Supporting Information for

Neuropeptide Y neurons mediate opioid-induced itch by disinhibiting GRP-GRPR microcircuits in the spinal cord

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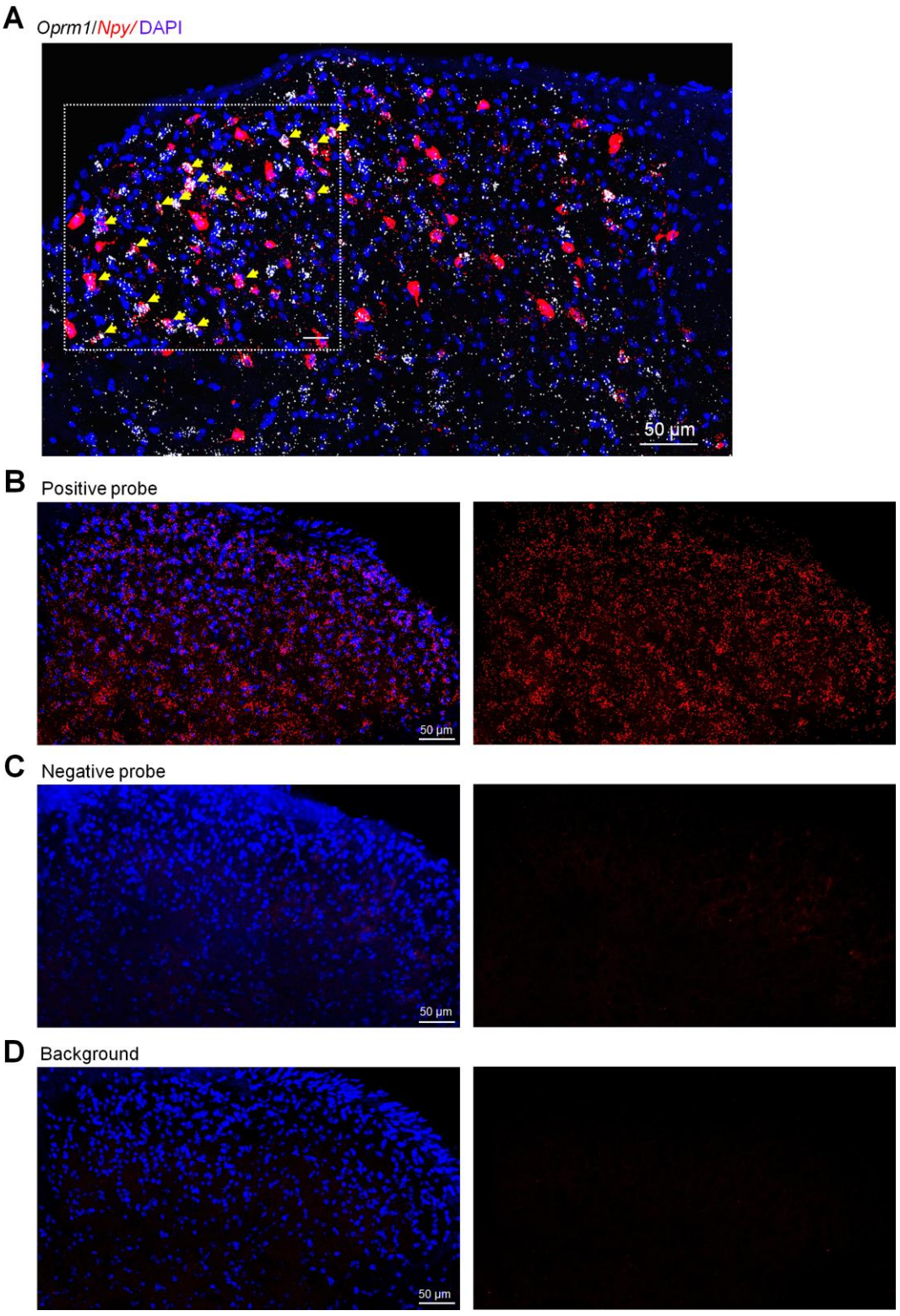

Figure S1. **The whole SDH images for Figure 1A and control probes. (A)** The whole SDH for Figure1A. Scale bar = 50 μm. In situ hybridization RNAscope images of MOR mRNA (*Oprm1*, white) and NPY mRNA (*Npy*, red) in mouse spinal dorsal horn (SDH). Yellow arrows indicate *Oprm1* double-labeled with *Npy*. 12 spinal cord sections from 6 mice were analyzed. **(B)** RNAscope images of positive probe (red) and DAPI (blue) in WT mice. 15 spinal cord sections from 3 mice were analyzed. Scale bar = 50 μm. **(C)** RNAscope images of negative probe (red) and DAPI (blue) in WT mice. 15 spinal cord sections from 3 mice were analyzed. Scale bar = 50 μm. **(D)** RNAscope background images of DAPI (blue) in WT mice. 15 spinal cord sections from 3 mice were analyzed. Scale bar = 50 μm.

