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Supplemental Materials:

Material and Methods:

Antibodies:
Primary antibodies were as follows: rabbit anti-gastrin releasing peptide (GRP, 1:2750; Biomol, now Enzolifesciences, Farmingdale, NY), guinea pig anti-substance P (SP, 1:900; Neuromics, Edina, MN), mouse anti-calcitonin-gene-related peptide (CGRP, 1:250; Enzo Life Sciences), mouse anti-protein gene product 9.5 (1:18; Abcam, Cambridge, MA), guinea pig anti-protein gene product 9.5 (PGP9.5, 1:200, Neuromics), chicken anti-neurofilament 200 (1:20,000; Pierce, Rockford, IL). Secondary antibodies, obtained from Jackson Immunoresearch (West Grove, PA), were raised in donkey and conjugated with biotin, horse radish peroxidase, or Dylight dyes.

Skin Specimens and Immunohistochemistry:
Human skin, free from skin-disease, scar or other signs of trauma, and without incidental lesions, was obtained from the dog ears of excision specimens from both benign and malignant lesions of 17 patients, otherwise without pruritic skin diseases (following approval by the IRB at Boston University to use this de-identified and otherwise discarded surgical tissue without informed consent) and according to Declaration of Helsinki Principles). Patient consent for experiments was not required because Some of these skin samples were taken from patients with non-melanoma skin cancers, and these patients can have both pain and itch (Mills et al.). While no specific itch or pain rating was performed prospectively on the human skin from this study, the dog ear excision specimens were asymptomatic. Mouse skin was obtained from the cheek of 3 male C57/Bl6 mice (following approval by the IACUC at Boston University). Human and mouse skin were placed directly into PLP fixative for 24 hrs, then cryoprotected with 20% sucrose in 0.1M Sorrenson’s buffer, frozen with dry ice, and then sectioned with either a sliding microtome or a cryostat. The immunohistochemical procedure to detect cutaneous nerves was similar to previous described methods (Kennedy and Wendelschafer-Crabb, 1993). Frozen sections 80 uM thick were processed for triple-labeled immunofluorescence localization as follows: No antigen retrieval was used. Sections were blocked for 2 hours in the 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.5% Tyramide Blocking Reagent (Perkin Elmer). Next, rabbit anti-GRP antibody was applied and then detected with donkey anti-rabbit biotin, followed by ABC reagent (Vector labs, Burlingame, CA), followed by fluorophore-conjugated tyramide detection (Perkin Elmer, Waltham, MA/Invitrogen, Grand Island, NY). Horse-radish peroxidase was inactivated with hydrogen peroxide incubation then the remaining primary antibodies were incubated as a cocktail, and either detected with a dylight–conjugated secondary
Timmes et al, GRP-positive nerves in human skin are Aδ and C-fibers (mouse anti-CGRP, guinea pig anti-SP, mouse anti-PGP9.5) or with a hrp-conjugated secondary followed by a second round of tyramide detection (chicken anti-NF200). In all cases, anti-GRP antibody was used first to ensure reactivity attributed to GRP could not be a product of antibody cross-reactivity. Moreover, for each antibody combination, appropriate negative controls omitting each primary antibody were carried out, confirming complete inactivation of the first hrp-conjugate as well as absence of cross-reactivity of the secondary antibodies. Furthermore, the specificity of the anti-GRP antibody was confirmed by preabsorbing the anti-GRP serum (1:2750) with full-length GRP peptide (50 μG/mL, American Peptide Company, Sunnyvale, CA, 46-1-70A) overnight at 4 °C with gentle agitation, followed by high speed centrifugation (~16,000 x g) for ten minutes (Fleming et al., 2012). The resulting supernatant was used for immunohistochemistry, as described above, and showed absence of GRP-nerve fiber immunoreactivity in the presence of SP(+) nerves (data not shown). Finally, the distinction between C- and Aδ-fibers utilized differential staining with NF-200. In this regard, Aδ-fibers can be identified by the simultaneous presence of NF-200 protein and absence of myelin basic protein (MBP). However, given both the paucity of MBP-positive nerves in human skin and their location predominantly in the deeper dermis, NF-200 expression in papillary dermal nerves effectively marks Aδ fibers (Reinisch and Tschachler, 2012).

