Supplementary Materials

Supplemental Results

**Figure S1.** Cutaneous innervation is eliminated 10 days following surgical axotomy. Unilateral experimental denervation was completed and the success of the surgical denervation was confirmed 10 days later. Representative images of PGP9.5+ nerve staining in skin from sham operated wildtype control littermates (a), sham operated KC-Tie2 animals (b) and axotomized back skin from the same KC-Tie2 mouse (c). Decreases in PGP9.5 expression were confirmed using western blotting (d). Scale bar = 50μM.

**Figure S2.** Cutaneous macrophage and mast cell numbers remain unchanged following surgical denervation. Sham and denervated skin sections were stained with antibodies targeting F4/80 (a; macrophages), or stained using toluidine blue (b; mast cells). F4/80+ and mast cells were counted under innervated sham control and denervated conditions and data is presented for individual animals.
Figure S3. Cutaneous angiogenesis remains constant following denervation and SP and CGRP inhibition. Skin sections taken from sham operated or denervated skin, and innervated skin treated for 30 days with PBS, SP inhibitor (RP67580) or CGRP inhibitor (CGRP_{8-37}) were stained with antibodies against the pan-endothelial cell marker, mouse endothelial cell antigen (MECA) and the number of blood vessels was quantitated as described previously (Wolfram et al., 2009). (a) The number of blood vessels remained unchanged between sham control and denervated skin 10 days following surgery; ELISA analyses of the pro-angiogenic growth factors VEGF (c) and IGF (d) from whole skin lysates also revealed no differences. (e) Thirty days following neuropeptide inhibition, no significant decreases were found between KC-Tie2 mice treated with PBS, SP inhibitor (RP67580) or CGRP inhibitor (CGRP_{8-37}). The hatched line represents control wildtype mouse levels.
Figure S4. Proliferation of keratinocytes is modulated by CGRP agonists and antagonists, but not SP agonists and antagonists. Immunohistochemistry against the cell proliferation marker Ki67 was performed on sham operated skin (a, c, e) and denervated skin that had been treated with PBS (b), SP agonist GR73632 (d), or CGRP peptide (f) (n=4 per group) as well as from skin of animals treated for 30 days with PBS (g), the SP antagonist RP67580 (h), or the GRGP antagonist CGRP_8-37 (i; n=4-6 for each treatment). Only mice receiving treatments targeting CGRP (f, i) showed changes in keratinocyte proliferation and levels of acanthosis. Scale bar = 100μM.
Table S1. Summary of statistically significant changes observed between innervated and denervated skin at time points following surgical axotomy of cutaneous nerves.

|                          | Day 1 | Day 3 | Day 5 | Day 7 | Day 10 |
|--------------------------|-------|-------|-------|-------|--------|
| CD11c⁺ cell numbers      | p=0.011 | p=0.001 | p=0.037 | p=0.004 | p=0.016 |
| Epidermal thickness      | p=0.66 | p=0.38 | p=0.36 | p=0.019 | p<0.0001 |
| CD4⁺ cell numbers        | p=0.93 | p=0.51 | p=0.62 | p=0.35 | p=0.029 |

Supplemental Materials and Methods.

Transgenic mice and surgical axotomy of thoracic cutaneous nerves.

In order to generate keratinocyte specific Tie2 expressing (KC-Tie2) transgenic mice (Wolfram et al., 2009), we crossed the pTET⁰-Tie2 responder and the keratin 5 tetracycline transactivator (K5tTA) driver lines (Diamond et al., 2000; Jones et al., 2001). Offspring were genotyped by PCR using DNA extracted from either tail or ear biopsies. DNA was prepared and PCR performed using primers as previously described (Diamond et al., 2000; Jones et al., 2001; Wolfram et al., 2009). For KC-Tie2 and control mouse comparisons, littermates that inherited one or no transgenes, showed no transgene expression and therefore served as experimental controls.