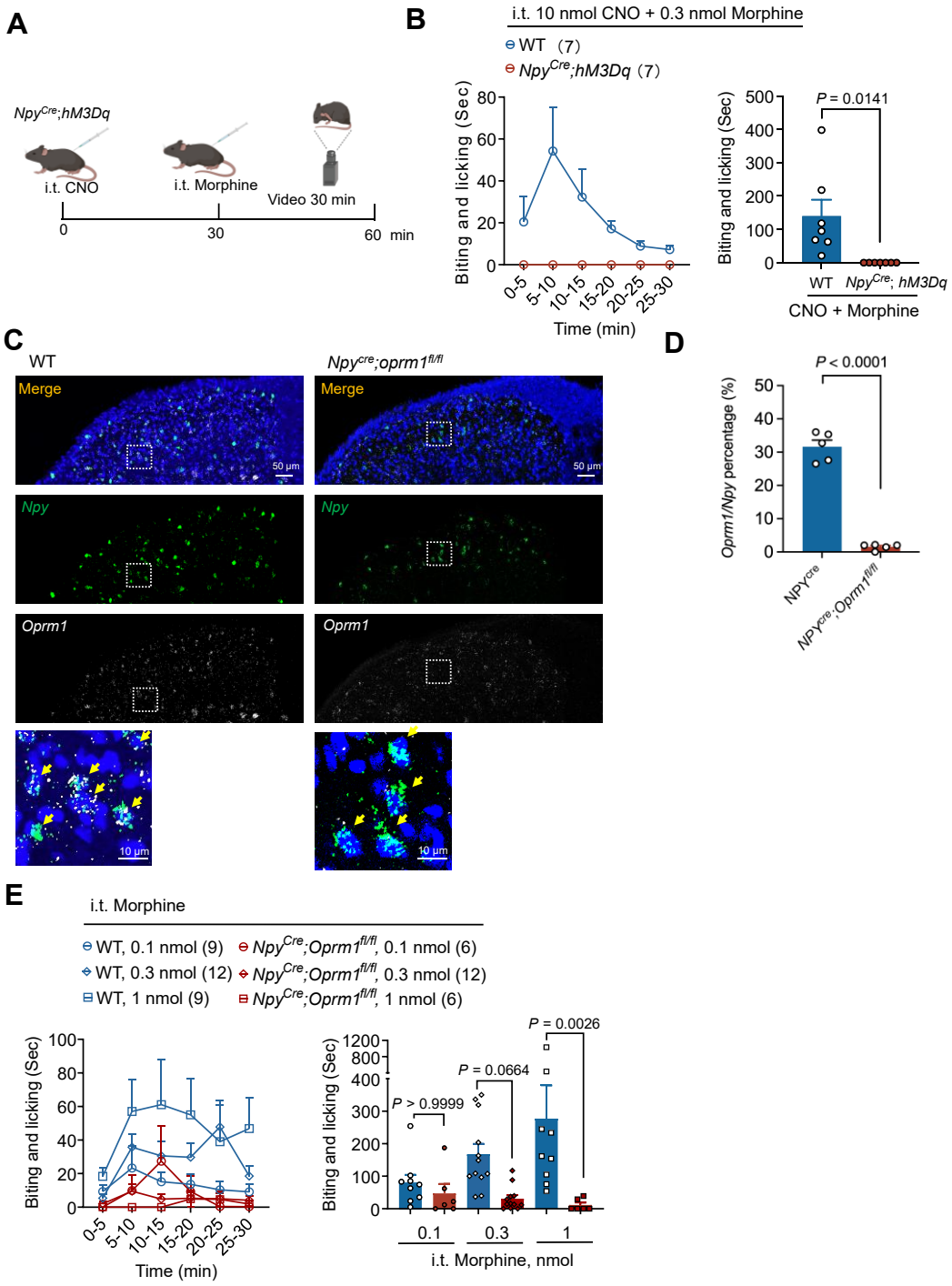

Figure S2. **Intrathecal injection of morphine-induced biting and licking were blocked in *Npy^{Cre};hM3Dq* and *Npy^{Cre};Oprm1$^{fl/fl}$* mice. (A)** Timeline for intrathecal injection of Clozapine N-oxide (CNO) (10 nmol) in *Npy^{Cre};hM3Dq* or WT mice. **(B)** Intrathecal injection of CNO (10 nmol) and morphine (0.3 nmol)-induced biting and licking were significantly inhibited in *Npy^{Cre};hM3Dq* mice. Student's unpaired two-tailed *t*-test. **(C-D)** RNAscope showed *Oprm1* (white) conditional knockout in *Npy*$^{+}$ (green) interneurons of *Npy^{Cre};Oprm1$^{fl/fl}$* mice, 15 spinal cord sections from 5 mice were analyzed, respectively. Student's unpaired two-tailed *t*-test. **(E)** Intrathecal injection of morphine (0.1, 0.3, 1 nmol)-induced biting and licking were blocked in *Npy^{Cre};Oprm1$^{fl/fl}$* mice, Two-way ANOVA, Bonferroni's multiple comparisons. Data are shown as means ± SEM. *P* values are indicated in the figures. Sample sizes are presented in parentheses. Source data are provided as a Source Data file. Created in BioRender. Wang, Z. (2025) https://BioRender.com/v1vbo0a

