



## Supplementary Material for The Cells and Circuitry for Itch Responses in Mice

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(available at [www.sciencemag.org/cgi/content/full/340/6135/968/DC1](http://www.sciencemag.org/cgi/content/full/340/6135/968/DC1))

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## Supplementary Materials:

### Materials and Methods:

All experiments using animals followed NIH guidelines and were approved by the National Institute of Dental and Craniofacial ACUC. The targeted JM8A3 ES-cell clone A04 with disruption of the *Nppb* gene was obtained from MBP, UC Davis and was used to generate chimeric mice. Chimeras were crossed with C57BL/6 mice and heterozygous offspring were mated to generate paired knockouts and controls. Tg(GRP-EGFP) animals (22) were employed to localize expression of GRP. Male C57BL/6 (8-14 weeks) mice were used for selective toxin ablation and GRP-antagonist itch experiments. Ablation of *Npra* and GRP-receptor-expressing spinal cord interneurons was accomplished by intrathecal (segment L3/4) injection of *Nppb*-saporin (5 µg in 10 µl; Advanced Targeting Systems) and GRP-saporin (2.5 µg) respectively. Experiments were initiated two weeks after toxin injection.

Itch inducing substances (see Table S2) were injected intradermally into the shoulder of mice and numbers of scratching bouts assessed over 30 minutes. Pruriceptive (itch) behavior was also elicited by lumbar 4-5 segment intrathecal injection of *Nppb* (5µg in 10µl) or GRP (1nM in 10µl); just like responses to pruritogens, responses to peptides exhibited delayed onset of approx. 5 minutes. Pretreatment with GRP antagonist deamino-Phe19,D-Ala24,D-Pro26-D-Phe27-GRP (23) (1 nM in 10 µl) was used to block GRP-receptor.

Thermal, mechanical, proprioceptive and pruriceptive behavioral responses were assessed as described previously (7). Thermal reactivity was determined using a hot plate (55 °C) or cold plate (-5 °C), with the time to the first lick or jump recorded. Mechanical sensitivity was measured using a semi-automated von-Frey apparatus and Randall-Sellito device. Proprioceptive responses were assessed using an accelerating rotarod. Statistical analysis used Prism Graph;  $P > 0.05$  values were considered non-significant.

*In situ* hybridization (ISH) was performed at high stringency (washed 30 min, 0.2x SSC, 70 °C) as described previously (24, 25). Detection of *MrgprA*-receptors used a mix of full-length *MrgprA3* and *MrgprA4* anti-sense probes. We examined *Nppb*-expression outside the somatosensory system and did not detect signal in other sensory systems or the brain; as expected *Nppb* is prominently expressed in the heart. Immunohistochemistry was performed with mAb anti-*Nppb*, rabbit anti-*MrgprC11*, and rabbit anti-*Npra* (from LifeSpan Bioscience); chicken anti-GFP was from Abcam and secondary antibodies were Jackson Immunolabs; tyramine FITC was used for HRP-signal amplification to visualize *Npra*-immunostaining. Fluorescent images (1 µm optical sections) were collected using confocal microscopy and were processed using Adobe Photoshop.

Total RNA was extracted from dorsal root ganglia and spinal cord using an RNAeasy kit and converted into cDNA. Quantitative real-time PCR was accomplished with commercially available TaqMan primer sets. Equal amounts of cDNA were used in duplicate and amplification efficiencies were validated and normalized against GAPDH, fold increases were calculated using the comparative threshold cycle method. Agilent whole genome arrays (mouse GE 44K V2) were screened with cRNA probes generated from DRG as recommended by the manufacturer; bioinformatic analysis was performed with Gene-Spring software.

