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Supplementary Information for

Pain and itch processing by subpopulations of molecularly diverse spinal and trigeminal projection neurons

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SUPPLEMENTARY METHODS (As described in Wercberger, 2019)

Immunoprecipitation and RNA preparation

To immunoprecipitate tagged ribosomes and their associated mRNA, we followed the protocol described by Ekstrand et al. (36) with the following minor modifications. Protein A Dynabeads (100 µl per IP, Invitrogen) were washed twice on a magnetic rack with Buffer A (10 mM HEPES [pH 7.4], 150 mM KCl, 5.0 mM MgCl2, 1.0% NP40), resuspended in Buffer A with 0.1% BSA and then loaded with either anti-GFP polyclonal antibody (25 µg per IP, abcam) or anti-HA-tag monoclonal antibody (2.0 µg per IP, Cell Signaling). Antibody-bead conjugates were mixed at 4°C overnight.

Mice were killed with an overdose of Avertin (2.5%) and dorsal spinal cord and TNC were rapidly dissected in ice-cold Buffer B (1xHBSS, 4.0 mM NaHCO3, 2.5 mM HEPES [pH 7.4], 35 mM Glucose) with 100 µg/ml cycloheximide (Sigma). The dissected pieces were pooled in 2 groups of 4 mice each (1 group of injected experimental mice, 1 group of non-injected controls), transferred to a glass homogenizer (Kimble Kontes 20) and homogenized in 1.5 ml ice-cold Buffer C (10 mM HEPES [pH 7.4], 150 mM KCl, 5.0 mM MgCl2) with 0.5 mM DTT (Sigma), 20 U/µl Superase-In (Invitrogen), 100 µg/ml cycloheximide, and protease inhibitor cocktail (Roche).

Tissue samples were homogenized 3 times at 300 rpm and 10 times at 800 rpm on a variable-speed homogenizer (Glas-Col) at 4°C. Homogenates were transferred to microcentrifuge tubes and clarified at 2,000g for 10 min at 4°C. 10% IGEPAL CA-630 (NP-40; Sigma) and 1,2-diheptanoyl-sn-glycero-3-phospho-choline (DHPC at 100 mg/0.69 ml; Avanti Polar Lipids) were then added to the supernatant for a final concentration of 30 mM and 1%, respectively. The solutions were mixed and centrifuged again at 20,000g for 15 min at 4°C. The resulting supernatants were transferred to new tubes and 50 µl of each cleared lysate was mixed with 50 µl Lysis Buffer (0.7 µl β-mercaptoethanol/100 µl Lysis Buffer; Agilent Absolutely RNA Nanoprep Kit) and stored at −80°C for later preparation as Input RNA. The remaining lysates (approximately 1.5 ml) were used for immunoprecipitation.

The beads incubating with GFP or HA antibodies were washed twice in Buffer A before the tissue lysates were added. We ran the IPs at 4°C for 5 min (GFP) and 10 min (HA). Beads were washed 4 times with Buffer D (10 mM HEPES [pH 7.4], 350 mM KCl, 5 mM MgCl2, 1% NP40) with 0.5 mM DTT, 20 U/µl Superase-In Plus and 100 µg/ml cycloheximide. Before removing the last wash solution the beads were transferred to a new tube. After the final wash, RNA was eluted by adding 100 µl Lysis Buffer and purified using the Absolutely RNA Nanoprep Kit (Agilent).

RNA yield and quality

RNA yield (ng/µl) and quality (RIN value) were quantified using an Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Pico Kit (Cat No. 5067-1513). IP RNA yields for the GFP IP ranged from 0.1-0.9 ng/µl and yields from the HA IP ranged from 2.5-3.5 ng/µl. Input RNA yield ranged from 50-90 ng/µl. We only analyzed samples with RIN values of 8.4 or greater.

For the Input control, we used total mRNA from homogenized dorsal spinal cord and TNC. Consistently, the RNA yields from the IPs performed on tissue from injected animals were at least one order of magnitude greater than IPs performed on tissue from uninjected controls. We only detected 18S and 28S rRNA peaks from the former samples, which indicates that the ribosomal tag was critical to the RNA pull down and that the IPs were specific for projection neurons.

In situ hybridization (ISH): RNAscope multiplex hybridization

To detect and confirm expression of candidate genes in spinal cord and TNC tissue we performed fluorescent *in situ* hybridization (ISH) using the RNAscope Multiplex Fluorescent Assay (Advanced Cell Diagnostics, cat no. 320850) and target probes. For the complete list of probes used, see SI Appendix File 4. For these experiments, we perfused adult C57BL/6J mice with room temperature 1X phosphate-buffered saline (PBS). The TNC and spinal cord were rapidly dissected out, frozen on dry ice, and kept at -80^{o}C. From these tissues, we cut 12 µm cryostat sections and stored the sections at -80^{o}C until use.

The day of ISH, frozen sections were immediately fixed at 4°C in 4% formaldehyde for 15 minutes, washed twice in PBS and dehydrated through successive ethanol steps (50%, 70% and 100% ethanol) for 5 minutes each and dried at room temperature (RT). After a 30 minute incubation step with protease IV, sections were washed twice in PBS and incubated at 40°C with RNAscope-labeled probes for 2h in a humidified chamber. Sections were then washed twice in washing buffer and incubated with 4 successive "signal amplifying" solutions at 40°C, for 15 to 30 minutes each. After two washes, the sections were dried and covered with mounting media containing DAPI.

For *Fos* induction studies: Mice were injected two weeks prior to stimulation with HSV-hEF1a-GFP-L10a or green Retrobeads (Lumafluor) into the LPb, to label LPb-projecting neurons. For pruritic stimulation, under anesthesia we injected chloroquine (CQ; 500 µg in 100 µL) into the left cheek, and performed ISH in the TNC for *Fos*, and each candidate gene. For noxious heat stimulation under anesthesia we submerged the left hindpaw in 50°C water for 30s and performed ISH on lumbar spinal cord. In mice in which projection neurons were labeled with HSV-hEF1a-GFP-L10a we also used a *Gfp* probe. All mice were injected i.p. with an anesthetizing dose of Avertin (1.25%) 20-30 minutes before stimulation, and killed 15-30 min after stimulation with a lethal Avertin dose.