Cutaneous denervation surgery was adapted from previously described techniques (Siebenhaar et al., 2008). Briefly, on the day of surgery, 8-12 week old mice were deeply anesthetized using the gas anesthesia, isoflurane; and a 2.5 to 3cm incision was made at the dorsal midline of the back skin. The dorsal cutaneous nerves (T3-12) were blunt dissected and exposed under a dissection microscope on both sides. On the left side, nerves were axotomized as close to their anatomical entry into the skin. The right side was kept as a sham-denervated control. Skin was then closed using steel wound
clips. The entire procedure from initiation of anesthesia to recovery took no longer than 30 minutes. Following recovery, awake and mobile mice had the area of skin denervation assessed and confirmed immediately by pinprick testing. 10 days after surgery, the area of denervated skin was confirmed and outlined on the surface of the skin using a marker. Mice were sacrificed and skin was harvested from the marked area of denervation. On the sham side, skin was harvested from a corresponding area. This approach allowed the direct comparison of outcome measures within the same mouse under innervated and denervated conditions. Only mice with confirmed acanthosis on their sham operated side were included in our analyses. Moreover, each mouse that underwent surgical denervation had loss of PGP9.5 expression confirmed at the histological and Western blotting levels (allowing analyses of adjacent skin for immunostaining and protein and RNA outcome measures, respectively) and only mice with a confirmed denervation were used for further analyses.

In vivo neuropeptide inhibitor and agonist experimental approach.

For SP and CGRP inhibitor experiments, two month old KC-Tie2 mice were used. For each animal, a pretreatment biopsy was taken, thus each animal could be used as its own control. Only animals with confirmed acanthosis were used. The highly selective NK-1R antagonist named RP67580 (Tocris) was injected intraperitoneally at a dose of 200 µg/200 µl PBS/mouse every other day from day 1 until day 29. The CGRP-1 receptor peptide antagonist, CGRP (8-37) (Tocris) was injected intraperitoneally at a dose of 1µg/200µL PBS/mouse every day from day 1 until day 29. At 30 days of treatment (24 hours after last injection) animals were sacrificed, and biopsies were taken adjacent to previous punches.
To restore SP and CGRP signaling to denervated skin, surgical denervations were performed as described above. CGRP peptide (Sigma) and SP agonist GR73632 (Tocris) were diluted in PBS. Starting 24 hours after surgery, 50µl intradermal injections of CGRP (1nmol per injection site), SP (5nmol per injection site), or PBS were performed on the denervated side of individual animals. 24 hours after last injections skin was harvested from injection site and from corresponding area on control side.

For all agonist and antagonist experiments, doses and administration routes were chosen based on previous reports showing in vivo effectiveness in mediating biological responses in skin (Arck et al., 2003; Costa et al., 2008; Fuller et al., 1987; Legat et al., 2004; Niizeki et al., 1999).

Tissue collection and histological and morphometric analyses.

After mice were euthanized, hair was shaved and skin from the back was harvested and processed for either frozen or paraffin sectioning. For paraffin sectioning, skin was placed in 10% buffered formalin (Surgipath Medical Industries, Richmond, IL), overnight at 4°C, followed by dehydration and embedding (Sakura Finetech, Torrance, CA). For frozen sectioning, skin was either fixed in Zamboni’s fixative (Newcomer supply, Middleton, WI) overnight at room temperature, transferred to 5% sucrose for 1 hour at 4°C and placed in 20% sucrose overnight at 4°C and then embedded; or fixed in the non-cross-linking fixative Histochoice (Amresco, Solon, OH) for 4 hours at 4°C, transferred to 5% sucrose for 1 hour at 4°C and placed in 20% sucrose overnight at 4°C and then embedded; or immediately embedded in Tissue Fixation Media in TBS (TFM; Triangle Biomedical), and then flash frozen in liquid nitrogen. Additional pieces of back skin from the same animal were flash frozen in liquid nitrogen and stored at -80°C for use in protein experiments.
DRGs and skin from the thoracic region were isolated and harvested from individual animals from a separate subset of KC-Tie2 and control mice and immediately flash frozen in liquid nitrogen and stored at -80°C for use in RNA experiments.

H&E and toluidine blue staining on innervated and denervated back skin from KC-Tie2 mice was completed on 5 µm thick paraffin sections using standard protocols. Images were captured using a Leica DM L82 microscope with an attached Q Imaging MicroPublisher 3.3 Mega Pixel camera and Q-capture Pro software. Epidermal thickness was quantified using Image Pro Plus software (MediaCybernetics, Bethesda, MD). For each animal, 5 measurements were taken from at least five different fields of view from one section (25 measurements per animal). Epidermal thickness was measured from the stratum basale to stratum granulosum, and excluded the stratum corneum. Similarly, mast cells were counted in at least 7 different fields of view from one section, and presented as an average number of mast cells/field of view per animal.

