Cell, Volume *139*

Supplemental Data

Sensory Neuron-Specific GPCR Mrgprs

Are Itch Receptors Mediating

Chloroquine-Induced Pruritus

Qin Liu, Zongxiang Tang, Lenka Surdenikova, Seungil Kim, Kush N. Patel, Andrew Kim, Fei Ru, Yun Guan, Hao-Jui Weng, Yixun Geng, Bradley J. Undem, Marian Kollarik, Zhou-Feng Chen, David J. Anderson, and Xinzhong Dong

Supplemental Experimental Procedures

Generation of *Mrgpr-cluster*$\Delta^{-/-}$ mice

For the *MrgprA1* construct, the PCR primer sequences for the 5' arm are 5'-AAGCTTGTTCCACTTGGTATC-3' and 5'-CAGGCGCGCCATGGTATTGTCCATTGGATTAG-3'. The PCR primer sequences for the 3' arm are 5'-GAGTTTAAACTGTTGGGTCCTGTTTACT-3' and 5'-CAGGCGCGCCTGATGAAGAGCCTTTGCCTGGC-3'. The lengths of the 5' and 3' arms are 3.8 and 3.0 kb, respectively. For the *MrgprB4* construct, the PCR primer sequences for the 5' arm are 5'-CAGGCGCGCCTGCTTAGGAATTTTCCACTGG-3' and 5'-CTGTACACCATAGTCTCTAGAAAGG-3'. The PCR primer sequences for the 3' arm are 5'-CAGGCGCGCCAGTAGTTGAGTGAGTCCCTGG-3' and 5'-CAGTTTAAACGATTTACCTGCAAACCTCCTG-3'. The lengths of the 5' and 3' arms are 4.3 and 3.0 kb, respectively. The *MrgprA1* targeting vector was constructed by inserting an *eGFPf/IRES-rtTA/loxP/Ace-Cre/PGK-neomycin/loxP* cassette between the 5′ and 3′ arms. For the *MrgprB4* targeting vector, a *PLAP/loxP/PGK-hygromycin* cassette was cloned between the 5′ and 3′ arms.

These two vectors were electroporated into mouse CJ7 embryonic stem (ES) cells by two rounds of electroporation. Correct recombination at both loci was verified by PCR with genomic DNA of the clones using primer sets flanking the 5' and 3' arms of the targeting construct. This was further confirmed by Southern blot hybridization using probes that flanked the 5' arms of the targeting constructs. A third round of electroporation with *CMV-Cre* was conducted in an ES cell clone with both *MrgprA1* and *MrgprB4* loci correctly targeted. The deletion of genomic DNA between the two loci (845 kb) in the ES cells by *Cre/loxP*-mediated recombination was confirmed by PCR using primers flanking the two loci and Southern blot. Chimeric *Mrgpr-cluster*Δ mice were produced by blastocyst injection of positive ES cells. *Mrgpr-cluster*$\Delta^{+/-}$ mice were generated by mating chimeric mice to C57Bl/6 mice. Manuscript describing the generation of GRP knockout mice is in preparation.

Behavioral Studies

Tail immersion test: Mice were gently restrained in a 50 ml conical tube into which the mice voluntarily entered. The protruding one third of the tail was then dipped into a water bath at 50 °C. Latency to respond to the heat stimulus with vigorous flexion of the tail was measured three times and averaged.

Hot plate test: A clear plexiglass cylinder was placed on the plate and the mice were placed inside the cylinder. The onset of brisk hindpaw lifts and/or flicking/licking of the hindpaw was assessed.

Cold plate test was carried out as previously described (Dhaka et al., 2007).

von Frey mechanical assay: Mice were placed under a transparent plastic box (4.5 × 5 × 10 cm) on a metal mesh. Mechanical sensitivity was measured with von Frey monofilaments using the frequency method (Mansikka et al., 2004) for the acute sensitivity test.

Acetic acid test: Mice were acclimated for 20 minutes in a transparent plexiglass box at room temperature. A diluted solution of acetic acid (0.6% acetic acid in saline) was injected intraperitoneally. Using 1 ml insulin syringe and 30G needle, 15 ml of diluted acetic acid was injected per kg body weight of the mouse. The number of writhings was recorded for 20 minutes.

The spinal nerve injury was carried out as previously described (Guan et al., 2007). Radiant heat (Hargreaves) test was performed as previously described (Caterina et al., 2000). The scratching behavior response to histamine, compound 48/80, and CQ was assayed as previously described (Green et al., 2006; Kuraishi et al., 1995; Sun and Chen, 2007).

