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Supplemental Information

Peptidergic CGRPα Primary Sensory Neurons Encode Heat and Itch and Tonically Suppress Sensitivity to Cold

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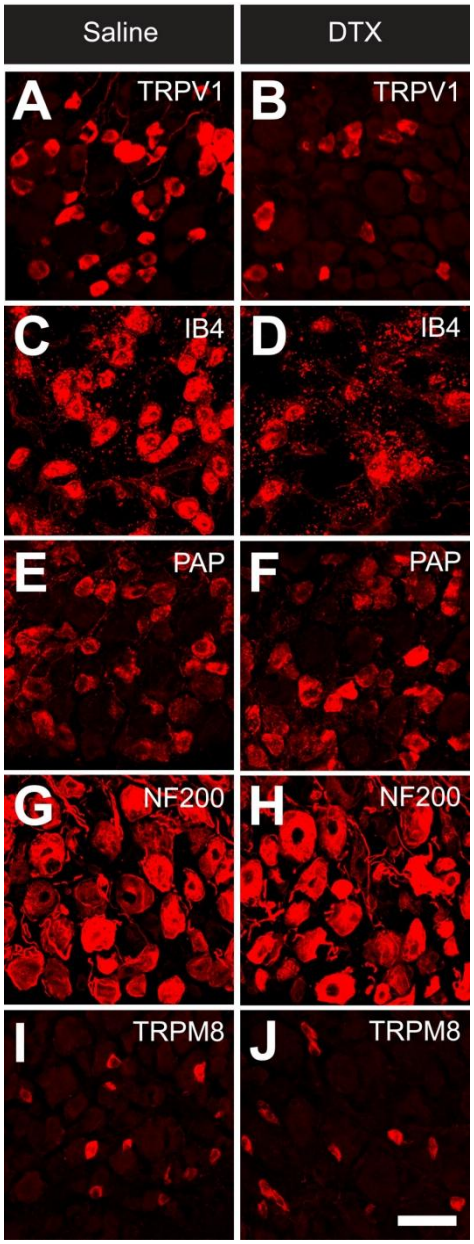

Figure S1, related to Figure 1. Sensory neuron markers in lumbar DRG from CGRPα-DTR$^{+/-}$ mice. Sections of lumbar DRG from CGRPα-DTR$^{+/-}$ mice treated with (A,C,E,G,I) saline or (B,D,F,H,J) DTX and immunostained with the indicated markers. Images were acquired by confocal microscopy. Scale bar in (J) is 50 µm. All tissue collected 7 days after second saline/DTX injection. n=3 male mice per condition.