In situ hybridization (ISH): RNAscope Hiplex12 hybridization

To simultaneously detect up to 7 genes in spinal cord tissue, we performed fluorescent ISH using the RNAscope Hiplex12 ancillary kit (Cat. No. 324140; Advanced Cell Diagnostics) assay, which is similar to the protocol used for RNAscope multiplex described above, with the following differences: 1) frozen sections were fixed for 60 rather than 15 minutes at RT; 2) sections were incubated for 2h with all 7 probes at once; 3) all 3 amplifying solutions were incubated for 30 minutes each; 4) after 2 washes, the sections were incubated for 15 min at 40°C with the Hiplex Fluoro T1-T4 solution. After 2 final washes, the sections were incubated with the RNAscope DAPI solution for 30 sec at RT and covered with Prolong Gold Antifade mounting media (Fisher Scientific #P36930). Because only 3 targets can be

concurrently visualized, due to a limited number of detection channels available for microscopy (488, 550 and 647 channels), we performed 3 successive rounds of detection/imaging/cleaving on 3 consecutive days, as follows. After the first imaging round, the sections were incubated in 4X SSC until the coverglass easily detached from the slide (up to 24h), briefly washed with 4X SSC and then incubated twice for 15 min at RT with 10% cleaving solution (with 2 washes in between). After 2 rinses in washing buffer, the sections were incubated for 15 min at 40^{o}C with the Hiplex Fluoro T5-T8 solution, washed twice, immediately covered with Prolong Gold Antifade mounting media and imaged. A final round of detection/imaging was performed on the 3rd day as described above, using the Hiplex Fluoro T9-T12 solution. Images from each round were merged together using the RNAscope Hiplex registration software 300065-USM and Photoshop to adjust intensity and contrast.

Imaging and image analysis

All images were taken with an LSM 700 confocal microscope (Zeiss) equipped with 405-nm (5mW fiber output), 488-nm (10mW fiber output), 55-nm (10-mW fiber output), and 639-nm (5-mW fiber output) diode lasers using a 20x Plan Apochromat (20x/0.8) objective (Zeiss).

To calculate the final percentages of gene expression and spatial distribution, we averaged counts and percentages across sections in each animal, and again across animals per experimental group.

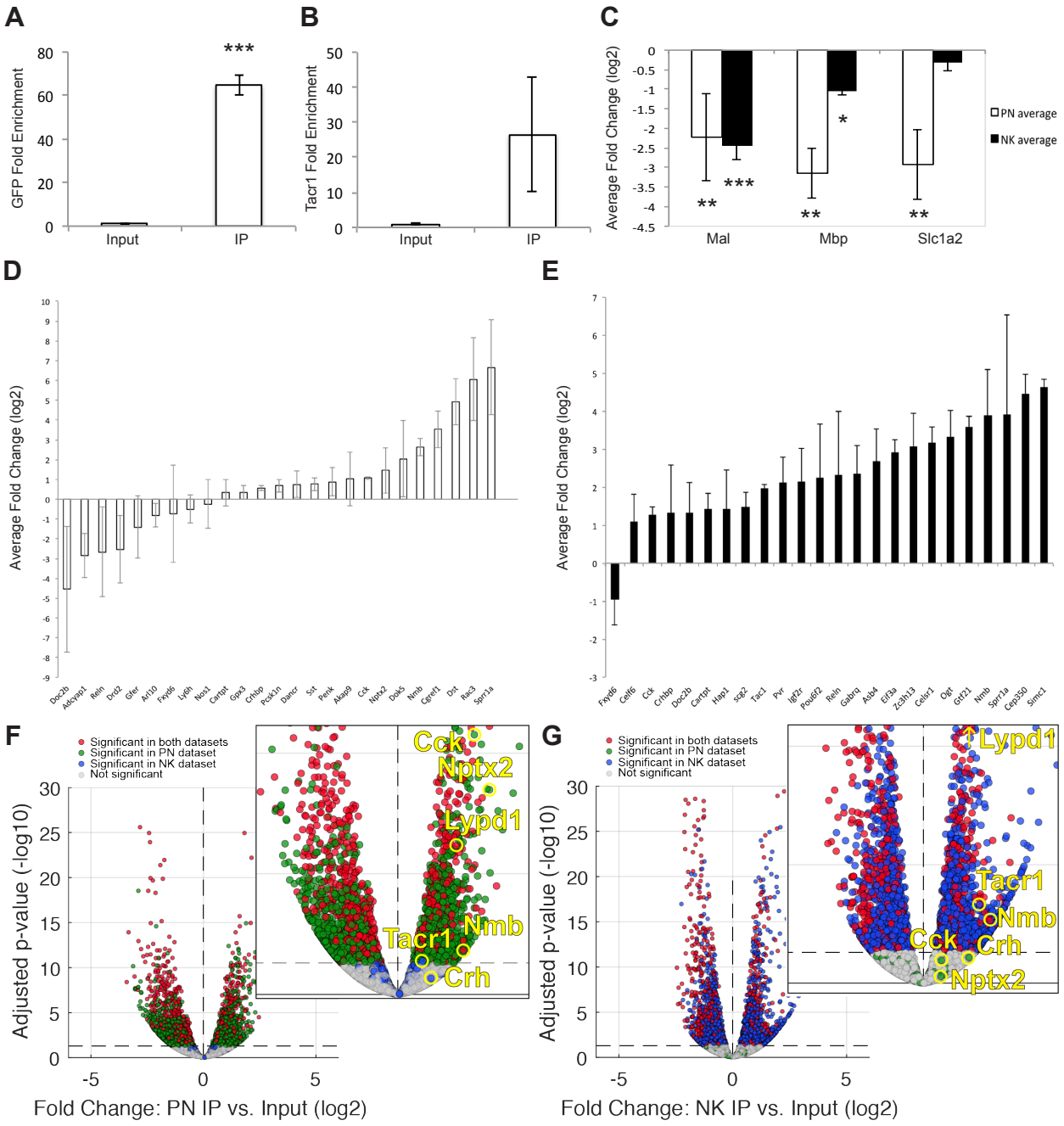

Figure S1: Validation of RNA purification, sequencing and Volcano plots of enriched and depleted genes in LPb-projecting neurons

(A) Bioanalyzer traces of projection neuron IPs from virus injected (top) and control (bottom) animals, for PN (HSV-GFPL10 injected, left) and NK (HSV-HAL10 injected, right) experiments. RIN, RNA Integrity Number. FU, Fluorescence intensity. (B) qPCR analysis showing average fold enrichment of *Gfp* mRNA in IP relative to Input from PN experiments. (C) qPCR analysis showing average fold enrichment of *Tacr1* mRNA in IP relative to Input from NK experiments. (D) qPCR analysis of average glial cell depletion in IPs relative to Input samples for PN and NK experiments. (E) qPCR analysis of enriched genes from PN RNAseq dataset (F) qPCR analysis of enriched genes from NK dataset (B-F) Data are normalized to Rpl27. n = 3 libraries. *p < 0.05 **p < 0.005 ***p < 0.0001 (G,H) RNAseq analysis represented as volcano plots for the PN (G) and NK experiments (H). Insets show enlarged area with genes of interest highlighted in yellow. Genes significantly changed in both PN and NK datasets are indicated in red, genes significantly changed PN but not NK are shown in green, and genes significantly changed in NK but not PN are shown blue. P < 0.05.