Whole-cell current-clamp recordings of cultured DRG neurons

Neurons plated on cover slips were transferred into a chamber with medium (the extracellular solution: ECS) of the following composition (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, Glucose 5, with pH adjusted to 7.38 using NaOH. The intracellular pipette solution (ICS) contained (in mM): KCl 135, MgATP 3, Na_2ATP 0.5, $CaCl_2$ 1.1, EGTA 2, Glucose 5, with pH adjusted to 7.38 using KOH and osmolarity adjusted to 300 mOsm with sucrose. Chloroquine was stored at -20°C and diluted to 1mM in ECS before use. Patch pipettes had resistances of 2-4 MΩ. In current clamp recordings, action potential measurements were performed with an Axon 700B amplifier and the pCLAMP 9.2 software package (Axon Instruments). Electrodes were pulled (Narishige, Model pp-830) from borosilicate glass (WPI, Inc). Neurons were perfused with 1 mM CQ for 20 sec. All experiments were performed at room temperature (~25°C).

Histamine analysis

To obtain total histamine content of mouse skin, we dissected abdominal skin from wild type and SASH mice, cut it into small segments and incubated for 60 minutes in 4% perchloric acid. The histamine released into the supernatant solution was analyzed by automated fluorometry as previously described (Siraganian, 1974).

Cultures of dissociated DRG neurons

Dorsal root ganglia from all spinal levels of 4-week old mice or rats were collected in cold DH10 (90% DMEM/F-12, 10% FBS, 100 U/ml penicillin, and 100 µg/ml Streptomycin, Gibco) and treated with enzyme solution (5 mg/ml Dispase, 1 mg/ml Collagenase Type I in HPBS without Ca^{++} and Mg^{++}, Gibco) at 37°C. Following trituration and centrifugation, cells were resuspended in DH10, plated on glass cover slips coated with poly-D-lysine (0.5 mg/ml, Stoughton, MA) and laminin (10 µg/ml, Invitrogen), cultured in an incubator (95% O_2 and 5% CO_2) at 37°C and used within 24 hours.

Culture HEK293 cells

HEK293 cells were cultured in growth medium consisted of 90% DMEM, 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml Streptomycin (Invitrogen) at 37°C in the presence of 95% O_2 and 5% CO_2. HEK293 cells were transfected with Mrgpr-expression constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Single Cell RT-PCR

PCR conditions: 95°C 15 min and 50 cycles of 30 sec at 94°C, 30 sec at 60°C and 60 sec at 72°C, followed by 10 min at 72°C. The MrgprA3-specific primers used were 5' CGACAATGACACCCACAACAA 3' and 5' GGAAGCCAAGGAGCCAGAAC 3'. The primers for β-actin were 5' GTGGGAATGGGTCAGAAGG 3' and 5' GAGGCATACAGGGACAGCA 3'.

RT-PCR analysis

Total RNA was extracted from various tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done using Superscript first strand (Invitrogen). PCR conditions: 94°C 3 min and 40 cycles of 15 sec at 94°C, 30 sec at 52°C, and 45 sec at 72°C. The MrgprA3-specific intron-spanning primers (to avoid genomic contamination) used are 5' TTCTGTAGTGACTGTATCCTTCCTTC 3' and 5' GCGGTTACTTAGATAACCATTA 3'.

