Supplementary Figure 1

Expression of activated BRAF (^{V600E}BRAF) in DRG of BRAF$^{Nav1.8}$ mice. Western blot shows 3 different RAF protein levels in DRGs of 4 weeks old of wild-type (left) and BRAF$^{Nav1.8}$ mice (right). ^{V600E}BRAF protein was absent in wild-type but markedly expressed in BRAF$^{Nav1.8}$ mice, whereas total BRAF and CRAF levels were comparable between two genotypes. *n* = 3 per genotype. Two bands of BRAF proteins represent two major splicing variants.

Supplementary Figure 2

pERK$^+$ staining in DRG neurons at different segmental levels of wild-type and BRAF$^{Nav1.8}$ mice. DRGs were collected and grouped according to segmental levels: cervical (A-B), thoracic (C-D) and lumbar (E-F). (G) The percentage of pERK$^+$ neurons (red, arrows) in total DRG neurons significantly increased in BRAF$^{Nav1.8}$ mice (B, D, and F) compared to control mice (A, C, and E); however, there was no significant difference among three levels in BRAF$^{Nav1.8}$ mice (G). ***P < 0.0001 *vs*. control, one-way ANOVA and Newman-Keuls *post-hoc* test. *n* = 3 per genotype. C, cervical; T, thoracic; L, lumbar. Scale bar: 40 μm.

Supplementary Figure 3

pERK$^+$ staining in skin of wild-type and BRAF$^{Nav1.8}$ mice. Mouse back hairy skin was dissected from the neck (cervical) and lumbar (L5-L6) level, exactly along the back midline (approximately 1.0 × 1.0 cm each)(n = 3). Hoechst 33258 staining (blue in A-D) was used as the fluorescent nuclear counterstain to show the epidermis and dermis structures. (E) The integrated density of pERK$^+$ fibers (red, arrows) in both sites significantly increased in BRAF$^{Nav1.8}$ mice (B and D) as compared to wild-type mice (A and C); there was no significant difference between rostrocaudal levels in BRAF$^{Nav1.8}$ mice. * P < 0.05, ***P < 0.001 *vs*. control, two-way ANOVA and Holm-Sidak *post-hoc* test. (*n* = 6 sections per region and genotype) Scale bar: 20 μm.

Supplementary Figure 4

Normal acute mechanical, thermal and inflammatory pain behavior of BRAF$^{Nav1.8}$ mice. **(A**-**B**) Mechanical threshold was comparable between wild-type and BRAF$^{Nav1.8}$ mice as measured by von Frey filaments (**A**) and by Randall-Selitto test (**B**). (**C**-**D**) Paw withdraw latency in the Hargreaves test (**C**), tail-flick latency to the radiant heat (**C**) and hot water assays (48°C, 50°C and 52°C, **C**), and paw withdrawal latency on a hot plate (50°C, 52°C and 55°C, **D**) did not differ between wild-type and BRAF$^{Nav1.8}$ mice. (**E**-**F**) Spontaneous pain responses, induced by intraplantar injection of formalin (**E**), mustard oil (**F**), or capsaicin (**F**), were comparable between wild-type and BRAF$^{Nav1.8}$ mice. In (**A**-**F**), all results from littermates at 4 ~ 6 weeks of age. *n* = 5~9 per genotype. Wild-type (white) compared to BRAF$^{Nav1.8}$ (black) mice in the same test, $P > 0.05$ for each test.

Supplementary Figure 5

Normal chronic inflammatory pain behavior of BRAF$^{Nav1.8}$ mice. **(A**-**D**) Heat and mechanical hypersensitivity in response to intraplantar injection of capsaicin (**A**,**B**) or Complete Freund's Adjuvant (CFA) (**C**,**D**) was assessed by Hargreaves (**A**,**C**) and von Frey (**B**,**D**) tests and was comparable between wild-type and BRAF$^{Nav1.8}$ mice at 4 to 6 weeks of age. **E**, Normal motor performance of wild-type and BRAF$^{Nav1.8}$ mice tested by accelerating rotarod test. In (**A**-**E**) repeated measures two-way ANOVA was used for analysis, *n* = 5~9 per genotype per test, $P > 0.05$ for each test.

Supplementary Figure 6

GRP^{+} staining in DRGs at different segmental levels in wild-type and BRAF$^{Nav1.8}$ mice. As in Supplementary Figure 2, mouse DRGs were grouped as cervical (A-B), thoracic (C-D) and lumbar (E-F). Quantitative analysis shows that the percentage of GRP^{+} neurons (red, arrows) in total DRG neurons significantly increased in BRAF$^{Nav1.8}$ mice (B, D, and F) compared to wild-type mice (A, C, and E), but there was no significant difference among segmental levels in BRAF$^{Nav1.8}$ mice (G). *n* = 6~8 per genotype, ** $P < 0.01$ *** $P < 0.001$ *vs*. control, one-way ANOVA and Newman-Keuls *post-hoc* test. C,

cervical; T, thoracic; L, lumbar. Scale bar: 40 μm.

Supplementary Figure 7

Expansion of *Mrgpra3*$^{+}$ cells in DRGs of BRAF$^{Nav1.8}$ mice. (**A**-**I**) Co-localization of pERK (**A**-**B**, brown), NF200 (**C**, brown), Peripherin (**D**-**E**, brown), P2X3 (**F**, brown), GRP (**G**-**H**, brown) and CGRP (**I**, brown), with *Mrgpra3* (**A**-**I**, blue) in DRG of wild-type (**A**,**D**,**G**) and BRAF$^{Nav1.8}$ mice (**B**, **C**, **E**, **F**, **H**, **I**). For simplicity, images of NF200, P2X3 and CGRP in DRGs of wild-type mice are not shown. In (**A**-**I**) arrows and arrowheads indicate *Mrgpra3*$^{-}$ and *Mrgpra3*$^{+}$ cells, respectively. (**J**-**K**) The percentage of double stained cells of the total *Mrgpra3*$^{+}$ cells (**J**) or the percentage of single stained cells co-expressing *Mrgpra3.* Note significantly increased GRP, CGRP and H1R expression in *Mrgpra3*$^{+}$ cells in BRAF$^{Nav1.8}$ mice (**J**), and up-regulation of *Mrgpra3*$^{+}$ in several major classes of DRG cells in BRAF$^{Nav1.8}$ mice (**K**). Scale bar: 20 μm in (**G**) for (**A**-**I**). In (**J** and **K**) *n* = 3 per genotype.