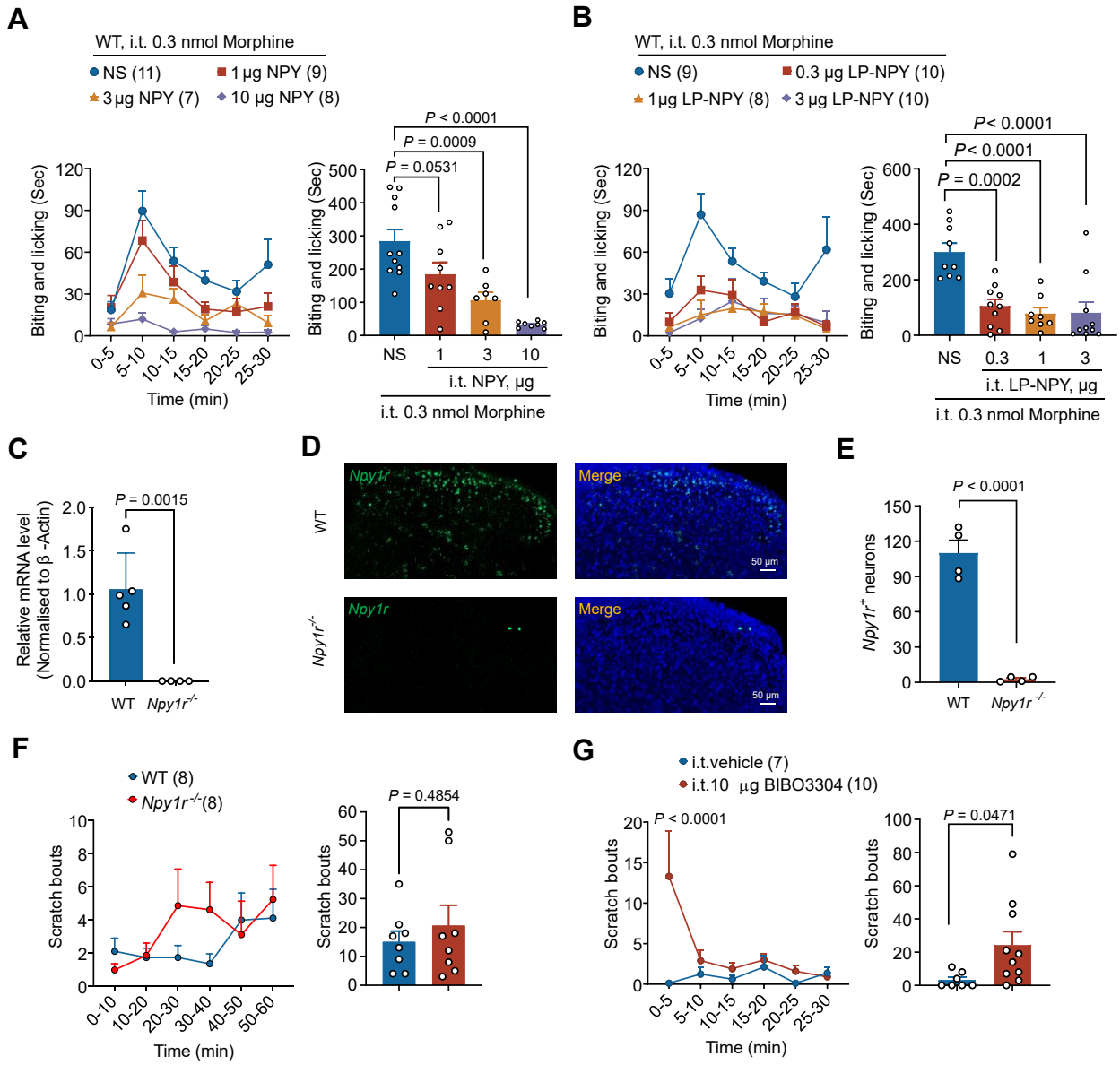

Figure S3. NPY-NPY1R modulates morphine-induced itch. (A) Intrathecal morphine-induced biking and licking were dose-dependently inhibited by NPY (1, 3, 10 μg). One-way ANOVA, Dunnett's multiple comparisons. **(B)** Intrathecal morphine-induced biking and licking were significantly blocked with the LP-NPY (0.3, 1, 3 μg). One-way ANOVA, Dunnett's multiple comparisons. **(C)** qPCR quantification of *Npy1r* expression in the spinal cord of WT and *Npy1r*$^{-/-}$ mice, n = 5 and 4 mice, respectively. Student's unpaired two-tailed *t*-test. **(D-E)** RNAscope showed *Npy1r* expression in the spinal cord of WT and *Npy1r*$^{-/-}$ mice, 10 spinal cord sections from 4 mice and 8 spinal cord sections from 4 mice were analyzed, respectively.. Student's unpaired two-tailed *t*-test. **(F)** Spontaneous itch in WT and *Npy1r*$^{-/-}$ mice. Student's unpaired two-tailed *t*-test. **(G)** Spontaneous itch after intrathecal BIBO3304 (10 μg) in WT mice. Student's unpaired two-tailed *t*-test. Data are shown as means ± SEM. *P* values are indicated in the figures. Sample sizes are presented in parentheses. Source data are provided as a Source Data file.