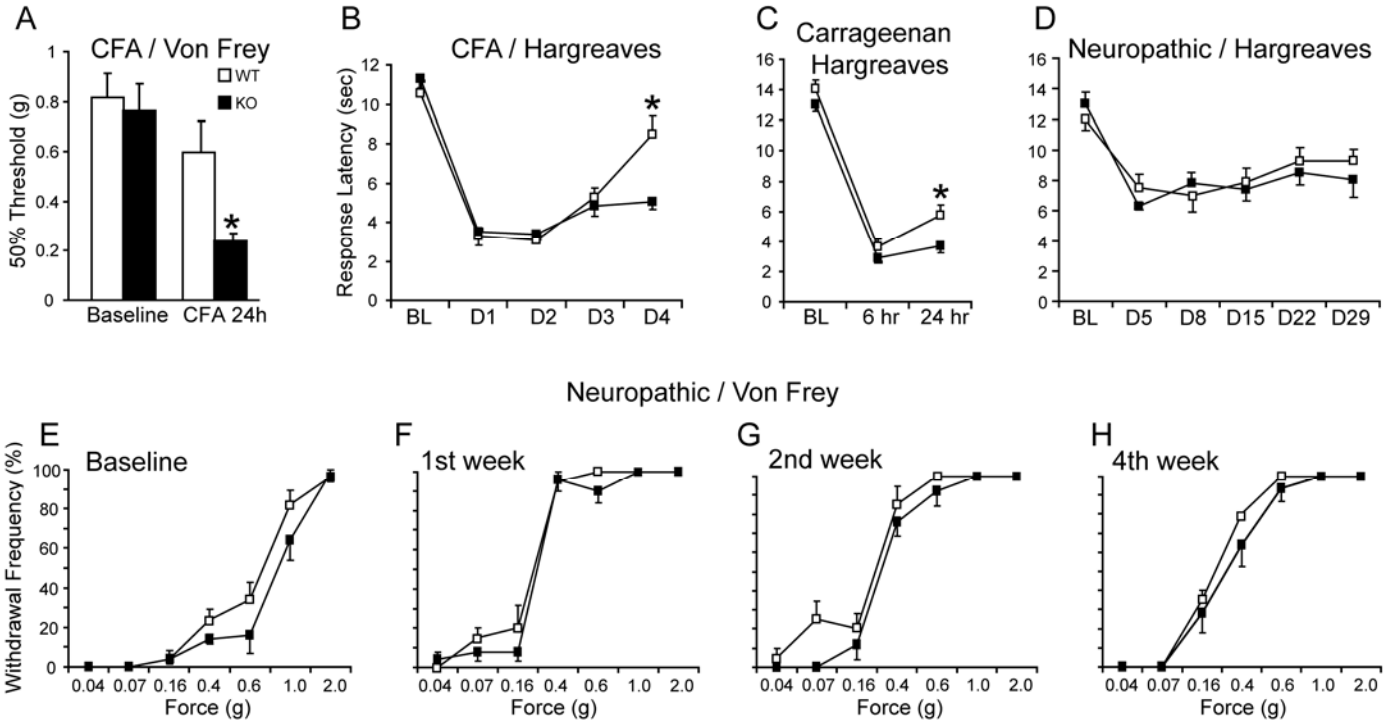

Figure S1. (**A-C**) *Mrgpr-cluster*$\Delta^{-/-}$ mice show enhanced inflammatory pain responses. (**A**) *Mrgpr-cluster*$\Delta^{-/-}$ mice displayed stronger mechanical allodynia 24 hours after intraplantar injection of complete Freund's adjuvant (CFA, 6 μl, 50%) as compared with WT (n = 13). (**B**) CFA-induced thermal hyperalgesia was indistinguishable between WT and *Mrgpr-cluster*$\Delta^{-/-}$ mice within three days after injection. However, on the fourth day, WT mice recovered compared to *Mrgpr-cluster*$\Delta^{-/-}$ mice (n = 13). (**C**) *Mrgpr-cluster*$\Delta^{-/-}$ mice showed stronger thermal hyperalgesia 24 hours after intraplantar injection of 1% carrageenen as compared with WT (10 μl, n = 14). (**D-H**) *Mrgpr-cluster*$\Delta^{-/-}$ mice show normal neuropathic pain responses. *Mrgpr-cluster*$\Delta^{-/-}$ mice displayed comparable thermal hyperalgesia (**D**) and mechanical allodynia (**E-H**) as WT (n = 10) within 4 weeks after lumbar L5 spinal nerve ligation surgery. The data are presented as mean ± SEM. *, $p<0.05$; two-tailed unpaired *t*-test.