Supplementary Figure 8

Ectopic ERK activation and expression of, GRP and *Mrgpra3* in DRG, and of GRPR in the spinal dorsal horn of mice with dry skin itch**. (A**-**I**) pERK (**A**-**C**), GRP (**D**-**F**) and *Mrgpra3* (**G**-**I**) in DRGs. (**J**-**L**) Expression of GRPR as indicated by GFP staining in the dorsal horn. In (**J**-**L**), GRPR-eGFP mice were used. All other panels are derived from C57BL/6J mice. Scale bars are 10 μm in (**B**) for (**A**, **B**, **D**, **E**) and 20 μm in (**H**) for (**G**, **H**, **J**, **K**). Results in (**C**, **F**, **I**, **L**) are quantitative comparisons between dry skin mice (black) and their controls (white), *n* = 3 per group.

Supplementary Figure 9

ERK was not activated in the dorsal horn in the dry skin and allergic contact dermatitis models of chronic itch during spontaneous itch. pERK staining in the cervical dorsal horn of wild-type mice (A), mice with ACD (B) and mice with dry skin (C). The spinal cord tissues were collected in the fourth week after DNFB challenge and 24 hrs after the last DNFB painting, or on the 8th day of AEW treatment (overnight after

last AEW treatment on 7th day). Although the same protocol of pERK staining was used as in DRGs, hardly any pERK^{+} cells were found (A-C). As a positive control, intraplantar injection of capsaicin induced ERK phosphorylation in the lumbar (L5-L6) dorsal horn of wild-type mice within 5 min (D). Scale bar: 40 µm. *n* = 3 per group.

Supplementary Figure 10

Expression of CGRP and IB4 in the spinal cord of ACD and dry skin mice. **(A**-**C**) Expression of CGRP^{+} (red) and IB4^{+} (green) fibers in cervical dorsal horn of mice with ACD (**B**) or dry skin (**C**) was comparable to their expression in wild-type mice (**A**, no treatment). Scale bar: 20 µm, *n* = 3 per group.

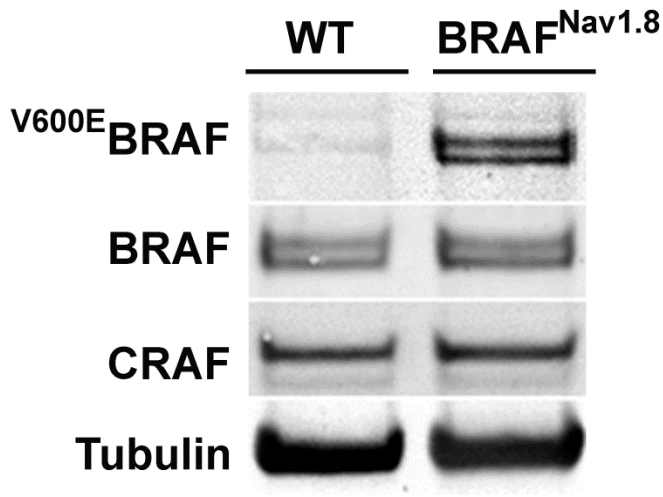
Supplementary Table 1

The table shows the electrophysiological properties of DRG cells of BRAF$^{Nav1.8}$ mice and wild-type mice. The mutant DRG cells exhibited altered distribution of firing properties. In both BRAF$^{Nav1.8}$ and wild-type cells (wild-type, *n* = 44 *vs*. BRAF$^{Nav1.8}$, *n* = 61), three firing patterns were routinely observed as, single spike (SS), delayed and tonic. BRAF$^{Nav1.8}$ mutants had an increased proportion of excitable, tonic firing cells and a decreased proportion of single spike (SS) cells compared to wild-type cells. * $P < 0.05$, ** $P < 0.01$, the chi squared test.