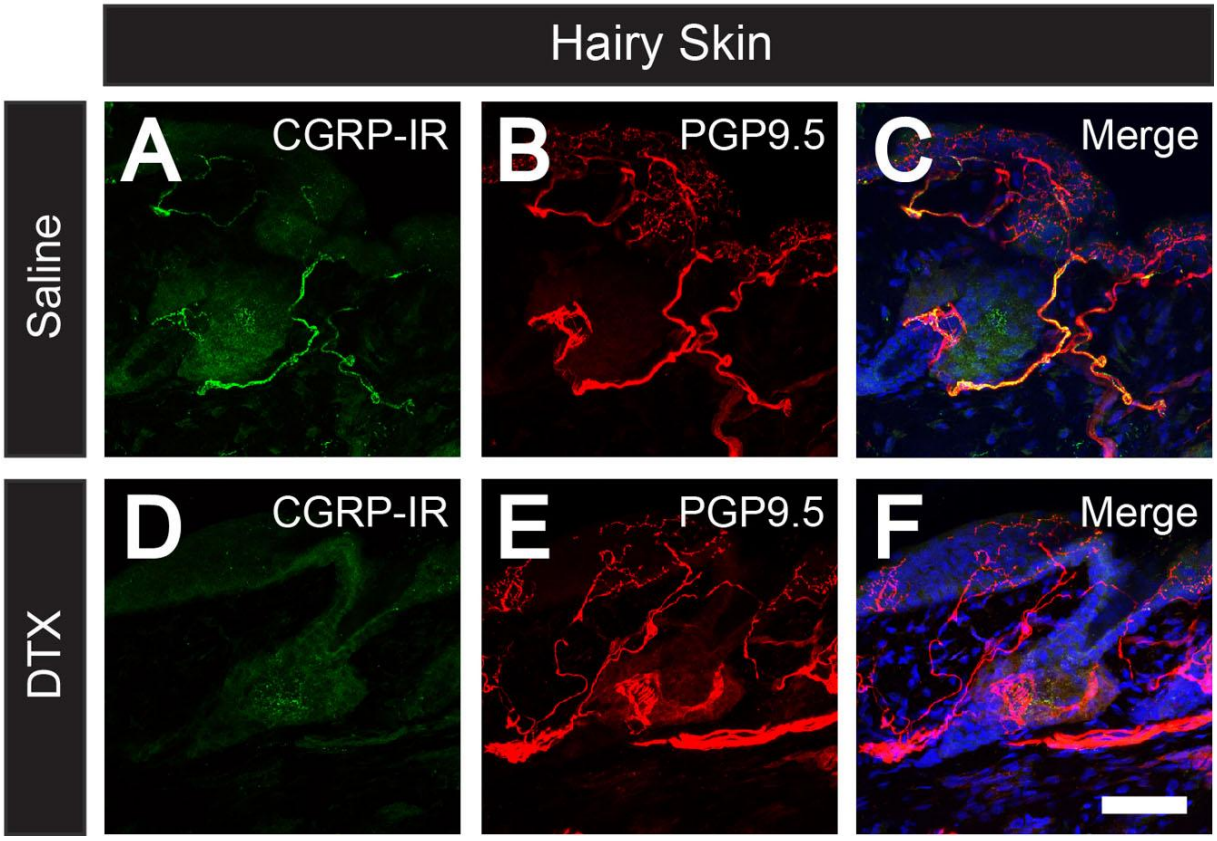

Figure S2, related to Figure 3. CGRP-IR afferents ablated in hairy skin. Sections of hindpaw hairy skin from saline- and DTX-treated CGRPα-DTR$^{+/-}$ mice were stained with antibodies to (A,D) CGRP and (B,E) the pan-nerve fiber marker PGP9.5. (C,F) Merged images were stained with the nuclear marker DRAQ5 to facilitate visualization of skin cells. Images were acquired by confocal microscopy. Scale bar in (F) is 100 µm.