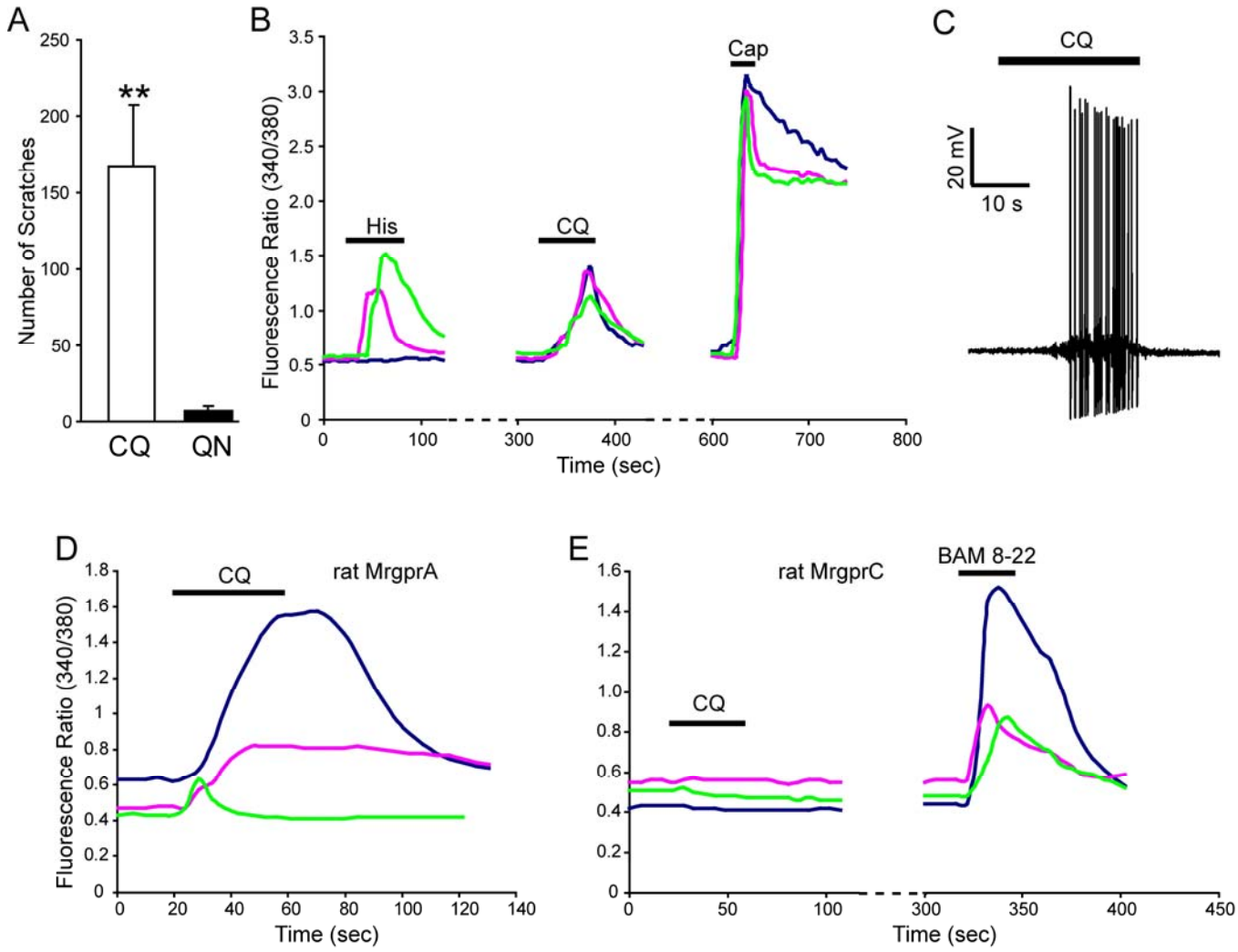

Figure S2. CQ induces neuronal and behavioral responses in rats. (**A**) The total scratching bouts during the first 30 min after CQ injection (500 μg/50 μl; n=8) were significantly more than those after quinoline (QN) injection (500 μg/50 μl; n = 7). (**B**) Most CQ-responsive rat DRG neurons also responded to histamine (50 μM) and capsaicin (1 μM) with increased $[Ca^{2+}]_i$ monitored by calcium imaging. 15% of total rat DRG neurons respond to CQ which is consistent with the percentage of rat neurons expressing MrgprA determined by in situ hybridization from our previous studies (Zylka et al., 2003). (**C**) CQ (2 mM) induced action potentials in rat DRG neurons. All CQ-sensitive neurons (as determined by calcium imaging, n=6) elicited a train of action potentials evoked by subsequent CQ treatment. In contrast, no CQ-insensitive neurons (data not shown, n=6) generated any action potentials to the drug. (**D**, **E**) show representative traces from 3 different *Mrgpr-cluster*$\Delta^{-/-}$ DRG neurons electroporated with rat MrgprA or MrgprC in calcium imaging assays. Rat MrgprA conferred CQ sensitivity to Mrgpr-deficient neurons (**D**, n=7) but rat MrgprC did not (**E**, n=10). However, rat MrgprC responded to BAM8-22 (10 μM) indicating it is a functional receptor. The data are presented as mean ± SEM. **, P<0.01; two-tailed unpaired *t*-test.