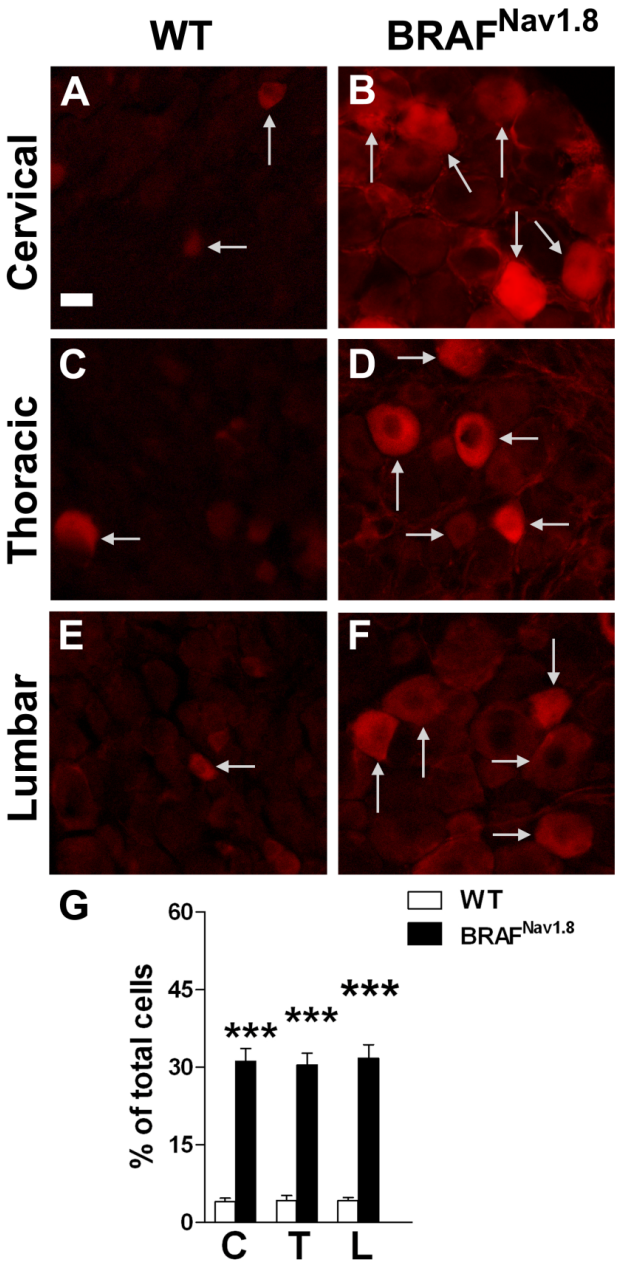
Supplementary Video 1

The video shows that the two 8-weeks old BRAF$^{Nav1.8}$ mutant mice display a pronounced scratching behavior.