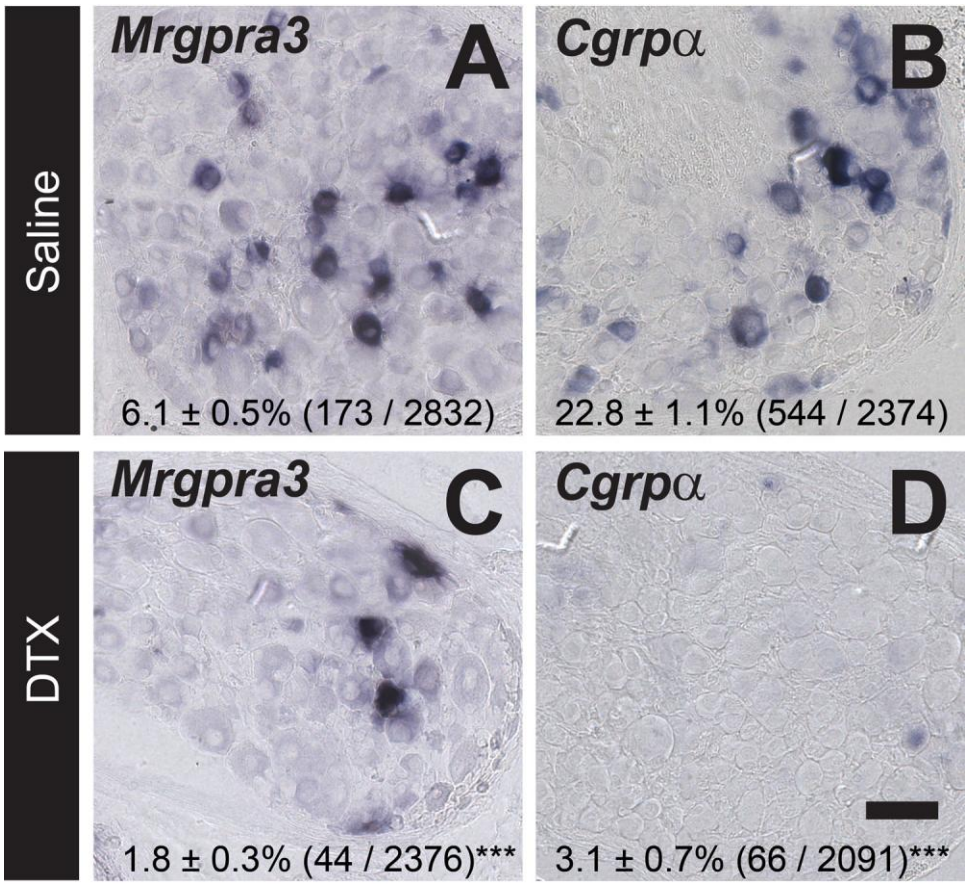

Figure S3, related to Figure 5. Ablation of *Mrgpra3*-expressing (chloroquine receptor) DRG neurons in CGRPα-DTR$^{+/-}$ mice. In situ hybridization with antisense probes to (A,C) *Mrgpra3* and (B,D) *Cgrpα* (*Calca*, positive control). Tissue was collected 7 days after second saline/DTX injection. Scale bar in (D) is 50 µm. Also shown: the percentage of neurons that were stained and, in parenthesis, the number of stained neurons / total number of neurons; presented as mean ± SEM. Cell counts were from representative sections of L3-L6 ganglia. ***p<0.0005.