**Table S1. Top 25 over-expressed genes in wild-type vs TRPV1-DTA mice.**

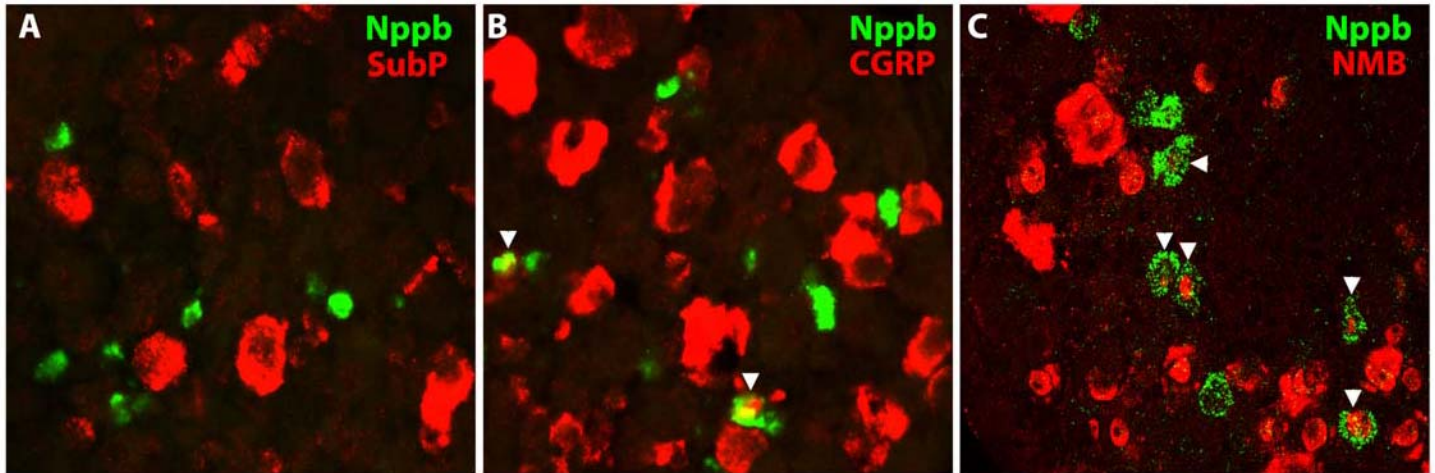
Data represents the means of data taken from n=3 arrays for each genotype, p value are indicated.

Gene name	Fold-change	P-value	Genbank acc #
Apod	105	3.8e-5	NM_007470
Trpv1	96	1.3e-4	NM_001001445
Myot	32	8.7e-6	NM_001033621
Zcwpw2	32	7.5e-2	XM_001473321
Gfra3	32	2.1e-6	NM_010280
Tnnt3	28	1.9e-6	NM_001163664
Osta	27	2.4e-5	NM_145932
Myl1	27	2.1e-4	NM_001113387
Ceacam10	26	1.4e-4	NM_007675
Wfdc2	26	2.8e-5	NM_026323
Kcnf1	25	6.2e-4	NM_201531
Trdn	25	4.3e-5	NM_001251987
Nppb	25	2.2e-6	NM_008726
Cacna2	25	9.7e-5	NM_001110843
Dgkk	25	2.1e-4	NM_177914
AW551984	25	1.0e-3	NM_001199556
Syt16	21	4.4e-5	NM_172804
Cacna1i	21	6.7e-6	NM_001044308
Gpr35	21	3.2e-4	NM_022320
9430021M05Rik	21	3.9e-5	NR_033569
Tnnc2	21	2.1e-6	NM_009394
Avpr1a	20	8.9e-4	NM_016847
Bex1	19	3.6e-6	NM_009052
Trpa1	19	8.1e-7	NM_177781
P2rx3	18	1.6e-6	NM_145526