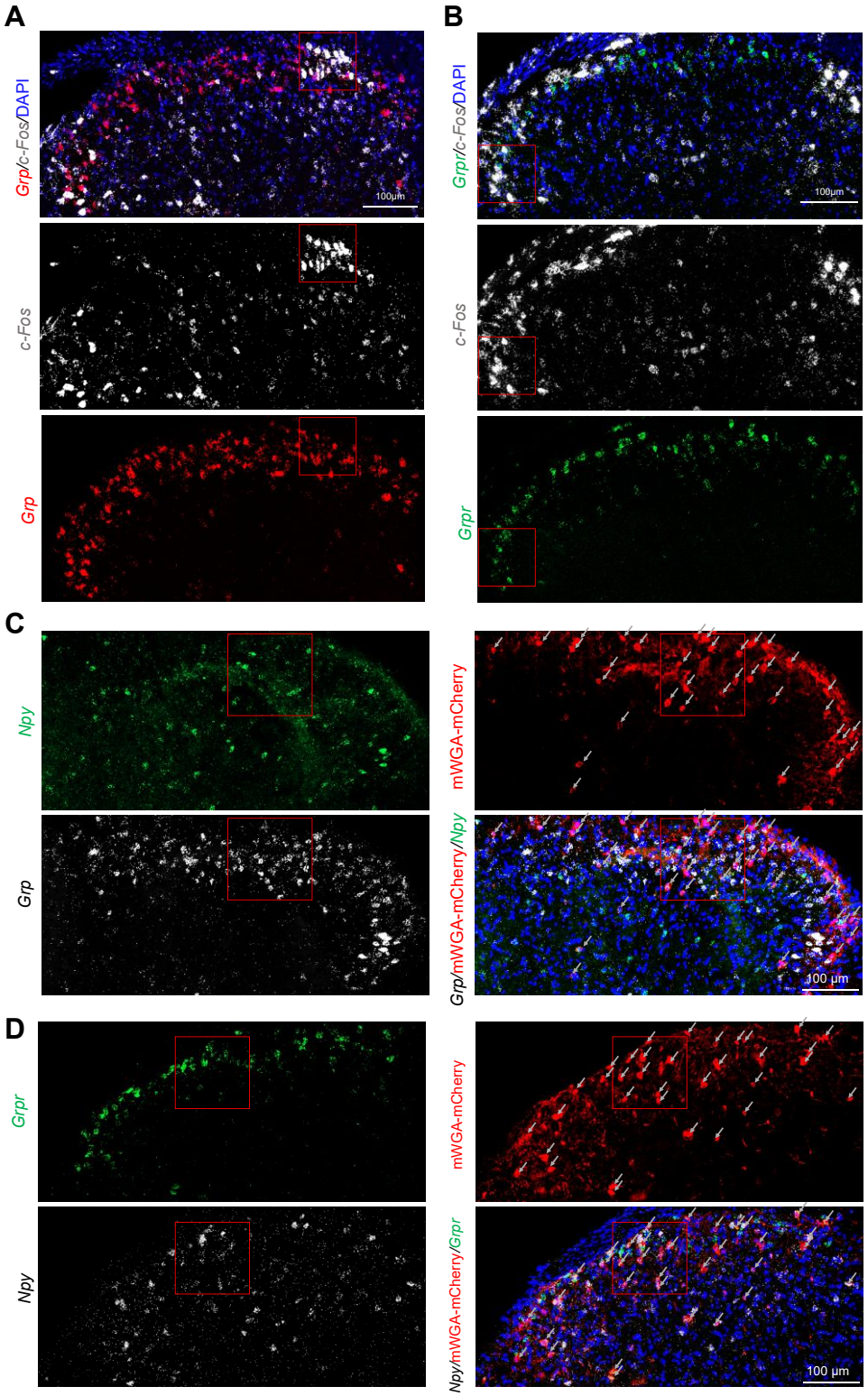

Figure S4. The whole SDH images for *c-Fos* and WGA in Figure 4. (A) The whole SDH for Figure 4A. Co-expression of *Grp* (red) and *c-Fos* (white) mRNA in the SDH after intrathecal injection of morphine. Scale bar = 100 μm. **(B)** The whole SDH for Figure 4D. Co-expression of *Grp* (red) and *c-Fos* (white) mRNA in the SDH after intrathecal injection of morphine. Scale bar = 100 μm. **(C)** The whole SDH for Figure 4H. mWGA-mCherry signals were detected in both *Npy*$^{+}$ (Green) and *Grp*$^{+}$ (White) interneurons.Gray arrows indicate mWGA-mCherry-labeled cells. Scale bar = 100 μm. **(D)** The whole SDH for Figure 4I. mWGA-mCherry signals were detected in *Npy*$^{+}$ (White), but only a few in *Grpr*$^{+}$ (Green) interneurons. Gray arrows indicate mWGA-mCherry-labeled cells. Scale bar = 100 μm.