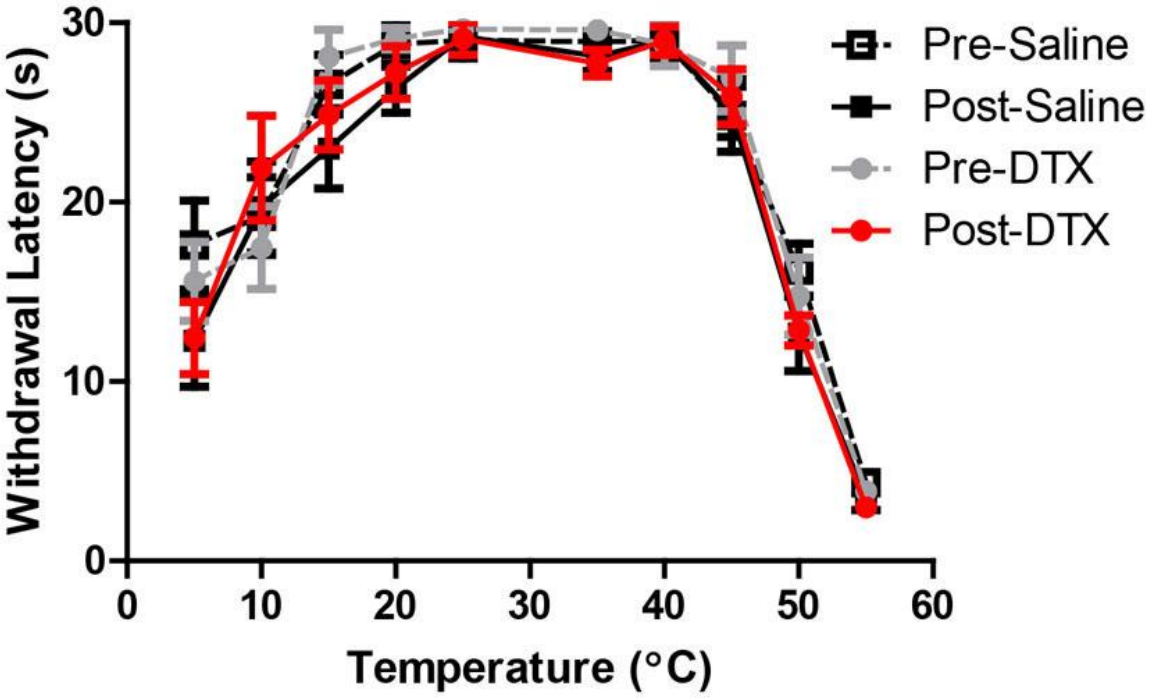

Figure S4, related to Figure 6. DTX-treatment does not affect cold or heat sensitivity in wild-type mice or body weight. Sensitivity to temperatures ranging from very cold to noxious heat was measured using a hindpaw withdrawal assay in wild-type male mice, pre- and post-saline/DTX treatment. n=10 male mice/group. Additionally, there were no significant differences in body weight between saline- and DTX-treated wild-type mice (21.2 ± 0.4 g versus 21.3 ± 0.5 g; 8 days after second saline/DTX-injection.)