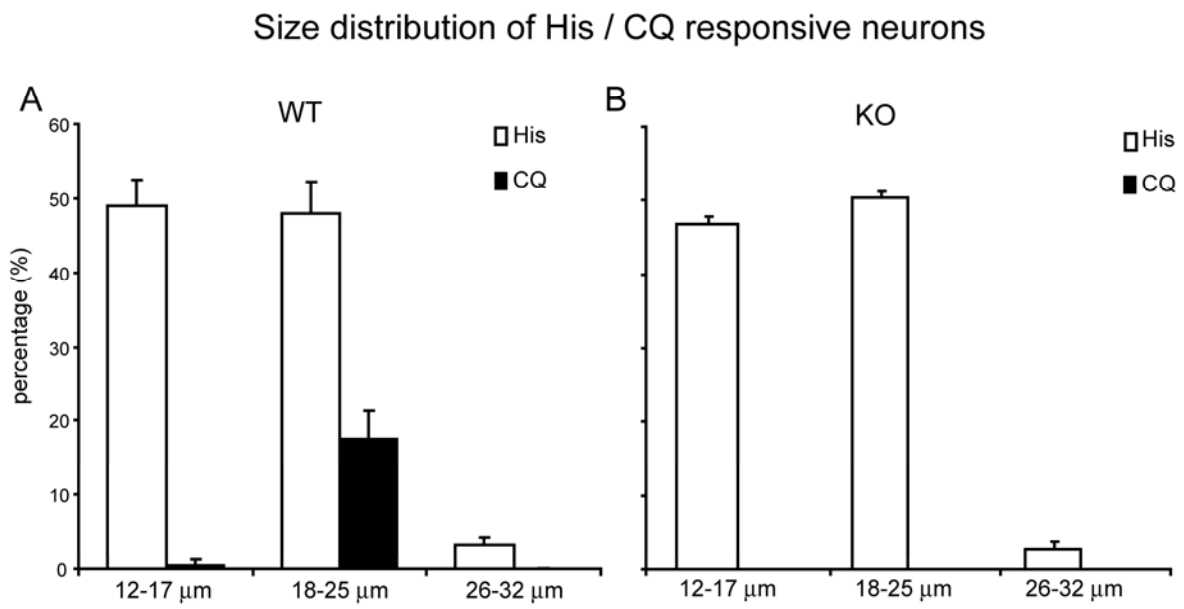

Figure S3. CQ-responsive neurons represented a specific subpopulation of histamine-responsive neurons. Histamine-sensitive neurons have a wide range of cell diameters from 12 to 32 μm whereas CQ-sensitive cells have a narrow range (19 to 22 μm, averaging 20.38 ± 0.18 μm, n=44). Histamine-responsive neurons were divided into three subgroups according to their cell body diameters (12-17 μm, 18-25 μm, 26-32 μm) (Breese et al., 2005; Brumovsky et al., 2006; Stucky et al., 2002). The first two populations accounted for 48.84 ± 3.65% and 47.82 ± 4.46% of total histamine-responsive neurons, respectively (**A**). Similar results were observed in *Mrgpr-cluster*$\Delta^{-/-}$ DRG neurons (**B**). These data provide additional evidence that deletion of Mrgpr genes does not affect DRG neuron survival. The black bars show the percent of histamine-responsive neurons that also responded to CQ. Most CQ-sensitive neurons fell into the subgroup of histamine-responsive cells with 18 to 25 μm diameter in WT, which account for 18% of all histamine-responding neurons (**A**). No CQ-sensitive neurons were found in *Mrgpr-cluster*$\Delta^{-/-}$ DRG (**B**, n = 3 per genotype).