**Table S2**

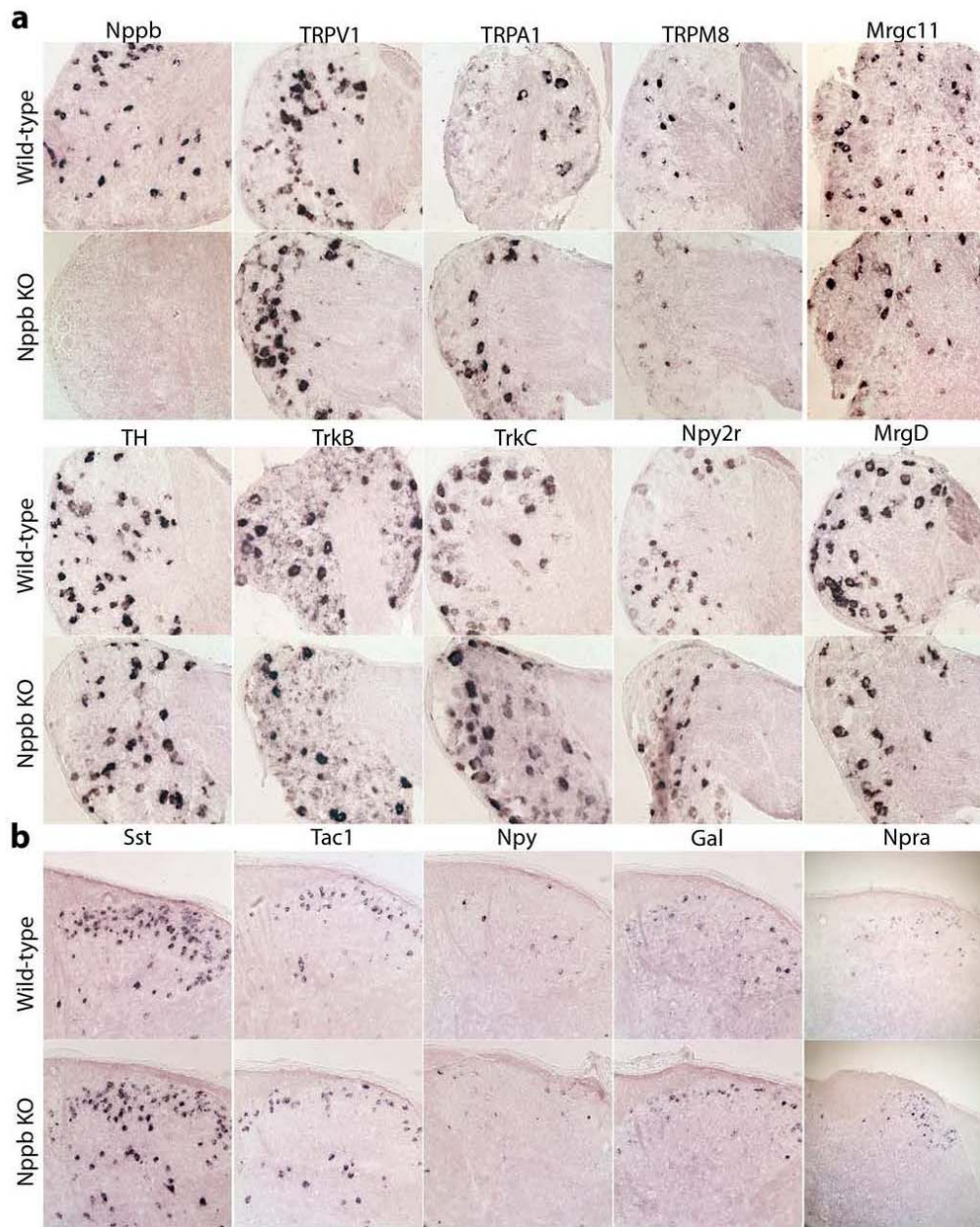
List of pruritic agents and dose injected subcutaneously; also indicated are the receptors or mechanisms believed to be activated by the various compounds in the itch response pathway.

Pruritogen	Dose	Receptor
Histamine	100 µg	Histamine (H1)
Chloroquine	100 µg	MrgprA3
Endothelin (ET-1)	25 ng	ETA
2-methyl serotonin	30 µg	5-HT
SLIGRL-NH2 (PAR2)	100 µg	MrgprC11
Compound 48/80	100 µg	Mast-cells



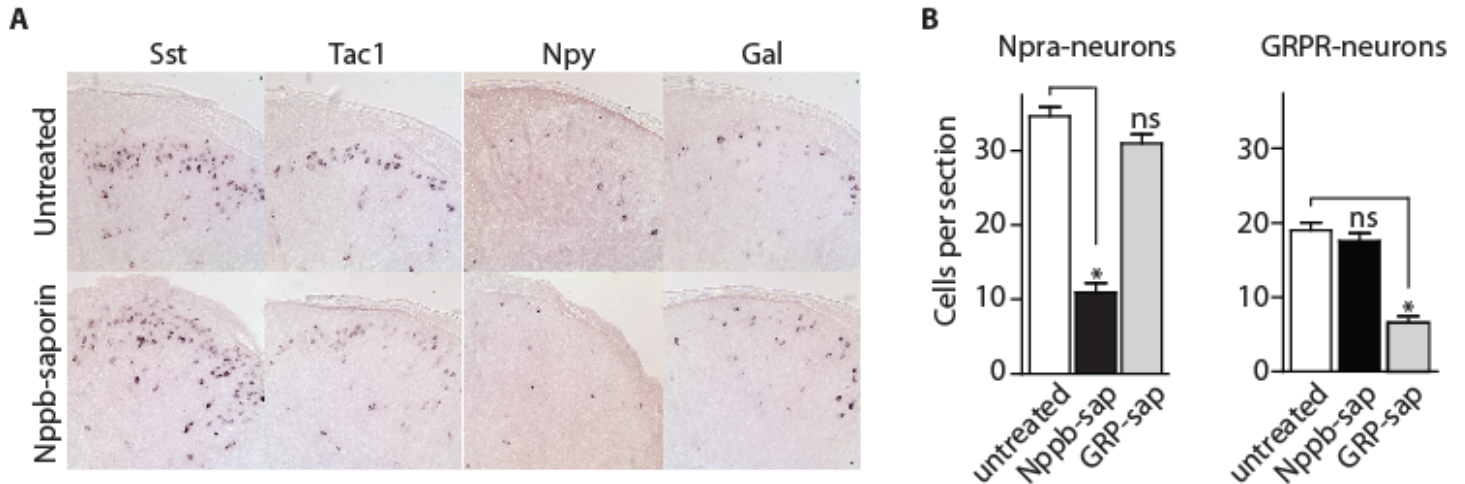
**Fig. S1. Overlap between expression of Nppb and peptides that trigger neurogenic inflammation.**