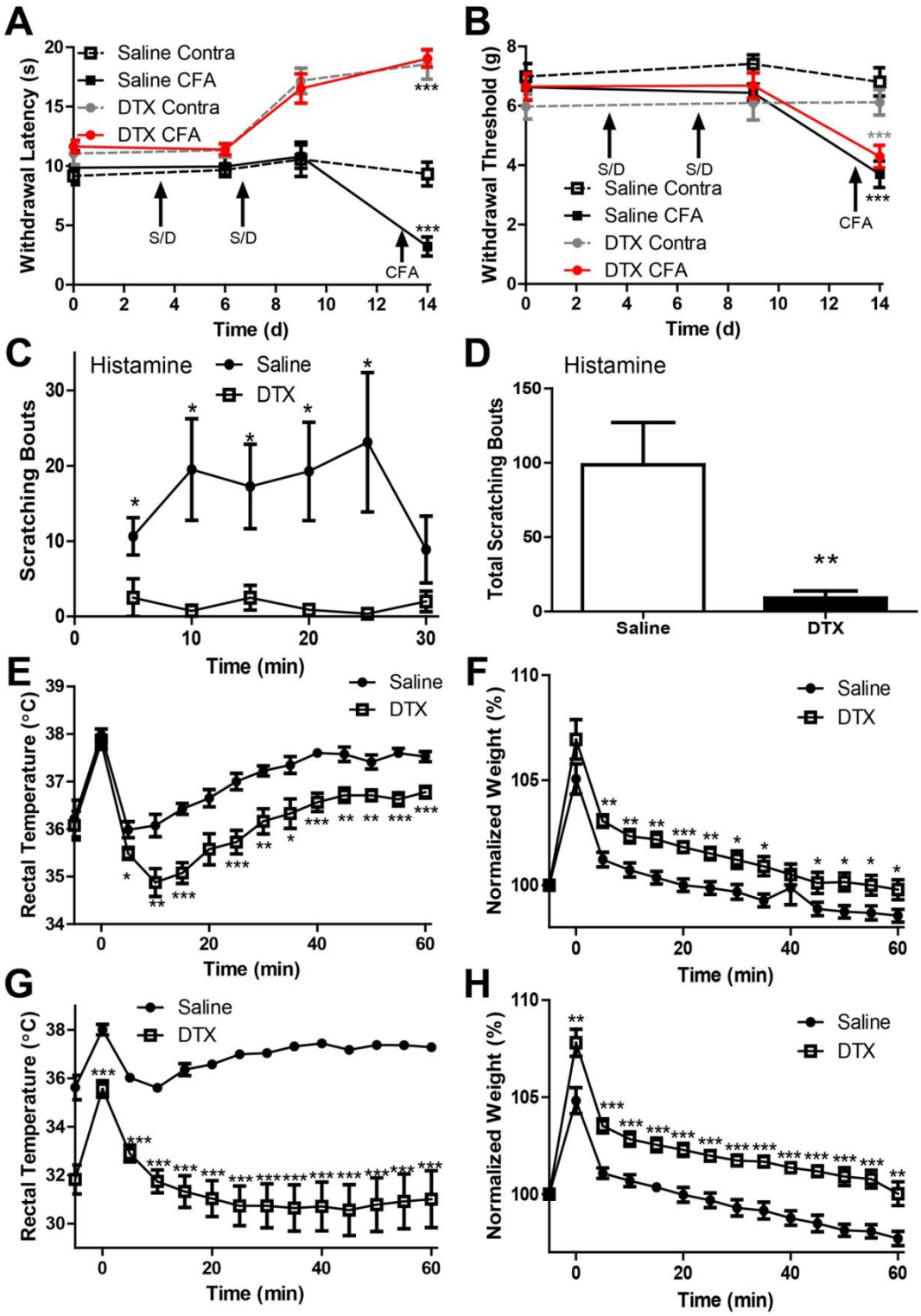

Figure S5, related to Figures 4, 5, 6 and Table 1. Behavioral and thermoregulatory phenotypes were reproduced when CGRPα-DTR$^{+/-}$ mice were treated with DTX

from a different vendor (Sigma-Aldrich). (A-B) CFA inflammatory pain model; (A) heat withdrawal latency, (B) mechanical withdrawal threshold. (C,D) Histamine-evoked itch. (E-H) Water repulsion assay (E,F) 3 days and (G,H) 6 days after the second saline/DTX injection. (E,G) Rectal (deep) body temperature and (F,H) body weight were monitored before (BL) and following brief (2 min) immersion of mice in 37°C water. Weights were normalized in (F,H) because DTX-treated mice weigh significantly less than saline-treated mice (F: 27.8 ± 0.5 g saline-treated versus 24.2 ± 0.6 g DTX-treated. H: 26.0 ± 0.5 g saline-treated versus 20.4 ± 0.5 g DTX-treated). Water retention phenotype apparent on day 3 and day 6 in these experiments versus day 6 in Figure 6D,F. T tests were used to compare responses at each time point between saline- and DTX-treated mice. (A-H) Data presented as means ± SEM. n=8 male mice/group. *p<0.05, **p<0.005, ***p<0.0005.

Table S1, related to Figure 7. Dorsal spinal cord neurons whose spontaneous EPSC frequency increased (responsive) or did not change (non-responsive) after pressure ejecting capsaicin (10 μM) or icilin (40 μM).

Neuron Type	Saline-Treated		DTX-Treated	
CAPSAICIN	***Responsive***	***Non-Responsive***	***Responsive***	***Non-Responsive***
Tonic	13	12	11	17
Transient (**)	8	4	0	9
Delayed	10	6	2	7
Other	0	4	4	3
Total (*)	31	25	18	37
ICILIN	***Responsive***	***Non-Responsive***	***Responsive***	***Non-Responsive***
Tonic	3	21	3	17
Transient	4	15	4	19
Delayed	0	13	1	16
Other	2	3	0	10
Total	9	52	8	62