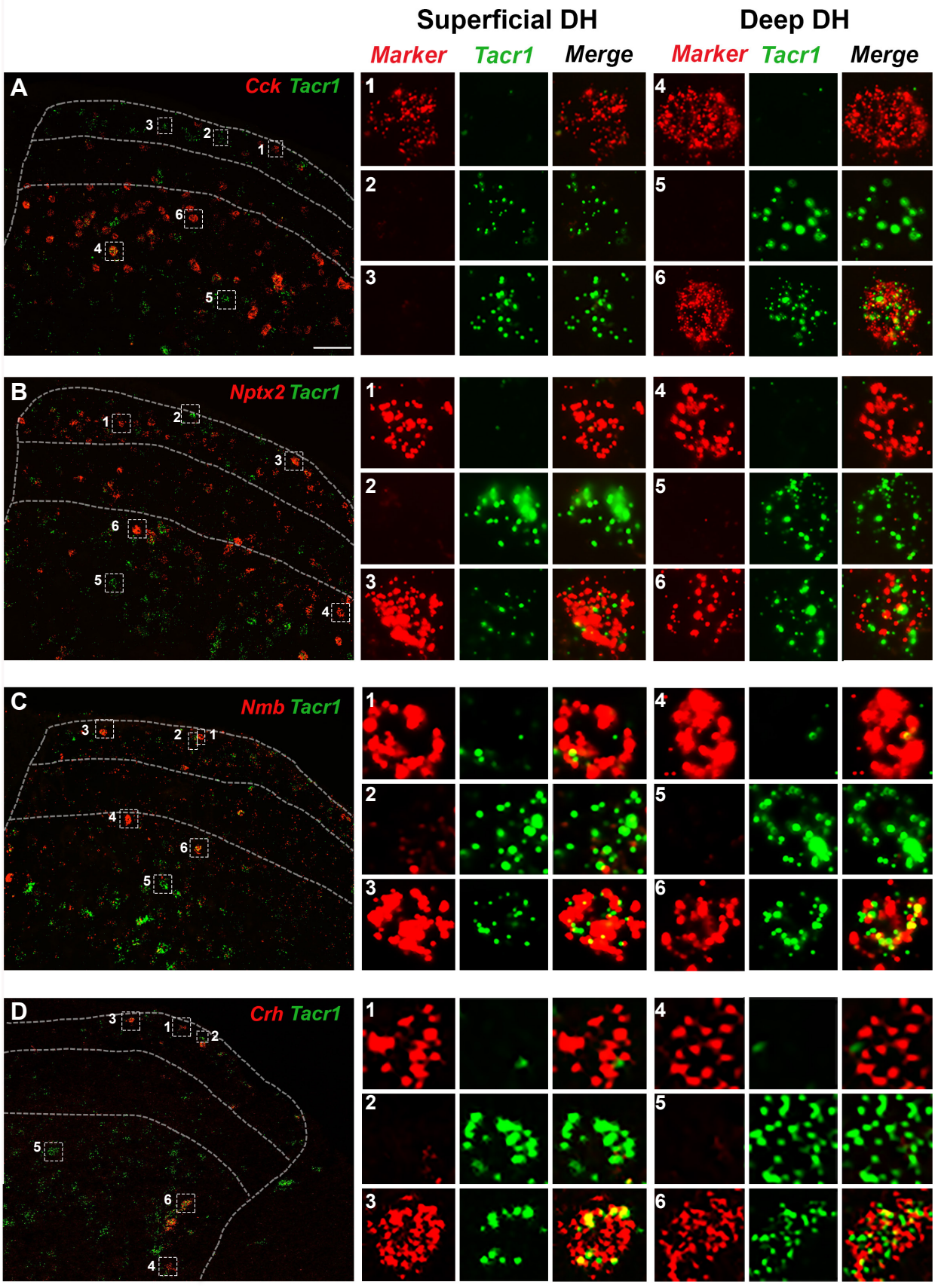
Superficial DH
Deep DH
Marker
Tacr1
Merge
Marker
Tacr1
Merge
A
Cck Tacr1
B
Nptx2 Tacr1
C
Nmb Tacr1
D
Crh Tacr1

Figure S2: Novel genes that mark subpopulations of NK1R-expressing cells
Representative sections from lumbar spinal cord showing coexpression of *Cck* (A), *Nptx2* (B), *Nmb* (C), or *Crh* (D) mRNA (red) with *Tacr1* mRNA (green) in both superficial and deep dorsal horn. Insets show magnified examples of single-labeled cells for each individual gene, or for *Tacr1*, as well as double-labeled cells, in superficial (middle panels) and deep (right panels) dorsal horn. Scale bars, 100μm.