Many itch inducing agents also trigger a peripheral inflammatory response. Therefore, double label in situ hybridization was used to determine whether Nppb-expressing neurons also contain neuropeptides that are known to be released in the periphery and cause neurogenic inflammation. Shown are sections through the DRG showing Nppb expression (green) and expression of inflammatory neuropeptides (red). (A) Very few Nppb-positive cells co-expressed substance P (Sub P). However, (B) about a quarter of Nppb-expressing neurons contained CGRP and (C) half the Nppb-neurons expressed neuromedin B (NMB); arrowheads denote double positive neurons. Quantification of the Nppb positive neurons revealed that 2 % co-labeled for Substance P, 24 %, for CGRP and 50 % for NMB (assessed from total neuronal counts of 147, 185, and 196 respectively).



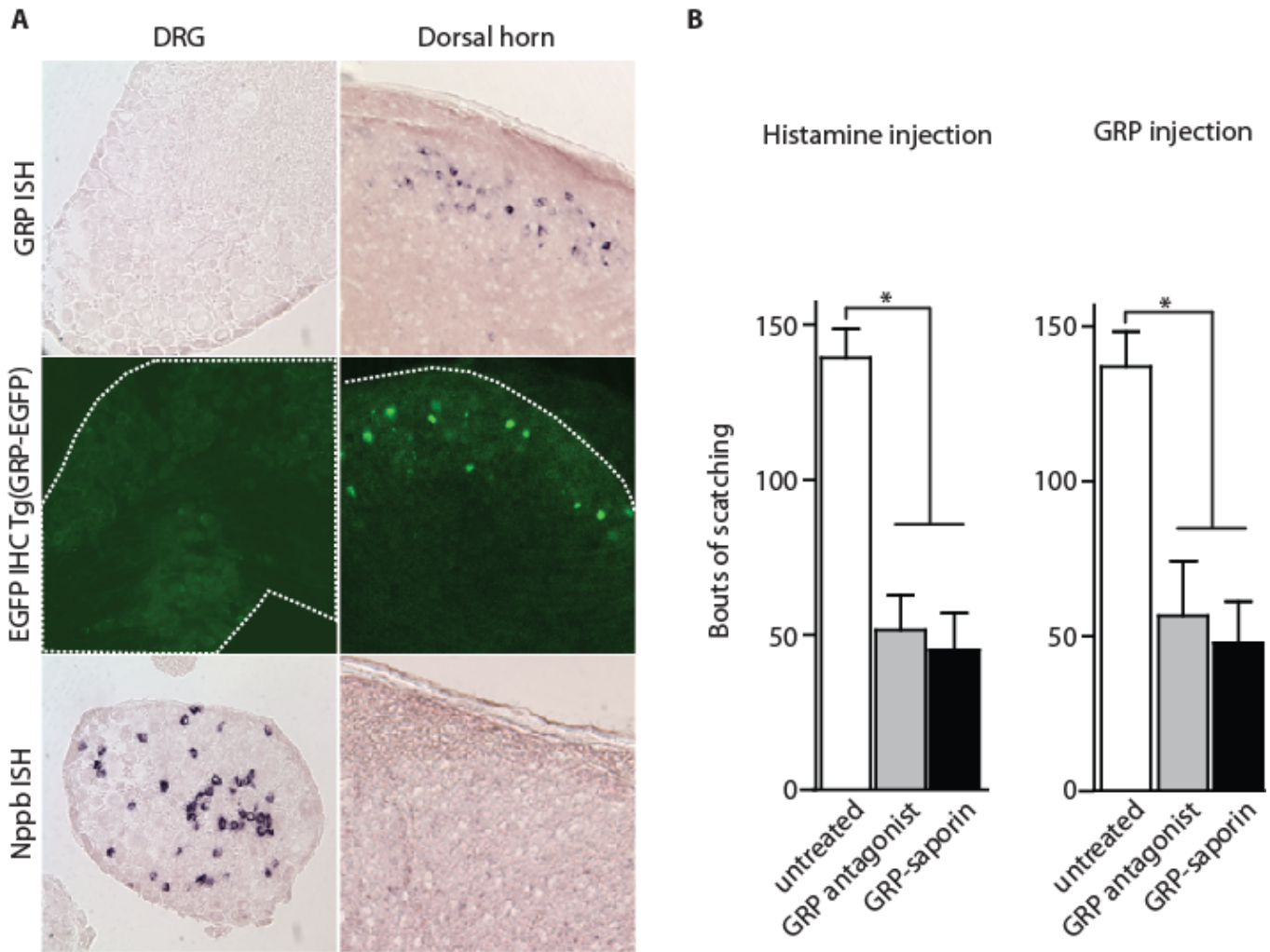
**Fig. S2. Expression of molecules involved in pruriception, nociception, proprioception, mechanical, and thermal sensation are unchanged in *Nppb*<sup>-/-</sup> mice.**