Image Analysis and Nerve Length Quantification:
For each specimen except one, at least 3 different sections were stained, with inter-section distance of at least 0.5 cm, resulting in random sampling of at least 1.5 cm of each tissue biopsy. For one hand specimen, only one section was studied. Specimens were examined with a laser scanning confocal microscope. Confocal images were acquired at 2 μm intervals throughout the depth of the specimen using a 10x lens (Z-series), separately for each fluorescent signal. In all cases, the imaging field had at its most rostral edge, the stratum corneum. Images contained the entire epidermis, papillary dermis, at least the superficial reticular dermis (including a depth of at least 200 μm beneath the dermal-epidermal junction). To quantify nerve lengths in sections stained with SP/PGP9.5/GRP or CGRP/PGP9.5/GRP, the entire length of each tissue section was imaged in consecutive and non-overlapping 10x fields to prevent double-tracing of nerves in adjacent 10x fields. To quantify nerve lengths in sections stained with SP/NF-200/GRP, at least 3 non-overlapping 10x fields containing GRP(+) nerves were randomly chosen per tissue specimen to image. Three-dimensional reconstruction of each Z-series was produced using Image J software (NIH) with the micro-manager plug-in. Each Z-series was then projected into a single in-focus image (Z-projection), and nerve fibers within the papillary dermis or at the DEJ, corresponding
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to single, double, or triple-labeled fibers were identified by manual comparison of the respective fluorescent Z-series. Neuron J software was used to manually trace nerve fibers in each Z-projection as well as to quantitate the resulting nerve length (Meijering et al., 2004). This measurement may underestimate the true PGP9.5 nerve length because small dermal nerve bundles composed of several fibers are assessed as a single dermal nerve fiber (Hirai et al., 2000). Nevertheless, in human papillary dermis, this overestimate is negligible for peptidergic nerves given their small caliber and predominance as single fibers in this anatomic location.

**Statistical Methods:**
Each tissue specimen had multiple tissue sections. The “% of (marker A) nerve length that is also positive for (marker B)” for a given tissue specimen was then calculated as follows: For each tissue section of a tissue specimen, 10x imaging fields were acquired as described in “Image Analysis and Nerve Length Quantification” above. For each 10x field, the total amount of a nerve length that co-expressed (markers A and B) was divided by the total amount of nerve length expressing (marker A). As these data are non-parametric, JMP10 statistical software was used to calculate, from the values of each 10x field per tissue section, the median % for that entire tissue section. These tissue section medians were used to generate the cumulative median with 25% and 75% quartiles, reported for the entire tissue specimen. When generating percentages for an anatomic grouping, that combines tissue specimens from different patients, cumulative medians per tissue section were each treated as one data point for further statistical analysis, as the tissue sections were not serially generated but were separated by at least 0.5 cm. To determine the statistical significance of differences between medians of different groups, the non-parametric Wilcoxon rank-sum test for two groups and the Kruskal-Wallis one-way analysis of variance test for three or more groups were used, with an alpha level of 0.05. If the Kruskal-Wallis one-way analysis of variance test for three or more groups showed significance, additional analysis with the Wilcoxon Each Pair test was done to compare medians between groups, also with an alpha level of 0.05.
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Table S1. Patient Demographics and % of PGP9.5(+) Papillary Dermal Nerves That Are GRP(+)