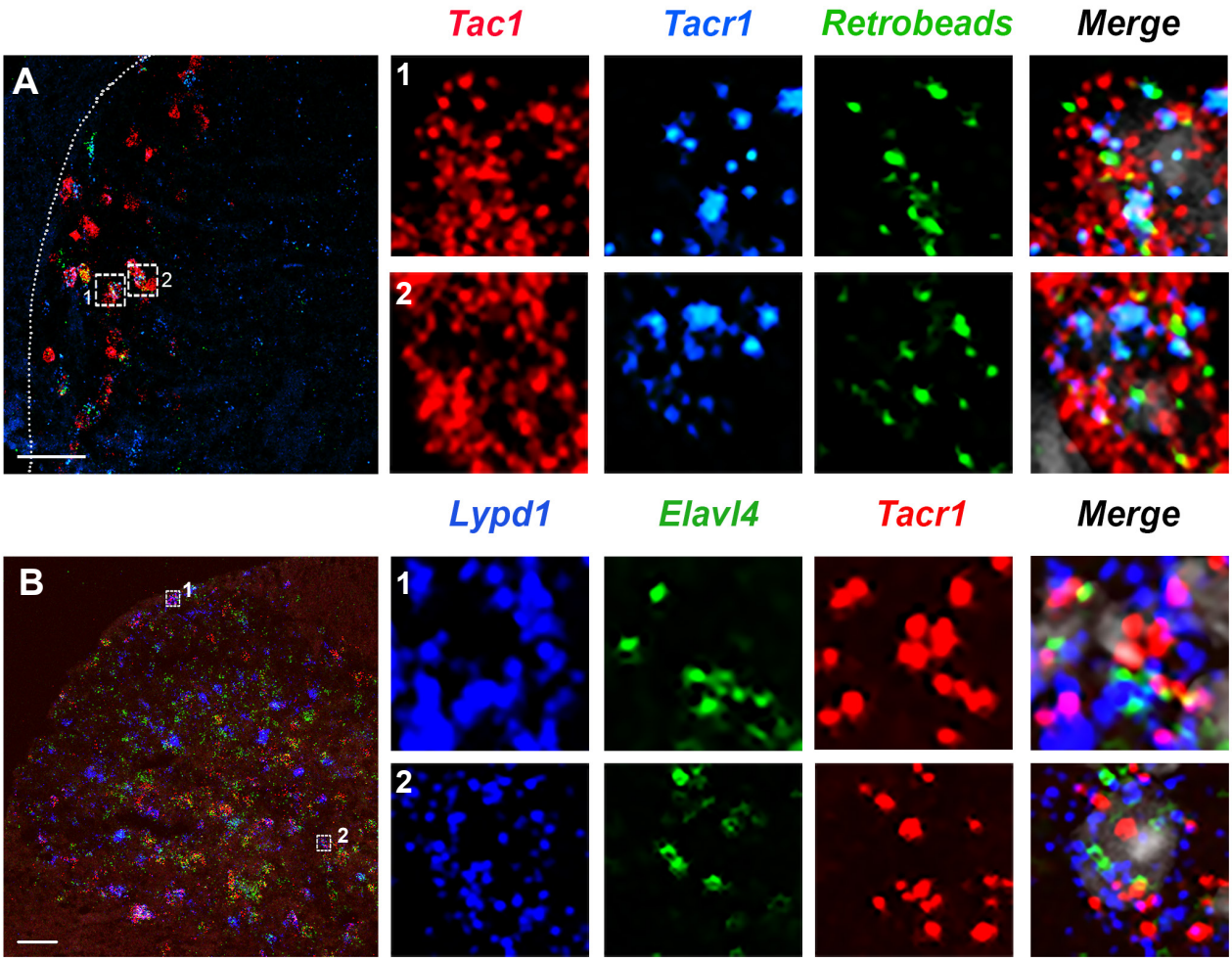

Figure S3: *Tac1*, *Lypd1* and *Elavl4* are expressed by subsets of NK1R-expressing neurons

(A) Representative section illustrates co-expression of *Tac1* (red) and *Tacr1* (blue) mRNA in Retrobead-labeled trigeminoparabrachial neurons (green). (B) Representative section illustrates co-expression of *Lypd1* (blue), *Elavl4* (green) and *Tacr1* (red) mRNA in lumbar dorsal horn neurons. Insets in A and B show enlarged examples of triple-labeled cells. Scale bars, 100μm.