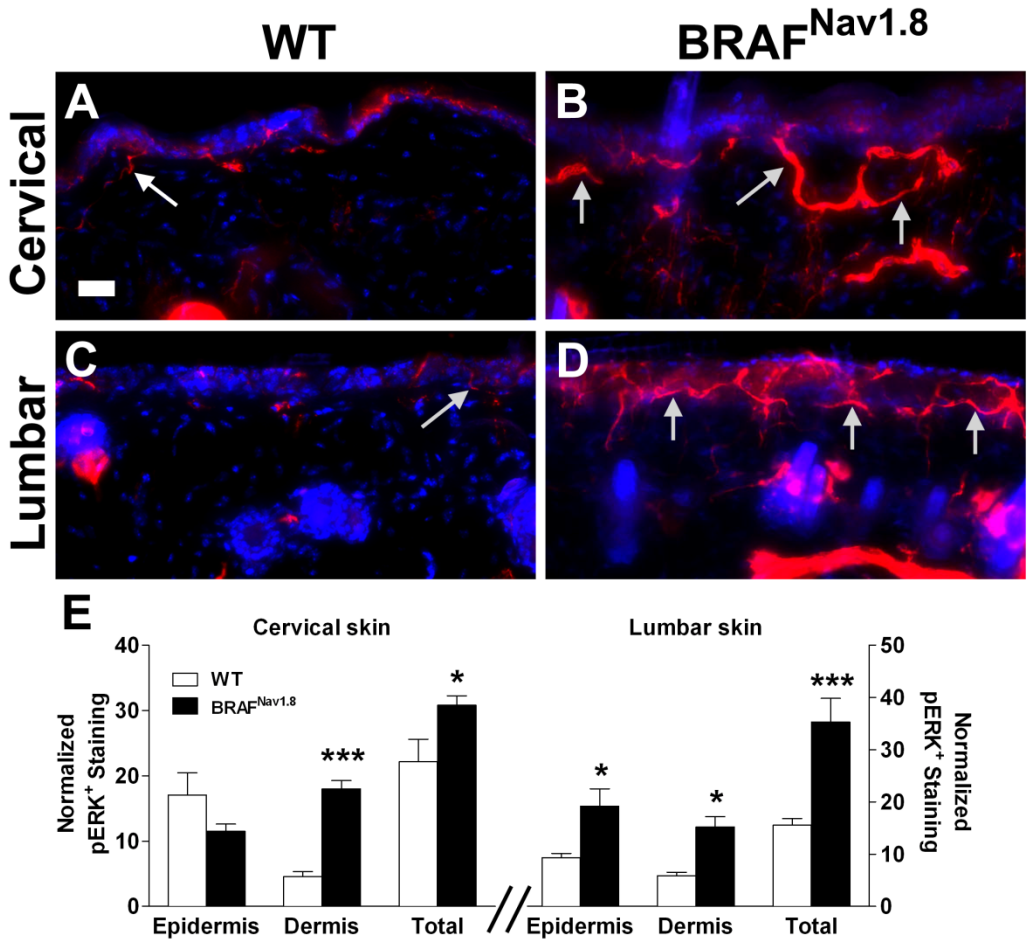
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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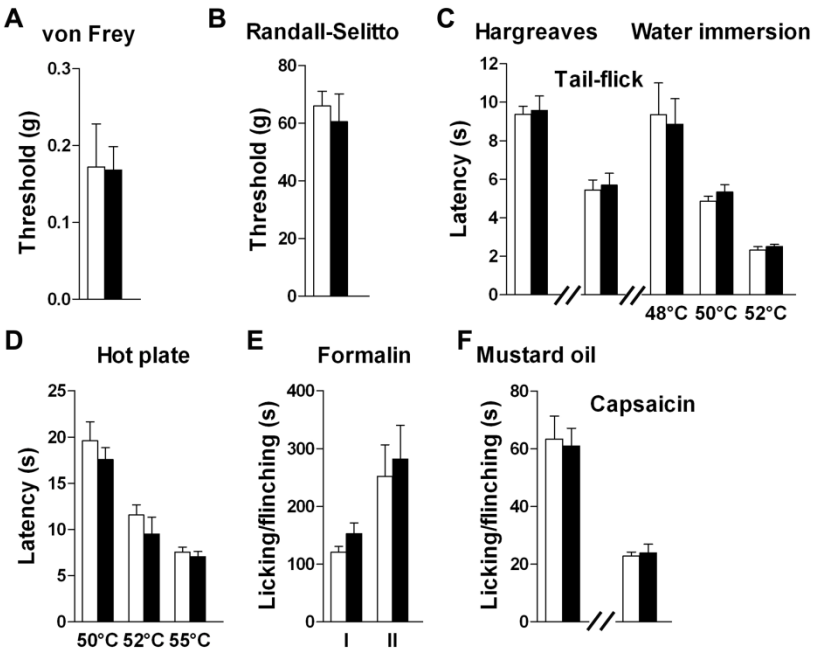
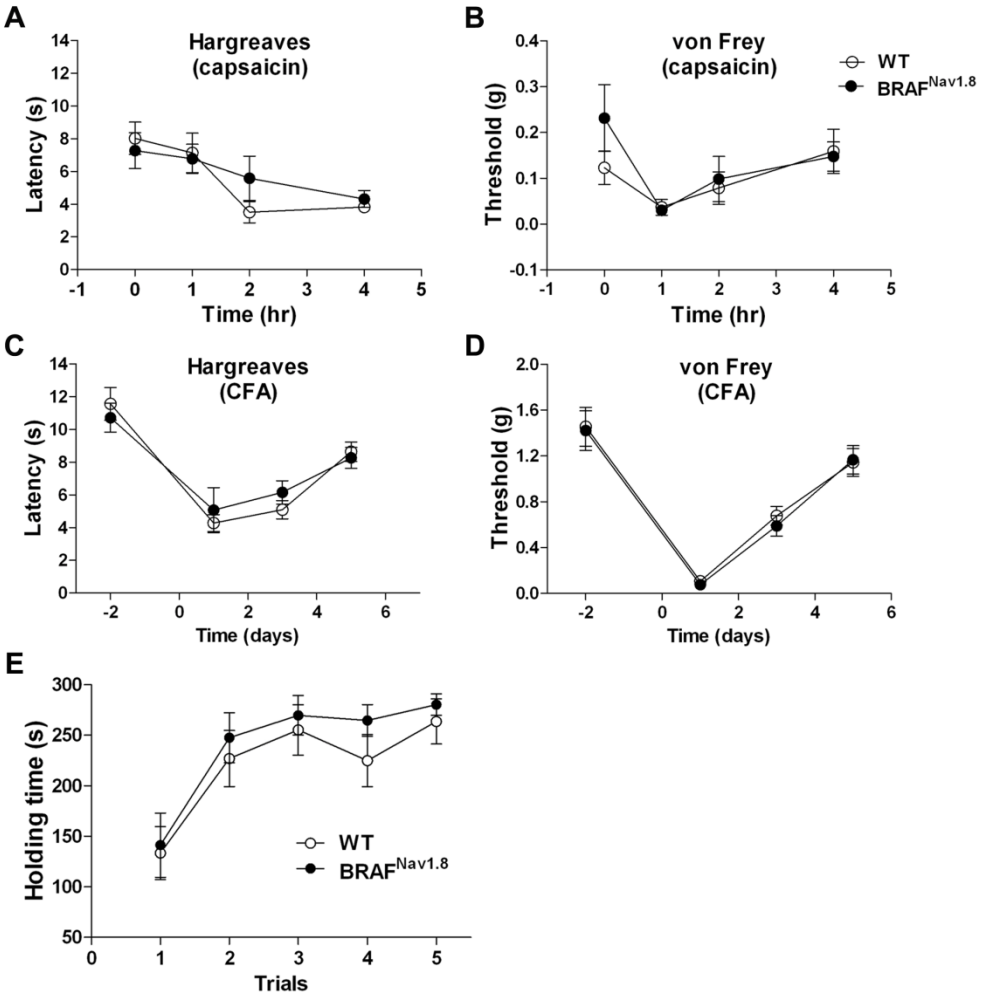
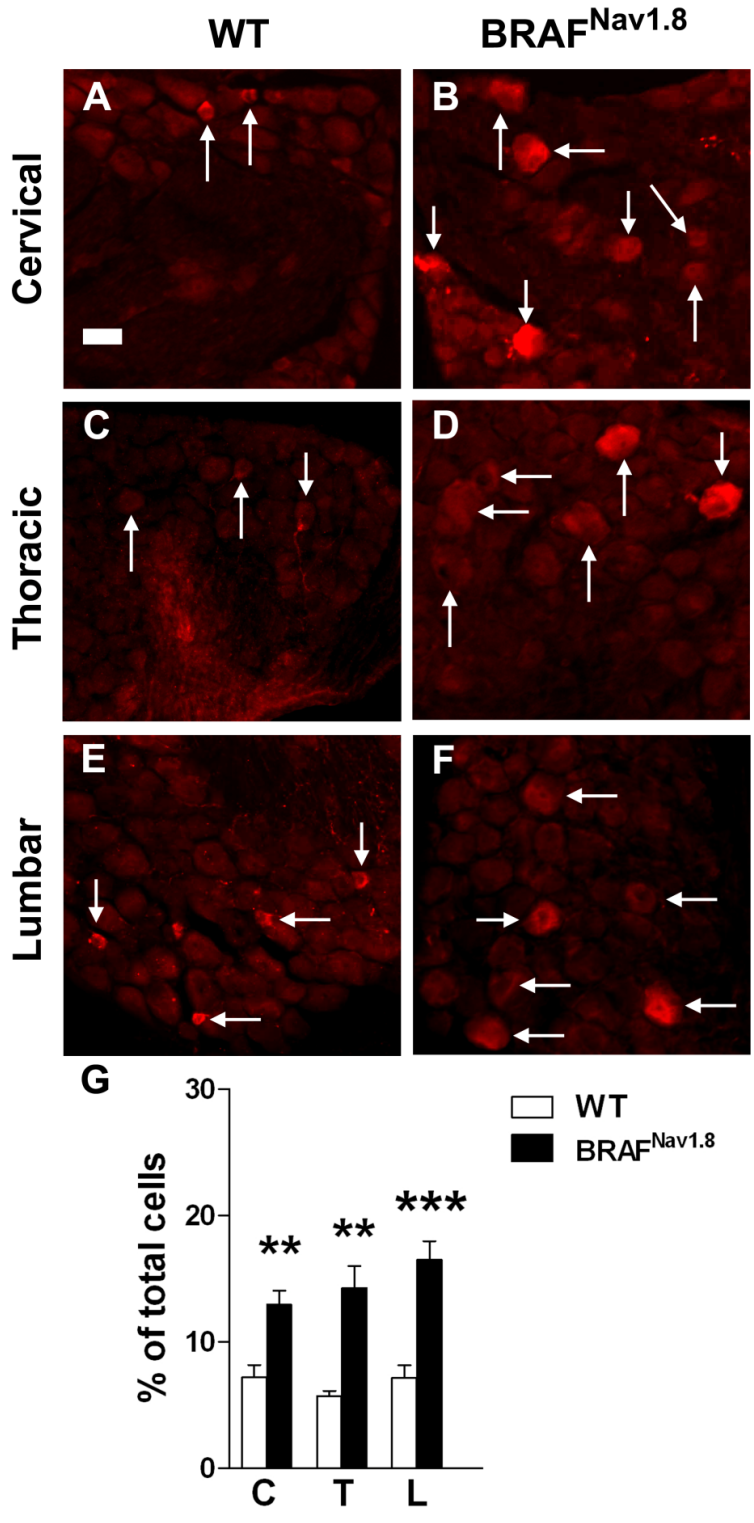


