ-control vs. CLZ-ablation: *P* = 0.0022 3D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 5D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 7D on CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 7D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 14D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 21D post CLZ-mCherry vs. CLZ-hM3D: *P* = 0.0022 Nonparametric ANOVA (Friedman test), followed by Dunn's multiple comparison test control pre vs. D1 on CLZ: *P* > 0.9999 control Pre vs. D2 on CLZ: *P* = 0.4633 control Pre vs. D3 on CLZ: *P* = 0.0354 control Pre vs. D5 on CLZ: *P* = 0.0027 control Pre vs. D7 on CLZ: *P* = 0.0008

		control Pre vs. D7 post CLZ: *P* = 0.0008 control Pre vs. D14 post CLZ: *P* = 0.0104 control Pre vs. D21 post CLZ: *P* = 0.0491
Extended Data Fig. 10c	eYFP+7DCLZ (n = 7, 4 males+ 3 females) hM3D+7DCLZ (n = 7, 4 males + 3 females)	Mann Whitney test Total distance eYFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.7104 % time in center: eYFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.0017
Extended Data Fig. 10d	eYFP+7DCLZ (n = 8, 4 males+ 4 females) hM3D+7DCLZ (n = 8, 4 males + 4 females)	Mann Whitney test EPM eYFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.003 TST eYFP+7DCLZ vs. hM3D+7DCLZ: *P* = 0.1949
Extended Data Fig. 10e	VPL+Po hM4D (n = 6, 3 males + 3 females) DCN-VPL hM4D (n = 6, 3 males + 3 females) WT (n = 5, 3 males + 2 females)	One-way ANOVA, followed by Holm–Šídák's multiple comparisons test WT vs. DCN to VPL: *P* = 0.0007 VPL+PO vs. DCN to VPL: *P* =0.0006 WT vs. SpC to VPL PO: *P* = 0.9207
Extended Data Fig. 10f	Saline vs. CLZ (n = 6, 3 males + 3 females)	Wilcoxon matched-pairs signed rank test Pre SNI saline vs. CLZ : *P* = 0.2188 2d post SNI saline vs. CLZ : *P* = 0.5 14d post SNI saline vs. CLZ : *P* = 0.5