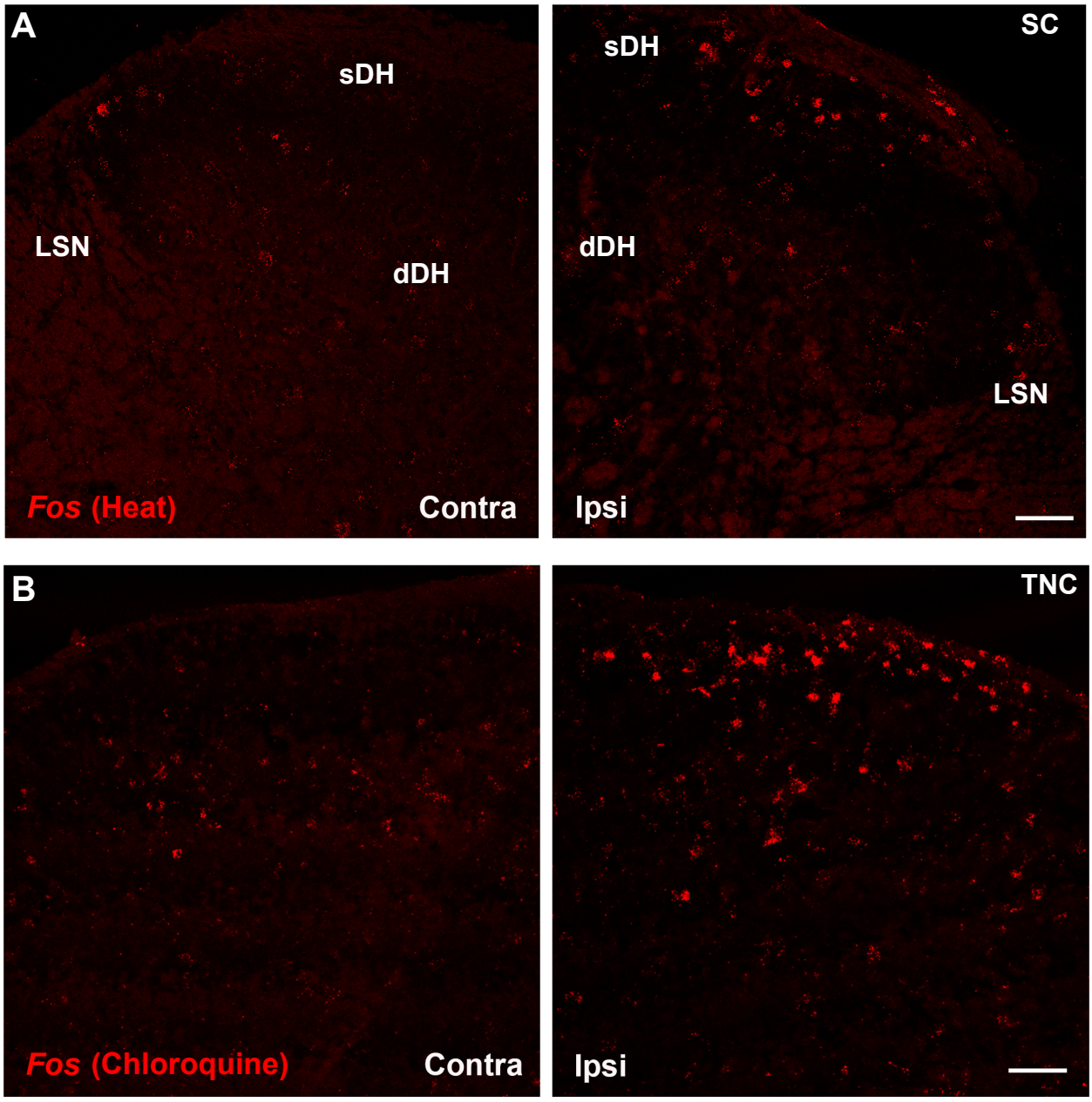

Figure S4: Algogen (heat) and pruritogen (chloroquine) stimulation evokes asymmetrical *Fos* mRNA in superficial dorsal horn and TNC
(A) Representative sections of lumbar spinal cord illustrate ipsilateral *Fos* expression in sDH in response to noxious heat (50^{o}C). (B) Representative sections of TNC illustrate ipsilateral *Fos* expression in response to chloroquine. sDH: superficial dorsal horn; dDH: deep dorsal horn; LSN: lateral spinal nucleus; SC: spinal cord; TNC: trigeminal nucleus caudalia. Scale bars, 100μm.

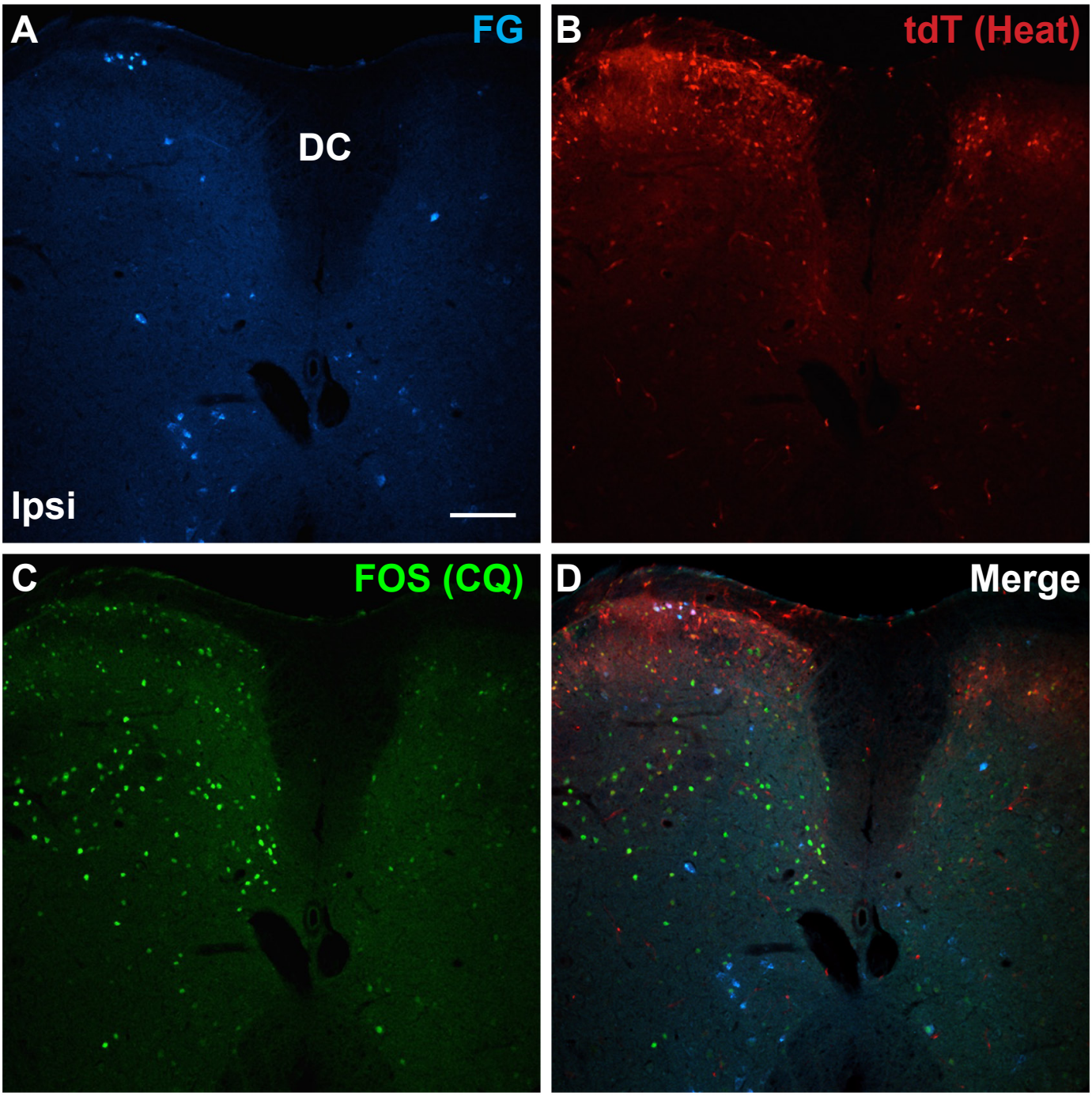

Figure S5: Permanent labeling of transiently activated spinal cord neurons via the TRAP2 (targeted recombination in active populations) assay

Representative sections of lumbar spinal cord from TRAP2-tdTomato mice illustrate (A) Fluorogold-labeled spinoparabrachial projection neurons (FG; blue), (B) heat-activated tdTomato (tdT; red) and (C) chloroquine (CQ)-activated Fos (green)-immunoreactive neurons. For multiple labeling, see Supp. Fig. 7. DC: dorsal columns. Scale bars, 100μm.