Immunohistochemistry against CD4, CD11c, F4/80, MECA-32, and Ki67 was performed on TFM-embedded frozen skin sectioned at 8 µm, using specific anti-CD4, anti-CD11c (BD Biosciences, San Jose, CA), anti-F4/80 (eBioscience, San Diego, CA), MECA-32 (Developmental Studies Hybridoma Bank, Iowa City, IA), or Ki67 (DAKO, Carpinteria, CA) antibodies. Antibodies were detected using either rabbit anti-rat IgG biotinylated (CD4, F4/80; MECA-32, Ki67; Vector Labs, Burlingame, CA) or goat anti-hamster IgG biotinylated (CD11c; Jackson Immunoresearch Labs, West Grove, PA), amplified with Avidin/Biotinylated Enzyme Complex (Vector Labs) and were visualized using the enzyme substrate diaminobenzidine (Vector Labs). The slides were counterstained with hematoxylin or eosin (Ki67). Images were captured as above. For quantification of MECA⁺, CD11c⁻ and F4/80⁺ cells, image analyses was completed in a blinded fashion using an automated Metamorph software program (Molecular Devices,
Sunnyvale, CA) from at least five fields of view per animal. For quantification of CD4⁺ cells were hand counted from at least five fields of view per animal.

Immunofluorescence against PGP9.5 was performed on free-floating Zamboni fixed tissue sectioned at 50µM using a specific antibody (Ultraclone, UK), followed by labeling with AlexaFluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). The slides were cover-slipped with Vectashield mounting medium (Vector Labs) and antigen-antibody complexes were detected under a Carl Zeiss Axiophot fluorescent microscope and images captured with Carl Zeiss Axiocam HRC. PGP9.5⁺ nerve fiber staining was quantitated using the Metamorph program and reported as number of PGP9.5⁺ nerves per field of view.

Quantitation of cutaneous blood vessel numbers was completed on MECA-32-stained tissue sections as described previously (Wolfram et al., 2009). Photographs were taken and image analyses completed in a blinded fashion using an automated Metamorph software program (Molecular Devices, Sunnyvale, USA). Background staining was minimal therefore colour thresholds were not altered between samples. Post analysis confirmation of blood vessel analysis demonstrated no false positive identification of blood vessels.

Protein isolation and analyses and RNA isolation and quantitation.

Frozen back skin samples taken from the adjacent areas of innervated and denervated skin from the same mice as used for histology and immunohistochemistry were homogenized using a Mikro-Dismembrator S (Sartorius Stedim Biotech, Edgewood, NY). Protein was isolated using PLC buffer (50 mmol/L HEPES pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10
mmol/L NaPPI, 100 mmol/L NaF, 1 mmol/L sodium orthovanadate, and 1 miniprotease inhibitor cocktail tablet Roche Applied Science, Indianapolis, IN) and protein was quantified using a microplate BCA assay (Thermo Scientific, Rockford, IL). For Western blotting, 20µg of protein per sample was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Temecula, CA). Membranes were blocked in 5% milk in TBS-T (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20) and probed with antibodies specific for IL-6 (HRP conjugated; R&D Systems, Minneapolis, MN), PGP9.5 (Ultraclone), GAPDH (Trevigen, Gaithersburg, MD) or actin (Sigma-Aldrich, Saint Louis, MO), washed, incubated with either goat anti-rabbit (for PGP9.5 and GAPDH blotting) or goat anti-mouse (for actin), IgG horseradish peroxidase secondary antibodies (Biorad, Hercules, CA), washed again, and then visualized with Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ). Measurement of the levels of pro-inflammatory cytokines was completed using ELISA according to the suggested protocol of the manufacturer: IL-17 and IFNγ, VEGF, IGF-1 (R&D Systems); and IL-23p19/40 (eBiosciences, San Diego, CA).

Frozen back skin or DRGs isolated from the thoracic regions of control and KC-Tie2 mice were homogenized using similar approaches as described above and RNA isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. First strand cDNA synthesis was achieved using MMLV reverse transcriptase (Invitrogen) following the manufacturer’s protocol. Realtime qRT-PCR was performed using StepOnePlus Taqman technology from Applied Biosystems (Foster City, CA). Probes and primers for CGRP, NPY, SP, VIP, somatostatin and 18S were obtained from Applied Biosystems. Expression levels were calculated relative to 18S using the comparative Ct method.
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