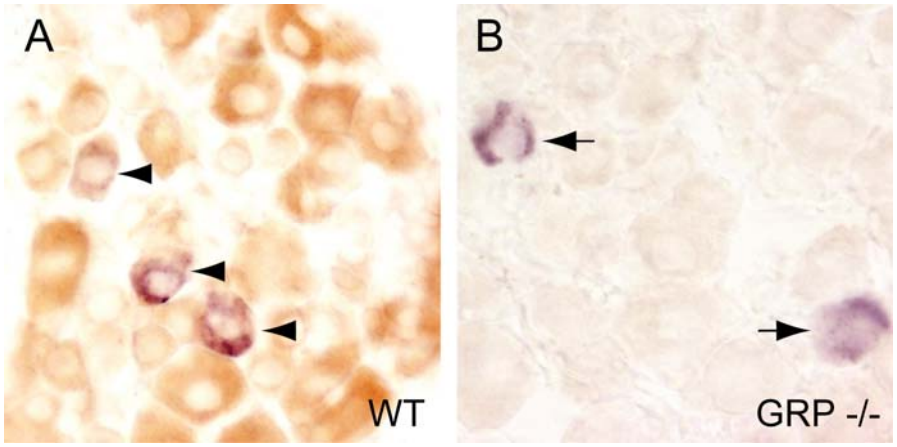

Figure S4. (**A**) A WT adult DRG section was doubly stained by in situ hybridization for MrgprA3 (blue) and immunostaining using anti-GRP antibody (brown). Arrowheads indicate MrgprA3/GRP co-expressing neurons. (**B**) The specificity of anti-GRP antibody staining was confirmed by using DRG sections from GRP knockout mice (GRP-/-) where GRP staining is completely gone but MrgprA3 expression remains intact. Arrows indicate MrgprA3^{+}/GRP^{-} cells.

Supplemental information for Figure 1C

To determine if neuronal survival is compromised in the absence of the *Mrgpr* gene cluster, we performed staining for NeuN (a pan-neuronal marker) and counted NeuN^{+} cells in lumbar (L5) DRG. The total number of L5 DRG neurons was comparable between WT and *Mrgpr-cluster*$\Delta^{-/-}$ mice (15844 ± 933 and 16396 ± 1037, respectively, n = 3), suggesting that Mrgprs are not required for the survival of primary sensory neurons. Next, we determined if Mrgprs are required for proper differentiation of DRG neurons. *Mrgprs* are specifically expressed in subsets of small-diameter primary sensory neurons (Dong et al., 2001; Han et al., 2002; Zylka et al., 2003). Small-diameter unmyelinated sensory neurons can be broadly divided into two classes: peptidergic and nonpeptidergic (Hunt and Mantyh, 2001). Peptidergic neurons express the neuropeptides substance P and CGRP while nonpeptidergic neurons do not express substance P but can be labeled with the lectin IB4. Most murine *Mrgpr*s are expressed in the nonpeptidergic subclass (Dong et al., 2001; Han et al., 2002; Lembo et al., 2002; Zylka et al., 2003). The proportion of these two subsets did not differ between WT and *Mrgpr-cluster*$\Delta^{-/-}$ mice (Figure 1C), suggesting that Mrgprs are not required for fate determination or differentiation of small-diameter sensory neurons.

Supplemental References

Breese, N. M., George, A. C., Pauers, L. E., and Stucky, C. L. (2005). Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. Pain *115*, 37-49.

Brumovsky, P., Hygge-Blakeman, K., Villar, M. J., Watanabe, M., Wiesenfeld-Hallin, Z., and Hokfelt, T. (2006). Phenotyping of sensory and sympathetic ganglion neurons of a galanin-overexpressing mouse--possible implications for pain processing. J Chem Neuroanat *31*, 243-262.

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., and Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science *288*, 306-313.

Dhaka, A., Murray, A.N., Mathur, J., Earley T.J., Petrus, M.J., and Patapoutian, A. (2007). TRPM8 is required for cold sensation in mice.

Guan, Y., Yaster, M., Raja, SN., Tao, YX. (2007). Genetic knockout and pharmacologic inhibition of neuronal nitric oxide synthase attenuate nerve injury-induced mechanical hypersensitivity in mice. Mol. Pain 3, 29

Hunt, S. P., and Mantyh, P. W. (2001). The molecular dynamics of pain control. Nat Rev Neurosci *2*, 83-91.

Mansikka, H., Zhao, C., Sheth, R. N., Sora, I., Uhl, G., and Raja, S. N. (2004). Nerve injury induces a tonic bilateral mu-opioid receptor-mediated inhibitory effect on mechanical allodynia in mice. Anesthesiology *100*, 912-921.

Siraganian, R. P. (1974). An automated continuous-flow system for the extraction and fluorometric analysis of histamine. Anal Biochem *57*, 383-394.

Stucky, C. L., Rossi, J., Airaksinen, M. S., and Lewin, G. R. (2002). GFR alpha2/neurturin signalling regulates noxious heat transduction in isolectin B4-binding mouse sensory neurons. J Physiol *545*, 43-50.