Supplementary Figure 5



Supplementary Figure 6



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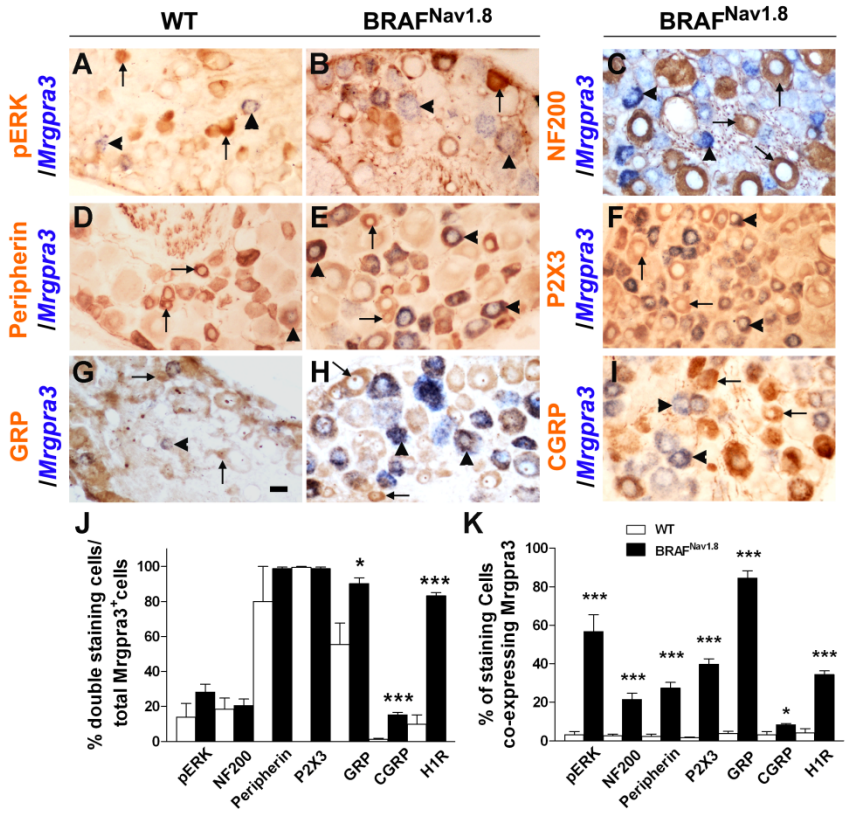

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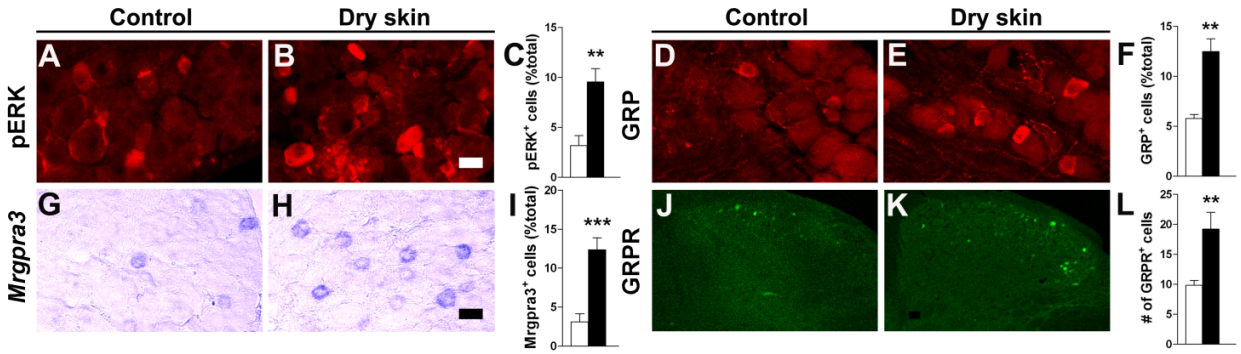