Section through (A) DRG and (B) spinal cord from wild-type control and *Nppb*<sup>-/-</sup> mice, were hybridized with probes as indicated (TH is tyrosine hydroxylase); other probes are designated by standard gene symbols. Except for *Nppb*, no significant changes between genotypes were observed in numbers of positive cells.



**Fig. S3. Nppb-saporin administration causes selective ablation of Npra-neurons in the spinal cord.**

(A) ISH reveals that intrathecal Nppb-saporin treatment (lower panels) has no effect on expression patterns of spinal cord neuropeptides. (B) Analysis of numbers of neurons ablated by Nppb-saporin (left panel) and GRP-saporin (right panel) administration shows that approximately 70% of Npra-cells are eliminated by Nppb-saporin administration. By contrast Nppb-saporin injection does not alter numbers of GRP-receptor positive neurons. ISH was performed on tissue from at least 4 control and 4 treated mice. Serial sections from >10 sections per mouse were hybridized and numbers of cells counted. Data are mean  $\pm$  s.e.m. Significant differences between treatment groups were determined using Student's t-test with \* indicating  $P < 0.0001$ .



**Fig. S4. GRP is found in a subset of spinal cord neurons and the downstream GRP-receptor expressing neurons are required for pruriception.**

(A) ISH (upper panels) and immunohistochemistry (IHC) of tissue from Tg(GRP-EGFP) animals (middle panels) show that GRP is expressed in a population of dorsal horn interneurons (right panels). In contrast, GRP is not expressed by primary sensory neurons (left panels). Outline of DRG and dorsal horn are highlighted by a dotted line in IHC images. The opposite expression pattern is observed for Nppb: ISH reveals that Nppb (lower panels) is expressed in DRG and is absent from the spinal cord. (B) In line with previously reports, (5, 6) we find that pretreatment with a GRP antagonist or ablation of GRP-receptor expressing neurons with GRP-saporin attenuates scratching following intradermal injection of histamine or intrathecal administration of GRP. Interestingly, knockout of the GRP-receptor (6) has a much less severe effect on behavioral-responses to histamine than pharmacological inhibition suggesting compensatory mechanisms may occur in GRP-R<sup>-/-</sup> animals; our results show that killing the GRP-R expressing cells with GRP-saporin (see also ref 6) more closely resembles pharmacological inhibition of the receptor. Data represent mean  $\pm$  s.e.m. ( $n \geq 5$  animals). Significant differences between treatment groups were determined using Student's t-test with \* indicating  $P < 0.001$ .



## **Movies S1-S3**

### **Movie S1. Intradermal injection of histamine in wild type and $Nppb^{-/-}$ mice.**

Intradermal injection of the puritogen histamine into the nape of the neck. Video shows the typical scratch response of wild type animals that begin approx. 5 min after injection and continue for about 25 – 30 minutes. As illustrated in the video  $Nppb^{-/-}$  mice exhibit dramatically reduced scratch responses compared to wild-type littermates.

### **Movie S2. Intrathecal injection of Nppb in wild type and $Nppb^{-/-}$ mice.**

Intrathecal injection of Nppb evokes scratching in mice starting about 5 minutes after stimulation and lasting approximately 30 minutes. Both wild type and  $Nppb^{-/-}$  mice exhibit equivalent responses that closely resemble responses of wild type mice to pruritogens.

### **Movie S3. Intrathecal injection of Substance P in wild type and $Nppb^{-/-}$ mice.**

For comparison, we also injected the nociceptive neuropeptide Substance P intrathecally. Substance P elicits a profoundly different behavioral response. Video illustrates the responses of wild type and  $Nppb^{-/-}$  mice. After Substance P injection, we never observed scratching but instead noted typical nociceptive withdrawal behavior (freezing, hunched posture and spasmodic twitching) with rapid onset and approx. 10 min duration.

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