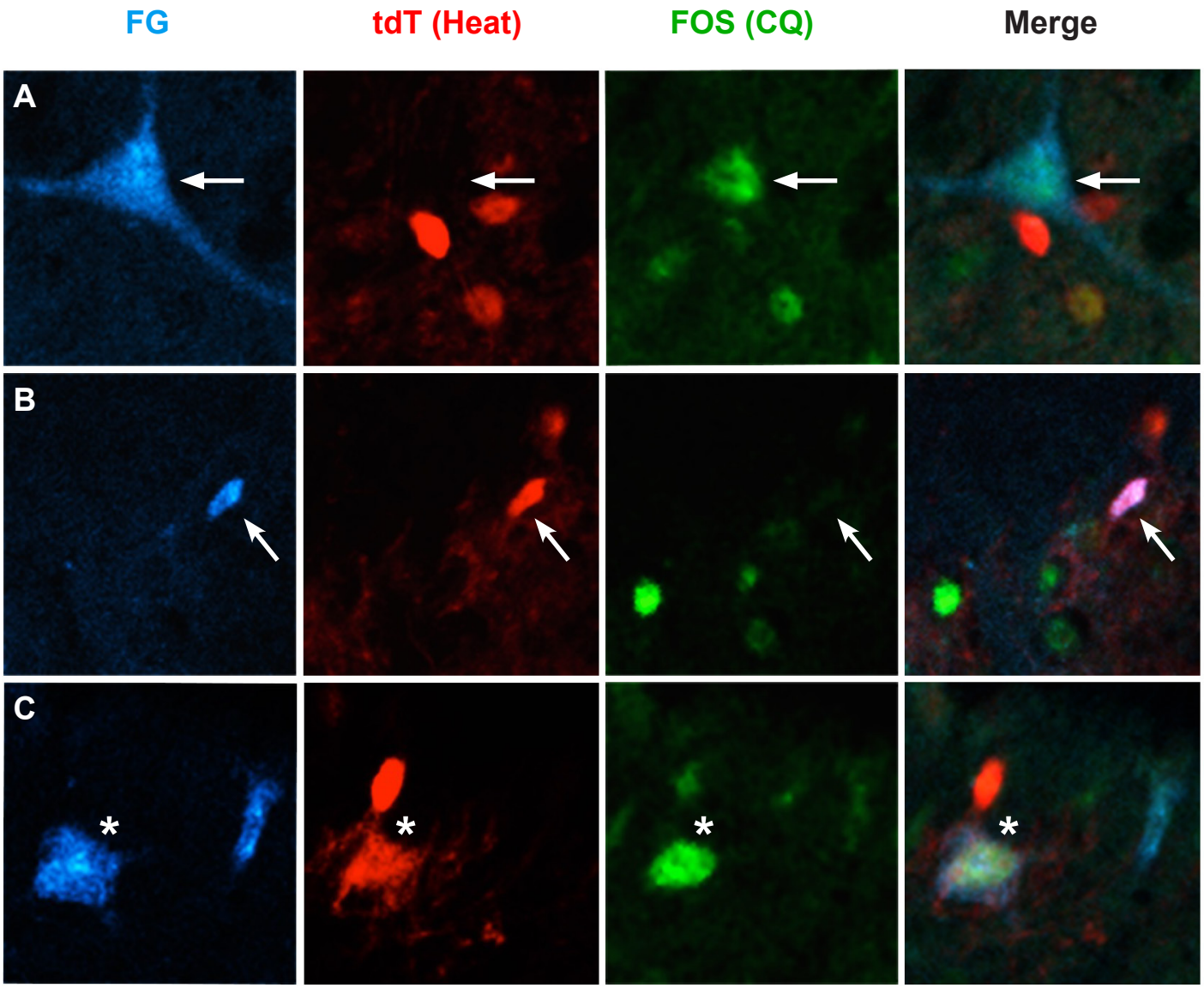

Figure S6: Retrogradely-labeled projection neurons respond to algogen (heat) and/or pruritogen (chloroquine) stimulation

Representative sections of lumbar spinal cord from TRAP2-tdTomato mice illustrate Fluorogold-labeled spinoparabrachial projection neurons (FG: blue), heat-activated tdTomato (tdT; red) and/or chloroquine (CQ)-activated Fos-immunoreactive (green) neurons. Arrows point to projection neurons that respond only to CQ (A) or heat (B). Stars point to a projection neuron that responds to both CQ and heat (C). Scale bars, 100μm.

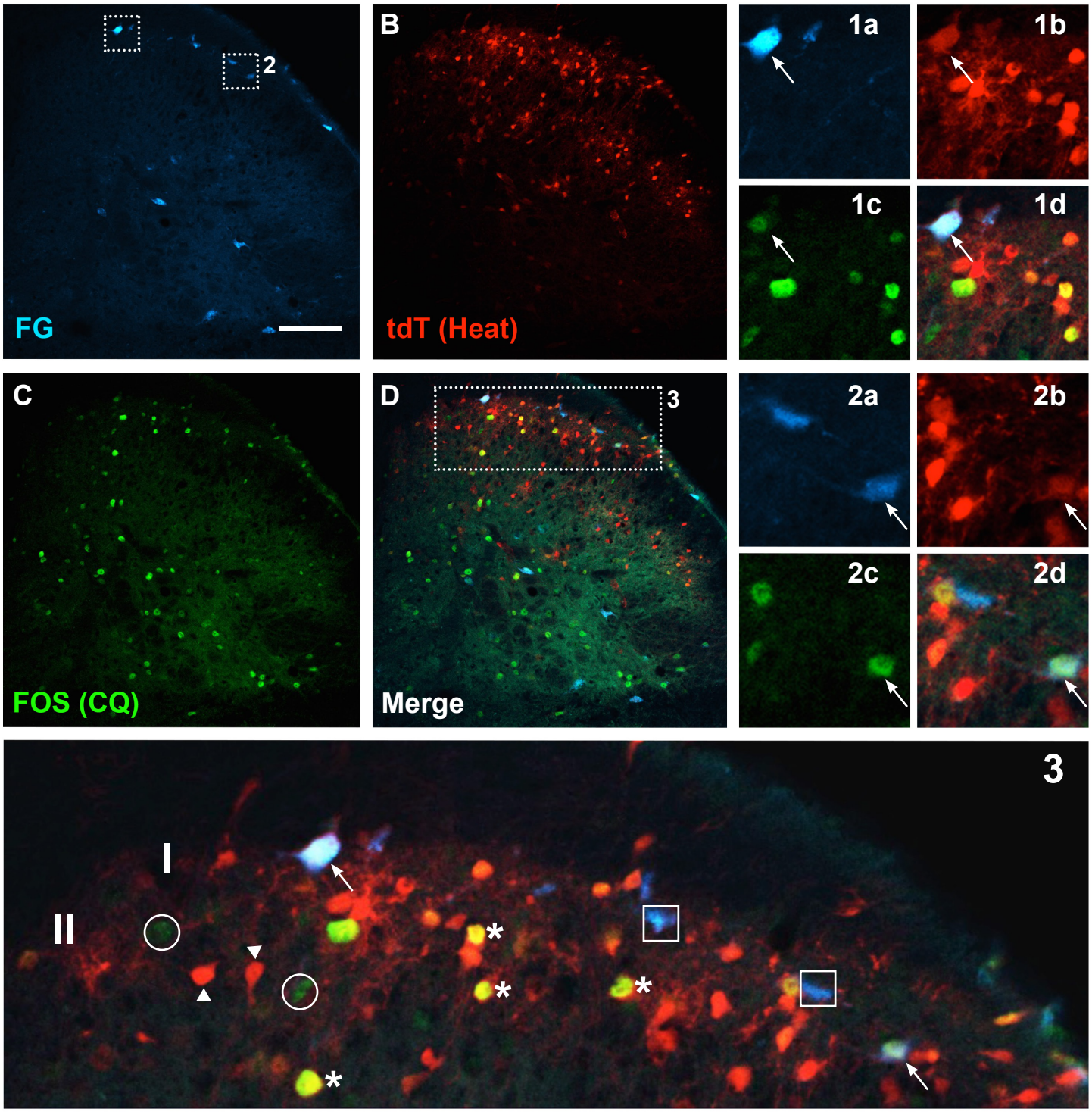
FG
2
B
tdT (Heat)
1a
1b
1c
1d
C
FOS (CQ)
D
3
Merge
2a
2b
2c
2d
3
I
II
E
% FG+ Projection Neurons
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Unresponsive
Responsive (TdT+ or Fos+)
PN (lam I)
F
% Responsive Projection Neurons
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Itch selective
Heat selective
Convergent
PN (lam I)