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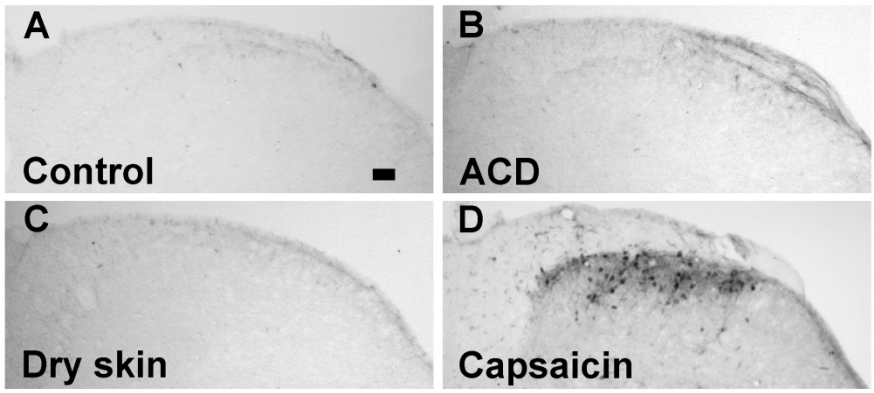

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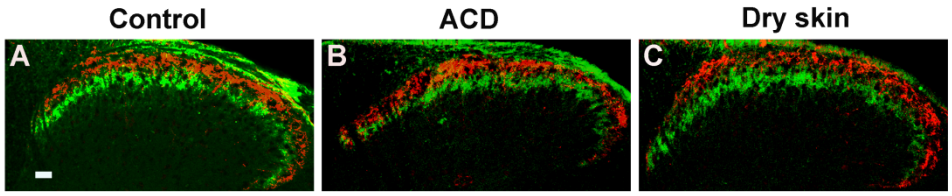

Supplementary Table 1

Cell Type	Cell Diameter (μm) WT/BRAF$^{NaV1.8}$	RMP (mV) WT/ BRAF$^{NaV1.8}$	Rheobase (pA) WT/ BRAF$^{NaV1.8}$	Input resistance (MΩ) WT/BRAF$^{NaV1.8}$	Proportion WT/BRAF$^{NaV1.8}$
SS	27±2/ 27±1	-55±2/ -52±3	363±73/ 160±46**	245±42/ 281±80	36%/ 16%*
Delayed	20±1/ 21±1	-47±1/ -45±1	50±3/ 52±5	554±87/ 533±57	37%/ 36%
Tonic	21±1/ 24±2	-43±2/ -42±1	24±4/ 23±2	731±120/ 677±86	25%/ 48%*

Supplemental methods

Mice. C57BL/6J mice, BRAF$^{Nav1.8}$mice, *Grp*$^{-/-}$ mice, *Grpr* KO mice and their wild-type littermate mice were used (56). GRPR-eGFP mice (MMRRC) were used to follow *Grpr* expression in the spinal cord and in SpVc in chronic itch models or after crossing with BRAF$^{Nav1.8}$ mice (referred as GRPR-eGFP/BRAF$^{Nav1.8}$ mice). BRAF$^{Nav1.8}$ mice also were crossed into *Grp*$^{-/-}$ or *Grpr* null background (referred as BRAF$^{Nav1.8}$/*Grp*$^{-/-}$ and BRAF$^{Nav1.8}$/G*rpr* KO mice, respectively) to define the role of GRP/GRPR in spontaneous scratching of BRAF$^{Nav1.8}$ mice. All experiments were performed in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain and were approved by the Animal Studies Committee at Washington University School of Medicine.

Generation of *Grp*$^{-/-}$ mice. *Grp*$^{-/-}$ mice were generated by replacement of exon 1 of the *Grp* gene with a neo cassette by homologous recombination in embryonic stem (ES) cells. The region of 5' homology consisting of a ~ 4 Kb *Xho*I/*BamH*I genomic fragment and the region of 3' homology consisting of a ~ 2 Kb *Spe*I/*EcoR*V genomic fragment were inserted into the X-pPNT vector (kindly provided by Dr. H. Westphal, National Institutes of Health). The *Not*I-linearized targeting vector was electroporated into ES cell line GSI-1 and recombinant clones were identified by Southern blot analysis with a 5' external 2.3 Kb *Hind*III/*Xho*I fragment probe. The primers for PCR analysis at the 3'-flanking region include Neo-3' oligo (5'-CTTCTTGACGAGTTCTTCTG) and the 3' external oligo SAEP-1rev (5'-GTTCCACTAAAGGGCAGGTT). The correctly targeted ES cell clone was used to establish germline transmission of the targeted allele and a congenic strain (B6.129X1-Grptm1Jfb) was created by backcrossing to C57BL/6J mice for more than 10 generations.

Drugs and reagents. Drugs were injected intradermally (i.d.) unless otherwise indicated. The drug doses were determined either from our previous studies (12, 13) or pilot experiments: chloroquine (nape, 200 μg, Sigma), histamine (nape, 500 μg,Sigma), compound 48/80 (nape, 100 μg, Sigma), endothelin-1

(ET-1) (nape, 25 ng, R&D Systems), capsaicin (intraplantar, 1 μg, Sigma), allyl isothiocyanate (AITC = mustard oil, 0.25%), (intraplantar, 2.5%, Sigma), formalin (intraplantar, 2%; Sigma) .

GRP^{18-27} (intrathecal (i.t.), 0.5 nmol, Bachem), Bombesin-saporin (i.t., 800 ng) and blank-saporin (i.t., 800 ng) (Advanced Targeting). GRP antagonist, 2-[(2-amino-6-chloro-4-pyrimidinyl)amino]ethanol (77427, intraperitoneal (i.p.), 30 mg/kg, Chembridge). Chlorpheniramine (CRP, i.p., 10 mg/kg, Sigma). MEK inhibitor U0126 (i.t., 10 nmol, Sigma). 2, 4-Dinitrofluorobenzene (DNFB, 0.15%, Sigma). Complete Freund's Adjuvant (CFA, Sigma).