| Body Site | Age | Gender | Anatomic Grouping | % of PGP9.5(+) nerves that are GRP(+)\(^1\) |
|-----------|-----|--------|-------------------|------------------------------------------|
| Cheek     | 81  | F      | Head and Neck     | 6.8, 3.0 – 13.2                          |
| Cheek     | 74  | F      | Head and Neck     | 11.4, 5.7 – 12.9                         |
| Forehead  | 77  | F      | Head and Neck     | 7.1, 1.2 – 8.4                           |
| Forehead  | 66  | M      | Head and Neck     | 3.8, 1.7 – 5.9                           |
| Temple    | 50  | F      | Head and Neck     | 12.2, 8.0 – 15.4                         |
| Nasal Tip | 66  | M      | Head and Neck     | 1.5, 0.0 – 3.0                           |
| Neck      | 47  | M      | Head and Neck     | 5.2, 5.0 – 12.6                          |
| Neck      | 65  | M      | Head and Neck     | 4.0, 2.5 – 7.6                           |
| Scalp     | 64  | F      | Head and Neck     | 9.8, 7.7 – 15.7                         |
| Forearm   | 48  | M      | Extremity         | 7.6, 6.6 – 9.4                           |
| Hand      | 72  | M      | Extremity         | 16.8                                     |
| Hand      | 78  | F      | Extremity         | 14.3, 8.8 – 21.0                         |
| Thigh     | 64  | M      | Extremity         | 12.5, 8.7 – 13.4                         |
| Shin      | 86  | F      | Extremity         | 11.4, 8.3 – 17.1                         |
| Back      | 66  | M      | Trunk             | 5.5, 3.5 - 10.4                          |
| Back      | 65  | F      | Trunk             | 9.5, 7.8 – 16.7                          |
| Shoulder  | 44  | F      | Trunk             | 5.6, 1.9 - 16.3                          |

\(^1\) median, 25% - 75% quartiles
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Table S2. Human Papillary Dermis Nerve Staining Results: PGP9.5/GRP/CGRP

| Body Site | Anatomic Grouping | % of PGP9.5(+) nerves that are GRP(+)<sup>1</sup> | % of PGP9.5(+) nerves that are CGRP(+)<sup>1</sup> | % of GRP(+) nerves that are CGRP(+)<sup>1</sup> | % of CGRP(+) nerves that are GRP(+)<sup>1</sup> |
|-----------|-------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Cheek     | Head & Neck       | 6.8, 3.0 – 13.2 | 14.0, 6.5 – 17.9 | 3.65, 0.0 – 39.6 | 2.1, 0.0 – 7.1 |
| Cheek     | Head & Neck       | 11.4, 5.7 – 12.9 | 10.6, 6.0 – 21.0 | 69.0, 34.5 – 94.3 | 42.0, 37.5 – 90.3 |
| Forehead  | Head & Neck       | 7.1, 1.2 – 8.4  | 16.5, 8.8 – 29.9 | 57.5, 29.8 – 67.6 | 10.6, 3.6 – 18.6 |
| Forehead  | Head & Neck       | 3.8, 1.7 – 5.9  | 13.2, 5.7 – 16.7 | 66.6, 12.3 – 96.0 | 29.3, 4.3 – 54.7 |
| Temple    | Head & Neck       | 12.2, 8.0 – 15.4 | 4.6, 2.9 – 5.3  | 18.9, 3.9 – 33.6 | 50.0, 8.0 – 80.2 |
| Nasal Tip | Head & Neck       | 1.5, 0.0 – 3.0  | 0                | 0                | 0                |
| Neck      | Head & Neck       | 5.2, 5.0 – 12.6 | 20.7, 8.6 – 52.4 | 73.8, 52.9 – 100 | 24.1, 13.2 – 42.6 |
| Neck      | Head & Neck       | 4.0, 2.5 – 7.6  | 10.4, 6.85 – 16.5| 76.1, 55.9 – 81.1| 27.9, 18.2 – 45.3 |
| Scalp     | Head & Neck       | 9.8, 7.7 – 15.7 | 30.2, 23.5 – 37.0| 18.2, 18.1 – 18.3| 6.3, 5.1 – 7.5  |
| Forearm   | Extremity         | 7.6, 6.6 – 9.4  | 12.2, 9.4 – 29.0 | 34.1, 18.5 – 56.8| 24.2, 6.5 – 39.9 |
| Hand      | Extremity         | 16.8            | 12.8             | 10.4             | 13.6             |
| Hand      | Extremity         | 14.3, 8.8 – 21.0| 27.8, 18.7 – 29.3| 83.7, 57.6 – 95.2| 35.6, 28.1 – 58.0|
| Thigh     | Extremity         | 12.5, 8.7 – 13.4| 8.9, 8.7 – 17.0  | 100, 71.4 – 100  | 100, 78.8 – 100  |
| Shin      | Extremity         | 11.4, 8.3 – 17.1| 33.3, 27.2 – 39.0| 71.2, 63.0 – 84.0| 35.7, 19.3 – 37.4|
| Back      | Trunk             | 5.5, 3.5 - 10.4 | 13.2, 12.7 – 24.0| 43.1, 25.8 – 97.4| 35.0, 23.3 – 37.6|
| Back      | Trunk             | 9.5, 7.8 – 16.7 | 29.2, 22.8 – 36.4| 84.9, 56.1 – 93.1| 39.1, 14.0 – 53.4|
| Shoulder  | Trunk             | 5.6, 1.9 – 10.5 | 11.6, 8.4 – 14.7 | 88.1, 80.0 – 97.3| 36.6, 35.8 – 37.3|