Figure S7: Activation of retrogradely labeled projection neurons first “Trapped” by noxious heat and later immunostained for Fos in response to choroquine (previous page). (A-D) Representative images of lumbar spinal cord from TRAP2-tdTomato mice illustrate Fluorogold-labeled spinoparabrachial projection neurons (A: FG; blue), heat-activated tdTomato (B: tdT; red) and chloroquine-activated Fos (C:green) positive neurons. Insets 1 and 2 are shown magnified to the right (a-d) and 3 below. The image below (3) highlights examples of single-labeled (circles, squares and arrowheads), double-labeled (asterisks) and triple-labeled (arrows) neurons. Scale bar, 100μm. (E) Histograms illustrate the percentage of lamina I projection neurons (PN) that responded (white bars) or did not respond (black bars) to one or both stimuli. (F) Histograms illustrate the percentage of lamina I projection neurons (PN) that responded only to CQ (bars), only to heat (blue bars) or to both stimuli (purple bars).

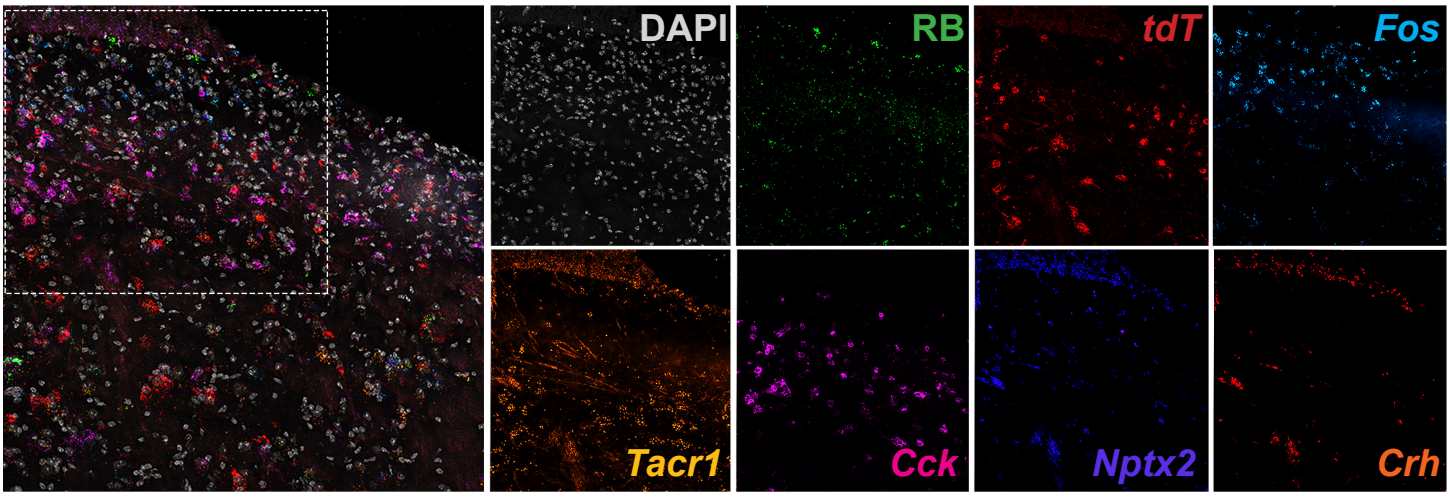

Figure S8: Highly multiplexed *in-situ* hybridization reveals widespread subsets of molecularly and functionally diverse projection neurons in the dorsal horn

(A) Representative section of lumbar spinal cord from Retrobead (RB)-injected TRAP2 mouse illustrates merge (A) and individual labeling for DAPI, RB, *Fos* mRNA (heat, 50^{o}C), tdTomato mRNA (*tdT*; chloroquine), and genes that mark subsets of projection neurons: *Tacr1, Cck, Nptx2* and *Crh*.