Capsaicin and 77427 were initially dissolved in ethanol, U0126 was dissolved in dimethyl sulfoxide (DMSO), followed by further dilution in sterile saline. The final concentration for ethanol or DMSO would be ~2% and ~10%, respectively. DNFB was dissolved in acetone. Other chemicals were dissolved in sterile saline.

Animal ages for behavior and anatomical regions for molecular analysis.

The onset of spontaneous scratching behavior of $BRAF^{Nav1.8}$ mice varies greatly. At 6 weeks of age, about 50% of the mutants showed obvious spontaneous scratching. At 8~10 weeks of age, about 80-90% of mice showed robust spontaneous scratching, which was maintained or increased with time (data not shown). Most pain behavioral tests, like Hargreaves, von Frey test, hot-plate test, etc, require mice in a resting or relative quiet status so that their hindpaw responses to the heat, von Frey filaments, or chemicals like capsaicin, formation, CFA, etc, can be measured. Therefore, pain behavior tests were carried out prior to the onset of spontaneous scratching, typically at the age of 4~6 weeks similar to a previous study (16). $BRAF^{Nav1.8}$ mice between 8~12 weeks of age were most suitable for observing the effect of a blockade of the spontaneous scratch behavior by genetic and pharmacological approaches, followed by the molecular studies. Since molecular marker changes correlate with spontaneous scratching response, results obtained from mice around 10 weeks of age were used as a representative time.

In most (but not all) staining we have used cervical DRGs or spinal cords for two reasons. 1) pruritic stimuli were usually applied to the nape of the neck which is equivalent to the cervical level, and scratching behavior with the hindpaws was used as an indicator of the sensation of itch. 2) One major phenotype of $BRAF^{Nav1.8}$ mice is that they exhibited spontaneous scratch directed to the neck and facial skin. Therefore, most of our analyses were focused on cervical DRGs.

Scratching behavior. All scratching behavioral tests were videotaped by using SONY HDR-CX190 digital video camcorders from a side angle.

Allergic contact dermatitis. The procedure was modified from (32). Mice were sensitized by applying 100 µl of 0.15% DNFB acetone solution on a ~2 cm^2 area of fur-shaved abdominal skin (sensitization)(day 1). On day 8, 50 µl of 0.15% DNFB acetone solution was topically applied twice a week (every 2~3 days) to the shaved nape of mouse back for over 3 weeks (challenge). To examine spontaneous itch, scratching was measured 24 hrs after each DNFB painting for 30 min. Pretreatment with vehicles or different drugs at the time indicated in figure legends. Three weeks after DNFB challenge, the back skin, blood, lymph nodes, and spleen were collected from the DNFB-treated mice and their controls (painted with acetone only) for a comprehensive pathological analysis as described (61), or their cervical DRG and spinal cord tissues were processed for immunostaining or molecular analysis.

Xerosis (dry skin). The dry skin model was implemented as described (40, 41). Briefly, the nape of mice at 8~12 weeks of age was shaved and a mixture of acetone and diethylether (1:1) was painted on the neck skin for 15 s, followed immediately by a 30 s distilled water application (AEW). This regiment was administrated twice daily for 2 weeks. Littermate control mice received water only for 45 s on the same schedule. Spontaneous scratches were examined for 60 min on the morning following the last AEW treatment. Pharmacological interventions were carried out during the second week after AEW treatment.

Cervical DRG and spinal cord tissues were isolated and processed for immunostaining or molecular analysis.

Pain behavior. All acute and inflammatory pain behavioral assays were performed as described (Sun, 2007 #1560;Sun, 2009 #2036}). Some of the details are as follows: Thermal and mechanical hypersensitivity was assessed by Hargreaves and von Frey tests after capsaicin or CFA injection. For capsaicin, the behavior tests were performed 1 hr before (baseline) or 1, 2, and 4 hrs after capsaicin injection. For the CFA data, behavioral tests were performed 2 days before (baseline) or 1, 3 and 5 days after CFA injection.

Dorsal rhizotomy. C57BL/6J male mice of 8~10 weeks of age were subjected to a unilateral rhizotomy at spinal lumbar level L5 (12). Laminectomy was performed to expose the L5 dorsal root, which was sharply transected. 14 days later the animals were terminally perfused and the lumbar spinal cord tissues collected for histology and immunostaining.

Rotarod test. Animal motor performance was assessed by using an accelerating rotarod treadmill (Ugo Basile) as described (59).

Immunohistochemistry and *in situ* hybridization (ISH). Immunohistochemical staining and ISH were performed as described (12, 59). The following primary antibodies were used for this study at the specified dilutions: anti-pERK (rabbit polyclonal, 1:500; Cell Signaling Technology), anti-GRP (rabbit polyclonal, 1:500; Immunostar), NeuN (mouse monoclonal,1:10,000; Millipore), NF200 (mouse monoclonal, 1:5000; Sigma), Peripherin (rabbit polyclonal, 1:1000; Chemicon), CGRP (rabbit polyclonal, 1:4000; Chemicon), TRPV1 (rabbit polyclonal, 1:1000; Neuromics) , P2X3 (rabbit polyclonal, 1:1000; Millipore), NK1R (rabbit polyclonal, 1:2000; Millipore). H1R (rabbit polyclonal, 1:300; Alomone Labs), Isolectin Griffonia simplicifolia (IB4) FITC conjugate (1:100; Sigma). GFP (rabbit polyclonal, 1:500; Life Technologies) and GFP (chicken polyclonal, 1:500; Aves Labs). In hairy

skin staining, Hoechst 33258 (Sigma, St. Louis, MO) was used as the blue fluorescent nuclear counterstain. To examine *Grpr*, *Mrgpra3* and *Trpv1* mRNA expression, ISH was performed. *Mrgpra3* or *Trpv1* ISH was followed by DAB double staining to evaluate cellular identities in DRG.