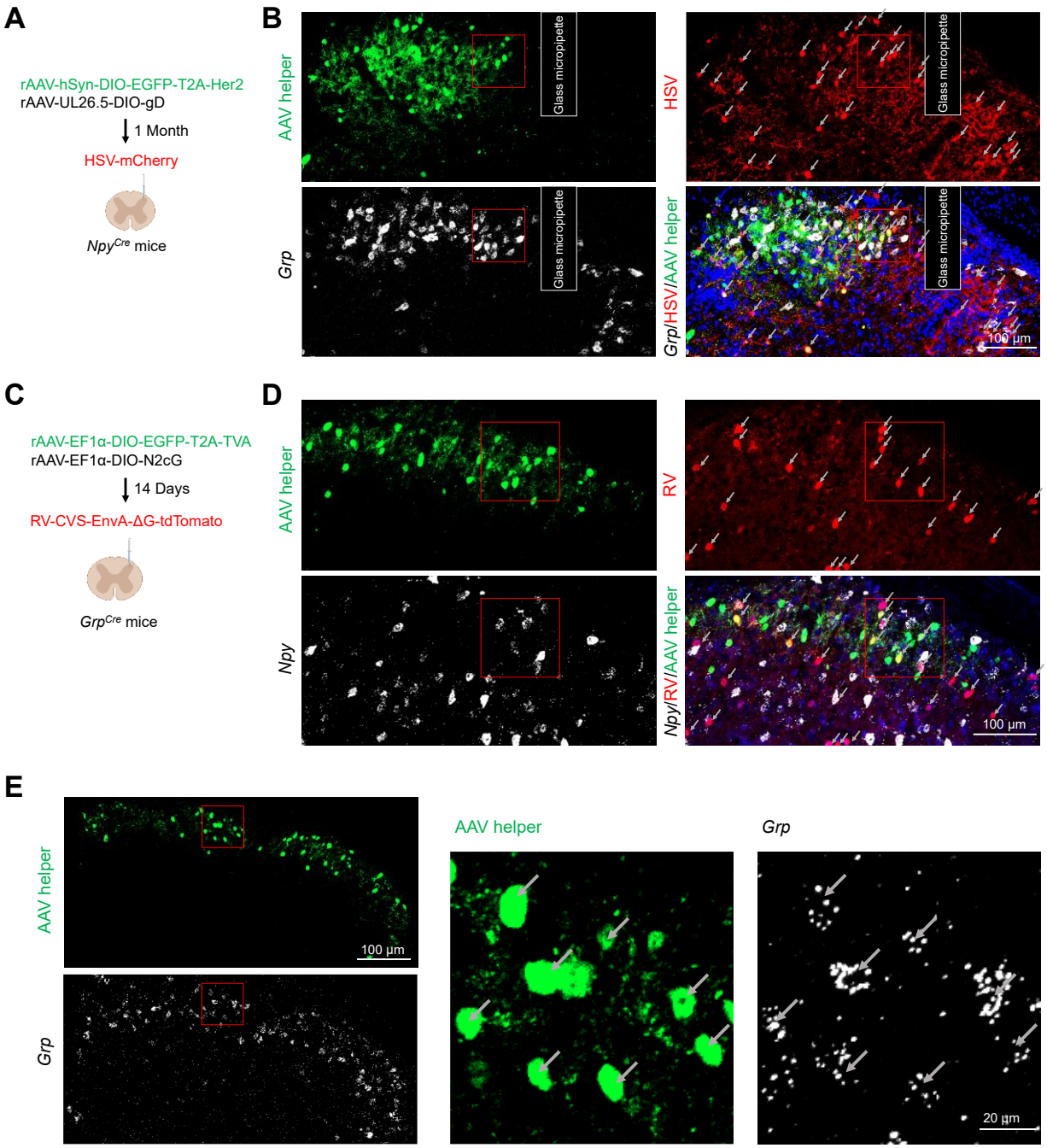

Figure S5. Supplementary figures for HSV and RV tracing in Figure 4. (A) Spinal dorsal horn injection (T_{13}-L_1 levels) of rAAV-hSyn-EGFP-T2A-Her2 and rAAV-UL26.5-DIO-gD helper virus 1 month ago, followed by HSV-mCherry virus in *Npy*^{Cre} mice, the spinal cords were dissected 6 days later for RNAscope. **(B)** The whole SDH for Figure 4L. HSV-mCherry signals were detected in *Grp*$^{+}$ (White) interneurons. Gray arrows indicate HSV-labeled cells. Scale bar = 100 μm. **(C)** Spinal dorsal horn injection (T_{13}-L_1 levels) of rAAV-EF1α-DIO-EGFP-T2A-TVA and rAAV-EF1α-DIO-N2cG helper virus 14 days ago, followed by RV-CVS-EnvA-ΔG-tdTomato virus in *Grp*^{Cre} mice, the spinal cords were dissected 6 days later for RNAscope. **(D)** The whole SDH for Figure 4N. RV-CVS-EnvA-ΔG-tdTomato signals were detected in *Npy*$^{+}$ (White) interneurons. Gray arrows indicate RV-labeled cells. Scale bar = 100 μm. **(E)** The AAV helper virus specifically infected *Grp*$^{+}$ neurons, gray arrows indicate AAV helper double-labeled with *Grp*. 15 spinal cord sections from 5 mice were analyzed. Scale bar: 100 μm (left), 20 μm (right). Created in BioRender. Wang, Z. (2025) https://BioRender.com/mxv2gwk

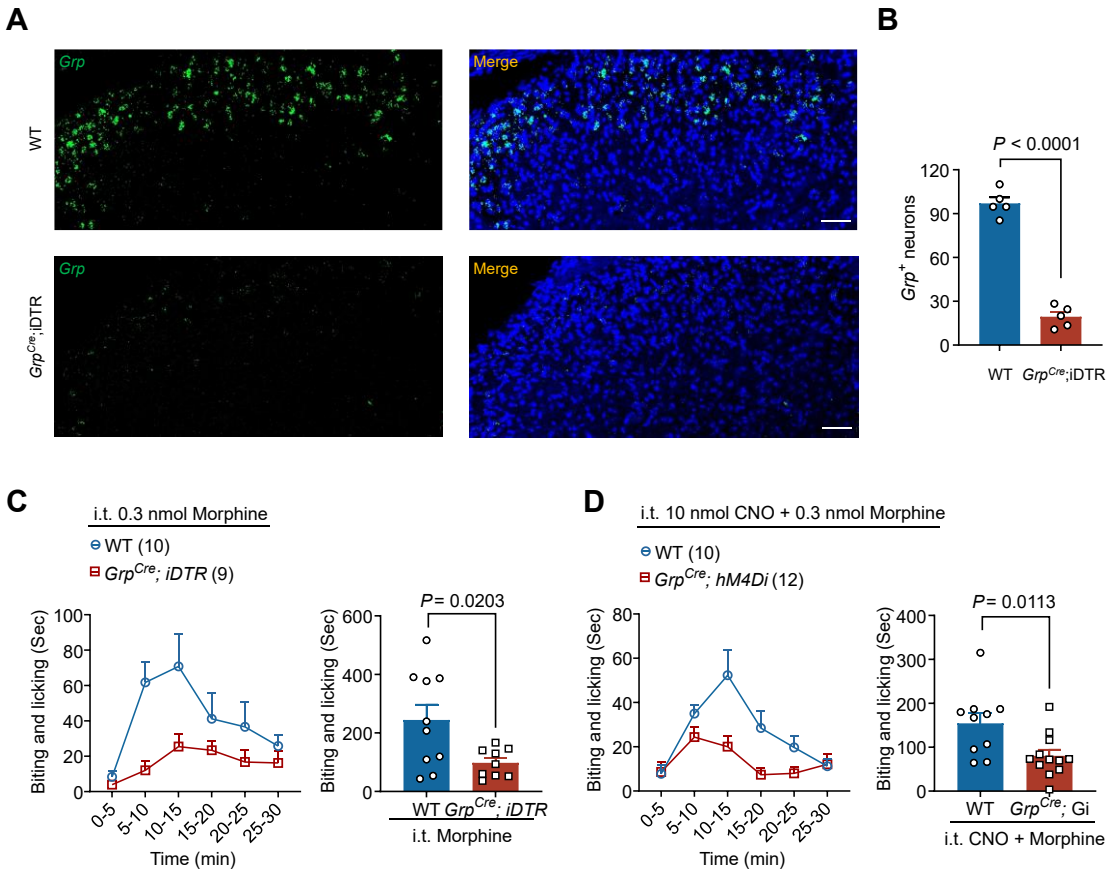

Figure S6. Intrathecal injection of morphine-induced biting and licking were attenuated in *Grp^{Cre};iDTR* and *Grp^{Cre};hM4Di* mice. (A-B) RNAscope data showed the ablation of *Grp*$^{+}$ (green) neurons in the SDH in *Grp^{Cre};iDTR* mice, scale bar = 50 μm, 13 spinal cord sections from 5 mice. cells containing ≥ 5 RNAscope puncta were considered positive expression. Student's unpaired two-tailed *t*-test. **(C)** Intrathecal morphine (0.3 nmol)-induced biting and licking were significantly inhibited in *Grp^{Cre};iDTR* mice, student's unpaired two-tailed t-test. **(D)** Intrathecal injection of CNO (10 nmol) significantly inhibited morphine (0.3 nmol)-induced biting and licking in *Grp^{Cre};hM4Di* mice, student's unpaired two-tailed *t*-test. Data are shown as means ± SEM. *P* values are indicated in the figures. Sample sizes are presented in parentheses. Source data are provided as a Source Data file.