<sup>1</sup>median, 25% - 75% quartiles
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Table S3. Human Papillary Dermis Nerve Staining Results: PGP9.5/GRP/SubP

| Body Site  | Anatomic Grouping | % of PGP9.5(+) nerves that are GRP(+)\(^1\) | % of PGP9.5(+) nerves that are SubP(+)\(^1\) | % of GRP(+) nerves that are SubP(+)\(^1\) | % of SubP(+) nerves that are GRP(+)\(^1\) |
|------------|-------------------|-------------------------------------------|-------------------------------------------|------------------------------------------|------------------------------------------|
| Cheek      | Head & Neck       | 6.8, 3.0 – 13.2                           | N.A.                                      | N.A.                                      | N.A.                                      |
| Cheek      | Head & Neck       | 11.4, 5.7 – 12.9                          | N.A.                                      | N.A.                                      | N.A.                                      |
| Forehead   | Head & Neck       | 7.1, 1.2 – 8.4                            | 2.4, 1.2 – 8.4                           | 17.4, 13.7 – 34.1                         | 100                                       |
| Forehead   | Head & Neck       | 3.8, 1.7 – 5.9                            | 3.8, 0.0 – 5.9                           | 100, 0.0 – 100                            | 100, 0.0 – 100                            |
| Temple     | Head & Neck       | 12.2, 8.0 – 15.4                          | 27.4, 0.3 – 32.9                         | 100, 47.9 – 100                           | 57.4, 35.9 – 100                         |
| Nasal Tip  | Head & Neck       | 1.5, 0.0 – 3.0                            | N.A.                                     | N.A.                                      | N.A.                                      |
| Neck       | Head & Neck       | 5.2, 5.0 – 12.6                           | N.A.                                     | N.A.                                      | N.A.                                      |
| Neck       | Head & Neck       | 4.0, 2.5 – 7.6                            | 8.2, 6.1 – 8.8                           | 94.5, 45.9 – 100                          | 56.1, 18.4 – 85.6                        |
| Scalp      | Head & Neck       | 9.8, 7.7 – 15.7                           | 14.2, 10.2 – 21.8                        | 76.3, 57.3 – 100                          | 65.0, 53.8 – 94.6                        |
| Forearm    | Extremity         | 7.6, 6.6 – 9.4                            | 8.1, 7.7 – 8.7                           | 100, 33.0 – 100                           | 95.1, 32.5 – 100                         |
| Hand       | Extremity         | 16.8                                      | N.A.                                     | N.A.                                      | N.A.                                      |
| Hand       | Extremity         | 14.3, 8.8 – 21.0                          | 19.0, 6.8 – 25.1                         | 98.2, 96.6 – 100                          | 98.5, 86.4 – 100                         |
| Thigh      | Extremity         | 12.5, 8.7 – 13.4                          | N.A.                                     | N.A.                                      | N.A.                                      |
| Shin       | Extremity         | 11.4, 8.3 – 17.1                          | 7.9, 4.3 – 11.4                          | 75.9, 51.7 – 100                          | 100                                       |
| Back       | Trunk             | 5.5, 3.5 - 10.4                           | 4.5, 2.5 – 6.25                          | 100, 7.7 – 100                            | 86.4, 7.9 – 95.1                        |
| Back       | Trunk             | 9.5, 7.8 – 16.7                           | N.A.                                     | N.A.                                      | N.A.                                      |
| Shoulder   | Trunk             | 5.6, 1.9 – 10.5                           | 11.7, 0.5 – 13.1                         | 0.0, 0.0 – 100                            | 0.0, 0.0 – 69.1                         |