Quantification of stained cells. Following fluorescent labeling or *in situ* hybridization double stains with antibody DAB staining, photographs were taken with a CCD camera attached to an Olympus microscope combined with ImageJ software from NIH Image (version 1.34e) for offline analysis. The total number of neurons in each section was counted by either using NeuN antibody fluorescent counterstaining or according to the nonspecific neuronal background. Only individual cells showing levels of expression or staining clearly above background with clearly visible nucleoli were counted. For GRP^+ and $H1R^+$ cell counting, only cells with specific and high level expression were counted. To ensure the accuracy of cell counts, labeling of all counted cells was also confirmed by direct visualization of cells through the microscope and counting in a blinded fashion. For each marker, 3~5 mice per group ("*n*" indicated in the figure legends) and at least 6 sections per mouse were counted. The number of immunolabeled cells is presented as a percentage of the total number in the DRG or TGs with images in a fixed magnification (i.e., 20x or 40x). GRP^+, $CGRP^+$ and $pERK^+$ immunoreactivity in the superficial dorsal horn or skin was scanned with the microscope and measured and calculated to normalize data with ImageJ.

Western blot analysis. Protein samples from cervical spinal cord and DRGs of 4 weeks (for RAF proteins) or 10~12 weeks of age (for MEK/pMEK/ERK/pERK proteins) wild-type and $BRAF^{Nav1.8}$ mice were isolated for western blot analysis as previously described (11). Primary antibodies used were, anti-^{V600E}BRAF (mouse monoclonal; 1:1000; Spring Bioscience), anti-BRAF (rabbit polyclonal; 1:1000; Santa Cruz), anti-CRAF (rabbit polyclonal; 1:1000; Santa Cruz), anti-alpha-tubulin (rabbit polyclonal; 1:2000; Millipore), anti-pMEK1/2 (rabbit polyclonal; 1:5000; Cell Signaling Technology), anti-MEK1/2 (rabbit polyclonal; 1:2000; Cell Signaling Technology), anti-pERK1/2 (mouse monoclonal; 1:5000; Cell Signaling Technology), anti-ERK1/2 (rabbit polyclonal; 1:10,000; Cell Signaling Technology) and anti-actin (mouse monoclonal, 1:10,000; Sigma-Aldrich).

Cell culture and calcium imaging. Cervical DRG tissues (10~20 DRGs per mouse, *n* = 3~4 mice per genotype of mice for cell culture) were dissected from adult $BRAF^{Nav1.8}$ mice and control littermates, placed in neurobasal media (Life Technologies) and incubated with 0.15% papain/0.25% trypsin in neurobasal at 37ºC for 20 min. Dissociated DRG cells were plated onto Poly-D-Lysine/laminin coated glass coverslips in a 24-well plate. The cells were loaded with fura 2-acetoxymethyl ester (Molecular Probes; Life Technologies) and imaged at 340 and 380 nm excitation to detect intracellular free calcium. Chemicals applied were chloroquine (100 μM), histamine (100 μM), capsaicin (1 μM) and KCl (30 mM). Each chemical was applied for 30 s and washed out for 30 to 60 s followed by the next chemical. Each experiment was done at least three times and at least six sister coverslips per drug were analyzed each time. The results were analyzed using the Prism software for cell activity tracing or population quantification.

Whole-cell current-clamp recordings of cultured DRG neurons. To evaluate the effect of pruritogens on neuronal excitation, patch clamp in current clamp mode was performed on dissociated cultures of cervical DRGs. The procedure was performed similarly as described (62). Current clamp commands, signal amplification and signal acquisition were made with pClamp software version 10.2, a multiclamp 700B amplifier and a Digidata 1440 analog-to-digital converter. Series resistance was monitored by applying a small voltage step in voltage clamp mode, and cells exhibiting a series resistance change greater than 20% were not analyzed. Input resistance was monitored by applying -20 pA current injections (500 ms duration) every 20 s. Cells having a resting membrane potential (RMP) more negative than -40 mV were studied. Action potential (AP) firing patterns and rheobase were observed and measured by injecting depolarizing current in steps of 500 ms duration. In addition to RMP criteria, cells were deemed healthy if AP amplitudes were greater than 70 mV. Firing patterns were assessed according to the response at rheobase. AP halfwidth was measured as the AP duration at one half of the AP amplitude.

Analysis of skin and immune system in mice with ACD. *Skin histology.* For hematoxylin and eosin (H&E) and toluidine blue staining using paraffin-embedded tissue sections, the skin was fixed in 4% paraformaldehyde in PBS, dehydrated with ethanol and embedded in paraffin. It was sectioned at 5~6 μm. The average number of mast cells in random 100× microscope fields was counted.

ELISA. Serum IgE was measured using Mouse IgE ELISA kit (Immunology Consultants Laboratory Inc.).

Hematological analysis. Blood samples collected from the mandibular vein were used for hematological analysis (Hemavet 950 analyzer; Drew Scientific Inc.), which included white blood cells (WBC) and neutrophils. Macroscopic examination of spleen and lymph nodes from the DNFB-treated mice and their controls was also performed.

RT-PCR. Skin TSLP levels were determined using mouse skin samples from control and DNFB-treated mice. The followings are primers used for PCR: 18sRNA, 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3'; TSLP, 5'-CCAGGCTACCCTGAAACTGA-3' and 5'-TCTGGAGATTGCATGAAGGA-3'.