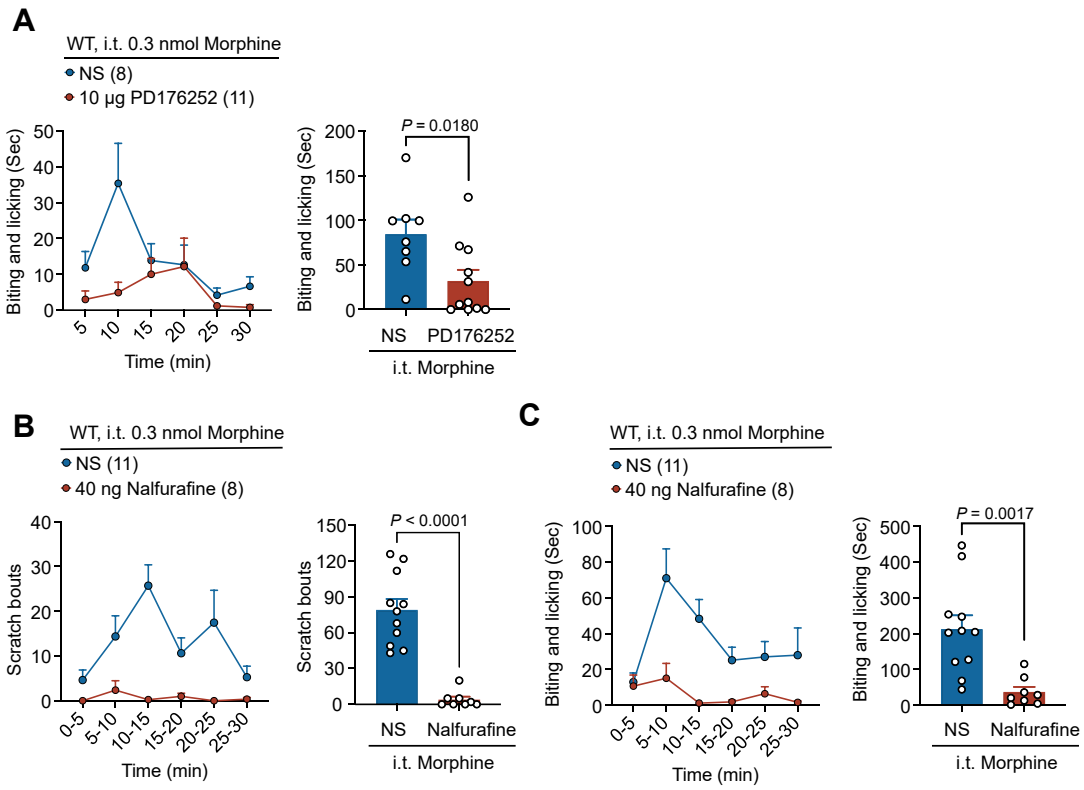

Figure S7. Intrathecal morphine-induced itch was inhibited by GRPR antagonist and KOR agonist. **(A)** Intrathecal morphine (0.3 nmol)-induced biting and licking were significantly reduced by intrathecal injected PD176252 (10 μg), student's unpaired two-tailed *t*-test. **(B-C)** Intrathecal morphine-induced scratch or biting and licking were significantly reduced by intrathecal injected nalfurafine (40 ng). Student's unpaired two-tailed *t*-test. Data are shown as means ± SEM. *P* values are indicated in the figures. Sample sizes are presented in parentheses. Source data are provided as a Source Data file.