\(^1\) median, 25% - 75% quartiles
\(^2\) N.A., not assessed
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Table S4. C57/Bl6 Mouse Papillary Dermis Nerve Staining Results

|       | % of PGP9.5 also GRP(+) | % CGRP(+) also GRP(+) | % GRP(+) also CGRP(+) | % SP(+) also GRP(+) | % GRP(+) also SP(+) |
|-------|-------------------------|-----------------------|-----------------------|---------------------|---------------------|
| Male  | 33.1%, 26.1 – 64.8%     | 35.3%, 32.6 – 83.8%   | 86.9%, 85 – 98.8%     | 81.4%, 61.6 – 90.1% | 86.2%, 79.4 – 91.6% |
| Cheek |                         |                       |                       |                     |                     |

1median, 25% - 75% quartiles
Figure S1. GRP is expressed in human skin by a subset of nerves innervating adnexal structures.

Figure S1. Immunohistochemical (a-f) double- and (g-i) triple-staining of human skin sections showing (a,d,g) GRP, (b, e) PGP9.5, or (c, f) overlay of GRP (red) and PGP9.5 (green); (h) CGRP, (i) overlay of GRP (red), CGRP (green), and PGP9.5 (blue). (c,f), yellow indicating GRP(+) / PGP9.5(+) staining. (i) pink pseudocolor indicates GRP(+) / CGRP(-) / PGP9.5(+) staining. (d,f), thick arrows indicate GRP(+) / PGP9.5(+) nerves. (g-i), thick arrows indicate GRP(+) / CGRP(-) / PGP9.5(+) nerve. Hair follicle (a-c), eccrine gland (d-f), epidermis/papillary dermis (g-i). (g), epi, epidermis; der, dermis. All scale bars: 50 μm.
Figure S2. Immunohistochemical double-staining of C56Bl/6 male mouse skin sections showing (a, d) GRP only, (b) SP only, (c) overlay of GRP (red) and SP (green), (e) CGRP only, and (f) overlay of GRP (red) and CGRP (green). (c, f), yellow indicates GRP(+)/SP(+) (c) or GRP(+)/CGRP(+) nerves (f). (a-f), arrow indicates GRP(+) nerves that co-stain for either SP (a-c) or CGRP(d-f). Arrowheads indicate GRP(-) and either SP(+) (b,c) or CGRP(+) (e,f) nerves. (e,f), thin arrows indicate GRP(-)/CGRP(+) intraepidermal nerve fibers. (a), e, epidermis; d, dermis. (d), epi, epidermis; der, dermis. All scale bars: 50 μm.