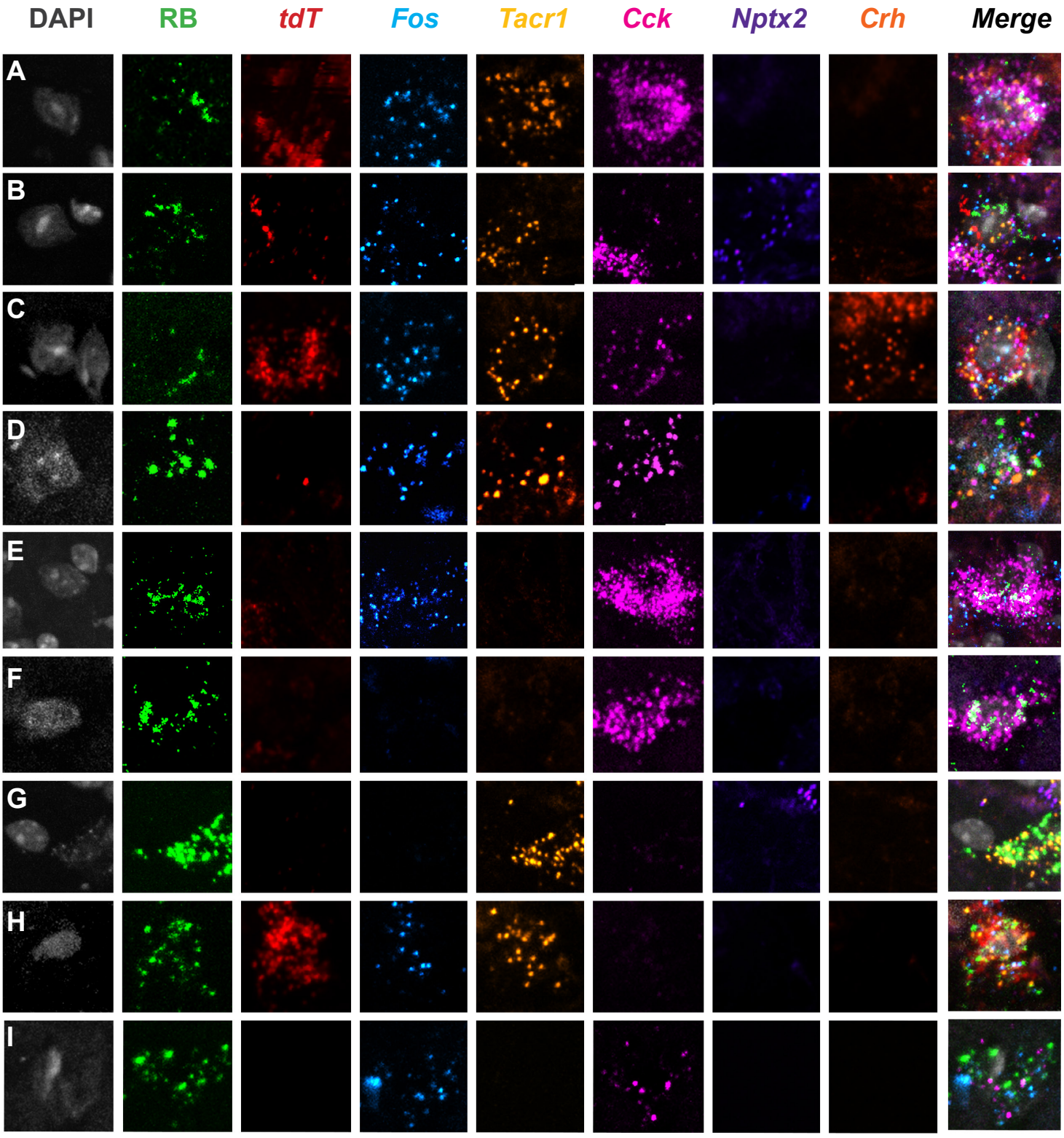

Figure S9: Highly multiplexed in-situ hybridization reveals subsets of molecularly and functionally diverse projection neurons in the deep dorsal horn and lateral spinal nucleus. Representative Retrobead (RB, green)-labeled projection neurons in deep dorsal horn (A-G) and lateral spinal nucleus (H-I) of TRAP2-stimulated mice. A subset of projection neurons (A-C, H) are activated by both 50oC heat (i.e., Fos mRNA+) and chloroquine (i.e., tdT+). Other subsets are activated by heat only (D-E, I), i.e. are td-Tomato negative or by neither stimulus (F-G). Each subset includes projection neurons that have varying combinations of the molecular markers: Tacr1, Cck, Nptx2, and Crh.

Table S1. List of ISH Probes Used

Probe Name	Cat No.	Channel
RNAscope® Probe- Mm-Cck	402271	C1
RNAscope® Probe- Mm-Crh	316091	C1
RNAscope® Probe- Mm-Fos	316921	C1
RNAscope® Probe- Mm-Lypd1	318361	C1
RNAscope® Probe- Mm-Nmb	459931	C1
RNAscope® Probe- Mm-Nptx2	316901	C1
RNAscope® Probe- Mm-Tac1	410351	C1
RNAscope® Probe- Mm-Tacr1	428781	C1
RNAscope® Probe- Mm-Cck	402271-C2	C2
RNAscope® Probe- Mm-Crh	316091-C2	C2
RNAscope® Probe- Mm-Elavl4	479581-C2	C2
RNAscope® Probe- Mm-Fos	316921-C2	C2
RNAscope® Probe- Mm-Gfp	409011-C2	C2
RNAscope® Probe- Mm-Nmb	459931-C2	C2
RNAscope® Probe- Mm-Tacr1	428781-C2	C2
RNAscope® Probe- Mm-Fos	316921-C3	C3
RNAscope® Probe- Mm-Gfp	409011-C3	C3
RNAscope® Probe- Mm-Lypd1	318361-C3	C3
RNAscope® Probe- Mm-Tacr1	428781-C3	C3
Not published		
RNAscope® Probe- Mm-Sprr1a	426871	C1
RNAscope® Probe- Mm-Vip	415961	C1
RNAscope® Probe- Mm-Grpr	317871	C2
RNAscope® Probe- Mm-Cartpt	432001-C3	C3
From Q4		
RNAscope® Probe- Mm-Casc4	435101	C1
RNAscope® Probe- Mm-Cdk16	423781	C1
RNAscope® Probe- Mm-Egr3	431101	C1
RNAscope® Probe- Mm-Lynx1	449071	C1
RNAscope® Probe- Mm-Mgat5	523961	C1
RNAscope® Probe- Mm-Neto2	434141	C1
RNAscope® Probe- Mm-Rnf10	496251	C1
RNAscope® Probe- Mm-Vegfb-CDS	424301	C1
RNAscope® Probe- Mm-Kcnh5	497691-C2	C2
RNAscope® Probe- Mm-Thra	519421-C2	C2
RNAscope® Probe- Mm-Tmtc1	518221-C2	C2
RNAscope® Probe- Mm-Cux2	469551-C3	C3
RNAscope® Probe- Mm-Gsg1l	478551-C3	C3
RNAscope® Probe- Mm-Necab3	428561-C3	C3

Dataset S1 (separate file). Differential Expression analysis of RNAseq hits from PN experiments

Dataset S2 (separate file). Differential Expression analysis of RNAseq hits from NK experiments

Dataset S3 (separate file). Q4 genes significantly enriched in PN dataset and depleted in NK dataset

References

1. Wercberger, R. (2019). DISSECTING PAIN AND ITCH CIRCUITS IN THE CENTRAL NERVOUS SYSTEM. *UCSF*. ProQuest ID: Wercberger_ucsf_0034D_11799. Merritt ID: ark:/13030/m5qv8kmn. Retrieved from https://escholarship.org/uc/item/7g59946x?