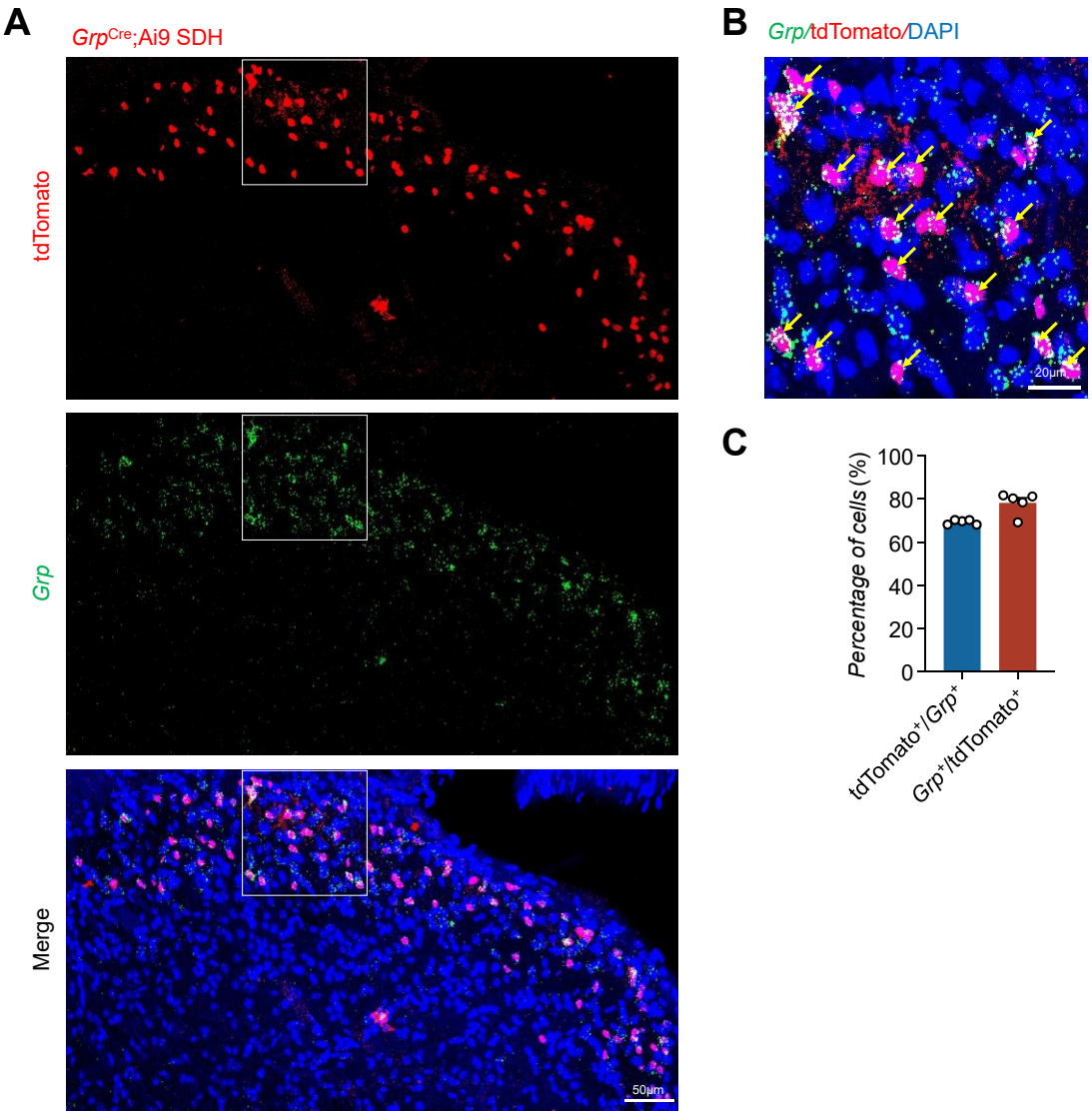

Figure S8. *Grp*$^{+}$ neurons labeling in *Grp*^{Cre};Ai9 mice. (A-B) RNAscope images of *Grp* mRNA (green) and tdTomato (red) in SDH of *Grp*^{Cre};Ai9 mice. Yellow arrows indicate *Grp* double-labeled with tdTomato reported. scale bar = 50 μm (left) and 20 μm (right). 10 spinal cord sections from 5 mice. **(C)** Double labeling of *Grp* RNAscope signals and tdTomato, Data are shown as means ± SEM. Source data are provided as a Source Data file.

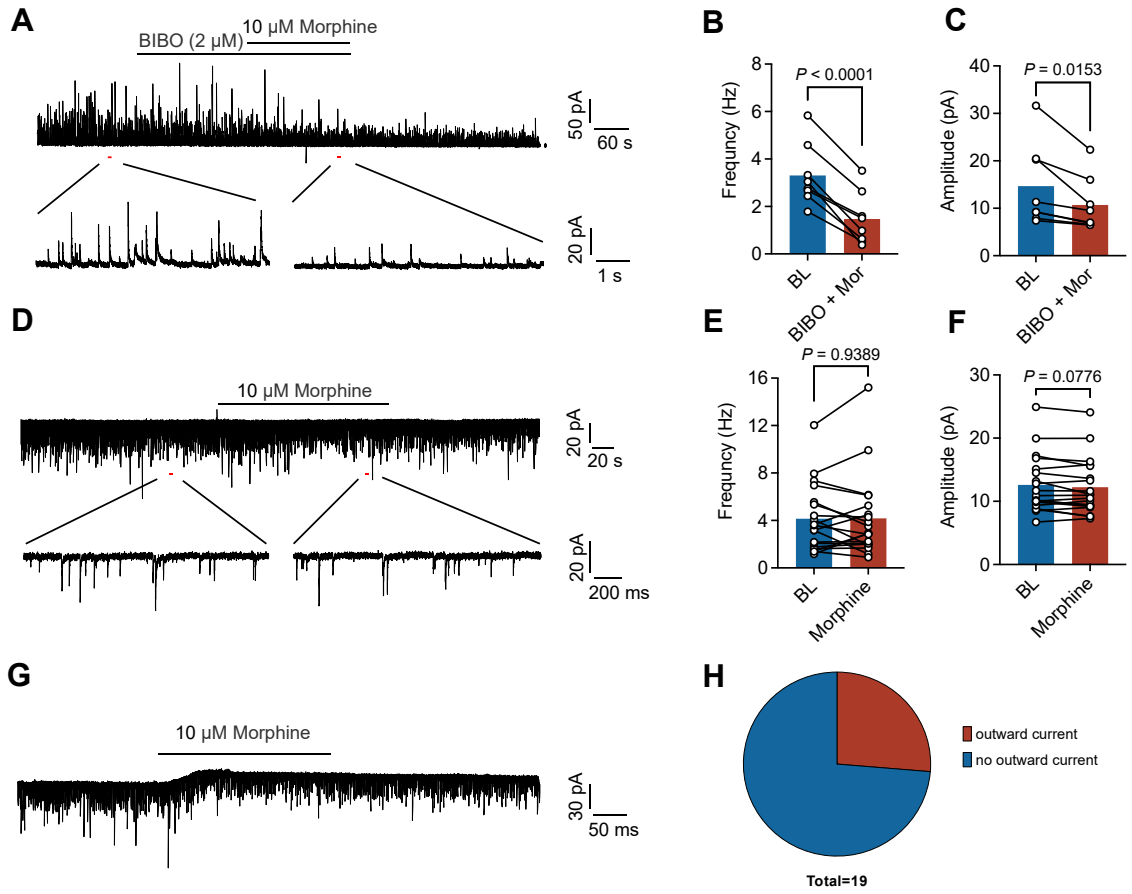

Figure S9. Morphine inhibits sIPSCs in GRP^{+} interneurons with BIBO3304 pretreatment, but not sEPSCs in the SDH. (A) Recording traces of spontaneous inhibitory postsynaptic currents (sIPSCs) of BIBO3304 (2 μM) pretreatment 3 min before morphine (10 μM) treatment in GRP^{+} interneurons of the spinal slices from *Grp^{Cre};Ai9* mice. **(B)** The sIPSC frequency before and after morphine treatment, 8 neurons from 3 mice were analyzed, paired two-tailed *t*-test. **(C)** The sIPSC amplitude before and after morphine treatment, 8 neurons from 3 mice were analyzed, paired two-tailed *t*-test. **(D)** Recording traces of spontaneous excitatory postsynaptic currents (sEPSCs) after morphine (10 μM) treatment in GRP^{+} interneurons of the spinal slices from *Grp^{Cre};Ai9* mice. **(E)** The sEPSC frequency before and after morphine treatment, 19 neurons from 6 mice were analyzed, paired two-tailed *t*-test. (**F**) The sEPSC amplitude before and after morphine treatment, 19 neurons from 6 mice were analyzed, paired two-tailed *t*-test. **(G)** Recording traces of morphine (10 μM) induced outward currents were recorded GRP^{+} interneurons in the SDH from *Grp^{Cre};Ai9* mice. **(H)** 5/19 of recorded neurons showed outward currents. *P* values are indicated in the figures. Data are shown as means with dots. Source data are provided as a Source Data file.