*p<0.05, **p<0.005

Supplemental Experimental Procedures

Histology

The following primary antibodies were used for overnight incubations: chicken anti-GFP (1:600; Aves, GFP-1020), rabbit anti-GFP (1:600; Invitrogen, A11122), rabbit anti-CGRP (1:750, Peninsula, T-4032), rabbit and sheep anti-CGRP (1:250 and 1:200; Enzo Life Sciences, CA1134 and CA1137, respectively), goat anti-HB-EGF (hDTR; 1:1,000; R&D Systems, AF-259-NA), mouse anti-NeuN (1:250; Millipore, MAB377), goat anti-TRPV1 (1:5,000; Santa Cruz, SC-21498), chicken anti-PAP (1:4,000; Aves), rabbit anti-TRPM8 (1:100; gift from Masatoshi Takeichi), rabbit anti-PGP9.5 (1:500; Ultraclone), rabbit anti-NF200 (1:500; Sigma, N4142), rabbit anti-PKCβII (1:600; Santa Cruz, SC-210), and rabbit anti-PKCγ (1:750; Santa Cruz, SC-211). Fluorescently tagged secondary antibodies were purchased from Invitrogen and used at 1:200. When appropriate, fluorescent conjugates of Isolectin B4 (IB4; 1:100; Invitrogen) and the nuclear stain DRAQ5 (1:10,000; Axxora) were added to secondary antibody incubations. PAP, TRPV1, and hDTR staining was performed using tyramide signal amplification. All images were obtained using a Zeiss LSM 510 confocal microscope. In situ hybridization was performed with *Mrgpra3* and *Calca* antisense probes (Zylka et al., 2003).

Behavioral Assays

Heat sensitivity was measured by heating each hindpaw once per day using the Plantar Test apparatus (IITC) with a cut-off time of 20 s. For the tail immersion assay, each mouse was gently restrained in a towel, and the distal one-third of the tail was

immersed into a water bath heated to 46.5°C or 49°C or into 75% ethanol cooled to -10°C (Wang et al., 1995). The latency to flick or withdraw the tail was measured once per mouse. The cut-off was set at 40 s, 30 s, and 60 s, respectively. For the hot plate test, the latency to jump, shake, or lick a hindpaw was measured within a 30 s cut-off time. For determining mechanical sensitivity, we used an electronic von Frey apparatus (IITC) with semi-flexible tips. Two measurements for each hindpaw were taken and averaged to determine the paw withdrawal threshold in grams. The tail clip assay (noxious mechanical) and cotton swab assay (innocuous mechanical) were performed as described (Garrison et al., 2012; Lariviere et al., 2002). For the acetone test (Bautista et al., 2007), each mouse was placed into a Plexiglas chamber with a wire mesh floor, 50 μL of acetone was placed onto the left hindpaw, and the time spent licking was measured for 1 min. Icilin (60 μg in 25 μL injection volume) was injected into one hindpaw and the number of flinches in 10 min was counted. The cold plantar assay was performed with mice resting on the glass surface of the Plantar Test apparatus (IITC) (Brenner et al., 2012). For the two-temperature preference assay, each mouse was placed into a Plexiglas chamber covering two metal surfaces that could be set at different temperatures (Bautista et al., 2007; Dhaka et al., 2007). The amount of time mice spent on each side over a 10 min period was recorded. Capsaicin (0.1 μg/μL in 0.9% saline/10% ethanol/0.5% Tween 80) was injected into the left hindpaw, and the amount of time spent licking was measured for 5 min. Hot and cold sensitivity was assessed on a metal plate heated/cooled to a range of temperatures (5-55°C), with a cut-off time of 30 s, as described (Gentry et al., 2010). For measuring itch responses, histamine (10 μg/μL), chloroquine (CQ; 4 μg/μL) or β-alanine (20 μg/μL)

dissolved in 0.9% saline was injected subcutaneously into the nape of the neck (50 μL injection volume). The number of scratching bouts was measured for 30 min in 5 min blocks. One bout consisted of a set of scratches at the injection site until the hindpaw was either licked or placed onto the floor. For the water repulsion assay (Westerberg et al., 2004), the mouse was immersed in a 37°C water bath for 2 min. The mouse was removed from the water and placed onto a paper towel for 5 s, then weight and rectal temperature (deep body temperature, T_b, measured using a digital thermometer, Acorn Temp TC Thermocouple) were measured every 5 min for 60 min. The CFA model inflammatory pain and the SNI model of neuropathic pain were performed as described (Shields et al., 2003; Zylka et al., 2008). Plasma extravasation with Evans Blue dye was performed as described (Green et al., 1992), except using hindpaw glabrous skin.

Supplemental References

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