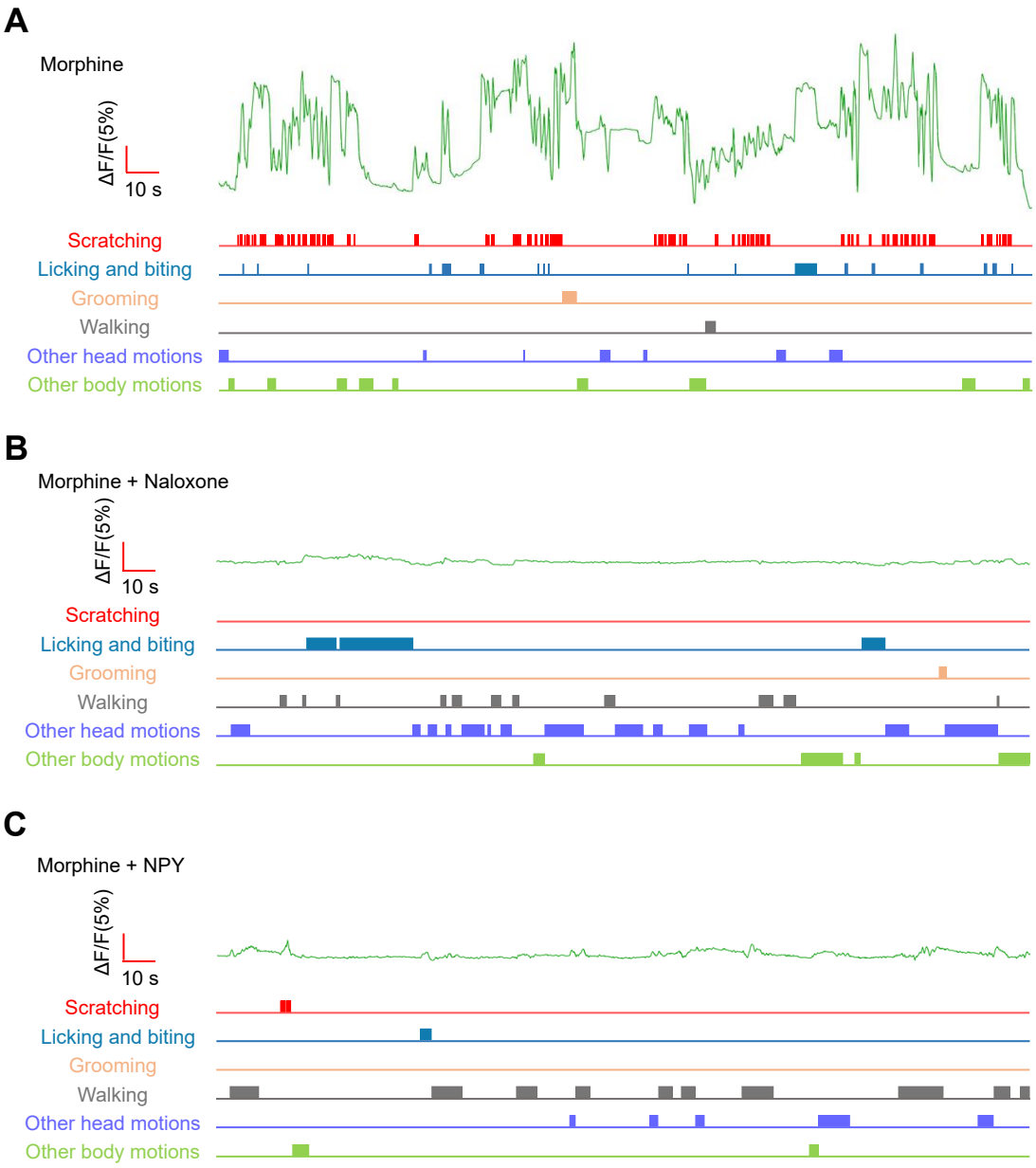

Figure S10. Example ΔF/F time series traces which correlated to the scratching behavior. (A) Example ΔF/F time series traces which correlated to the scratching behavior after intrathecal injected morphine (0.3 nmol). **(B)** Example ΔF/F time series traces after intrathecal co-injected morphine (0.3 nmol) and naloxone (10 nmol). **(C)** Example ΔF/F time series traces after intrathecal co-injected morphine (0.3 nmol) and NPY (10 µg). n = 5 mice per group.

Tables S1. List of probes and viruses

RNA Probes		
Mm-Oprm1	Advanced Cell Diagnostic	315841- C3
Mm-Npy	Advanced Cell Diagnostic	313321-C2
Mm-Npy1r	Advanced Cell Diagnostic	427021
Mm-Grp	Advanced Cell Diagnostic	317861-C2
Mm-Grp	Advanced Cell Diagnostic	317861-C3
Mm-Grpr	Advanced Cell Diagnostic	317871
Mm-Fos	Advanced Cell Diagnostic	316921-C3
Virus		
rAAV-GAG-DIO-mCherry	Brain Case	BC-1227
RV-CVS-EnvA-ΔG-tdTomato	Brain Case	BC-RV-CVS
rAAV-EF1α-DIO-EGFP-T2A-TVA	Brain Case	BC-0041
rAAV-EF1α-DIO-N2cG	Brain Case	BC-0442
H129-dgD-hUbC-mCherry-P2A-scHer	Brain Case	BC-HSV-Hs06
rAAV-hSyn-DIO-EGFP-T2A-Her2	Brain Case	BC-1663
rAAV-UL26.5-DIO-gD	Brain Case	BC-1356

Table S2. List of primers

Genes	Primer sequence (5'-3')	
β-actin	FP:GGCTGTATTCCCCTCCATCG	RP:CCAGTTGGTAACAATGCCATGT
Npy1r	FP:ACAGGCTGTCTTACACGACTCTCC	RP:CATGATGTTGATTCGCTTGGTCTC

Note: FP: forward primer; RP: reverse primer.