Published in final edited form as:

J Pain. 2017 May ; 18(5): 511–525. doi:10.1016/j.jpain.2016.12.008.

A local inflammatory renin-angiotensin system drives sensory axon sprouting in provoked vestibulodynia

Zhaohui Liao¹, Anuradha Chakrabarty¹, Ying Mu¹, Aritra Bhattacharjee¹, Martha Goestch^{2,3}, Catherine M. Leclair^{2,3}, and Peter G. Smith^{1,4}

¹Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS 66160

²Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon, 97239

³Co-senior authors

⁴Senior and corresponding author: psmith@kumc.edu

Abstract

Vestibulodynia is a form of provoked vulvodynia characterized by profound tenderness, hyperinnervation, and frequently inflammation within well-defined areas of the human vestibule. Prior experiments in animal models show that inflammatory hypersensitivity and hyperinnervation occur in concert with establishment of a local renin-angiotensin system (RAS). Moreover, mechanical hypersensitivity and sensory axon sprouting are prevented by blocking effects of angiotensin II on AT2 receptors. This case-control study assessed whether a RAS contributes to hyperinnervation observed in human vestibulodynia. Vestibular biopsies from asymptomatic controls or patients' nontender areas showed moderate innervation and small numbers of inflammatory cells. In women with vestibulodynia, tender areas contained increased numbers of mechanoreceptive nociceptor axons, T-cells, macrophages and B-cells, while mast cells were unchanged. RAS proteins were increased due to greater numbers of T-cells and B-cells expressing angiotensinogen, and increased renin-expressing T-cells and macrophages. Chymase, which converts angiotensin I to angiotensin II, was present in constant numbers of mast cells. To determine if tender vestibular tissue generates angiotensin II that promotes axon sprouting, we conditioned culture medium with vestibular tissue. Rat sensory neurons cultured in control-conditioned medium showed normal axon outgrowth, while those in tender tissue-conditioned medium showed enhanced sprouting that was prevented by adding an AT2 antagonist or angiotensin II neutralizing antibody. Hypersensitivity in provoked vestibulodynia is therefore characterized by abnormal mechano-nociceptor axon proliferation, which is attributable to inflammatory cell-derived angiotensin II (or a closely related peptide) acting on neuronal AT2

Disclosures: Supported by NIH R01HD049615 (PGS), with core facility support by U54 HD 090216 and P30 HD002528. The authors have no conflicts of interest to declare.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

receptors. Accordingly, reducing inflammation or blocking AT2 represent rational strategies to mitigate this common pain syndrome.

Perspective: This study provides evidence that local inflammation leads to angiotensin II formation which acts on the angiotensin II receptor type 2 to induce nociceptor axon sprouting in vulvodynia. Preventing inflammation and blocking AT2 therefore present potential pharmacological strategies for reducing vestibular pain.

Keywords

Vulvodynia; Peripheral nociceptors; Neural plasticity; Inflammation; Angiotensin II; Quantitative histology; Cell culture

Introduction

Vulvodynia comprises a family of disorders characterized by genital hypersensitivity (allodynia) with a prevalence of 8–15%²⁶. Vulvodynia may manifest as diffuse pain (generalized or ‘dysesthetic’ vulvodynia), or more commonly as localized pain occurring with mechanical stimulus to the vulvar vestibule⁴³ referred to as vestibulodynia or provoked vulvodynia (PVD, formerly vulvar vestibulitis). Vestibulodynia may present as penetrative pain occurring at the time of first attempts at intromission (primary) or appear after years of pain-free intercourse (secondary). PVD can severely compromise life quality and is estimated to cost the US economy as much as \$70B per year⁶². Non-invasive therapies may be helpful, but patients seen in programs offering expert vulvar care may elect to undergo surgical excision of the painful tissue (vestibulectomy) in order to obtain more effective relief^{9, 23}.

While the etiology of vestibulodynia remains unclear, several risk factors have been proposed including adolescent physical or sexual abuse⁴⁴, juvenile exposure to high-progesterone oral contraceptives^{4, 34}, and vulvovaginal yeast infections⁶¹. Interestingly, mice subjected to repeated vaginal candidiasis develop a vulvodynia-like phenotype, thus supporting a causal role of infection in this animal model¹⁹.

PVD is characterized by distinct histological changes within the painful zone⁵⁹. Studies using pan-neuronal markers consistently reveal excessive numbers of axons (hyperinnervation) within the tender dermal/epidermal tissue^{8, 10, 24, 57, 60}, although axonal subtypes involved have not been defined. Histological studies also frequently report evidence of inflammatory cell infiltration. For example, accumulations of T-cells^{12, 33} with smaller numbers of B-cells and monocytes¹² have been reported. While some investigators note increased numbers of mast cells^{10, 13} others do not^{33, 41}, and some failed to find any evidence of inflammatory cell infiltration¹⁸. Hence, while the preponderance of evidence suggests that vestibular pain correlates with axonal and inflammatory cell proliferation, questions remain as to the nature, degree and significance of these changes.

A question unexplored is whether a causal relation exists between inflammatory cells and hyperinnervation. Inflammatory cells produce many neuroactive substances including the octapeptide angiotensin II (ANGII). ANGI is formed by cleavage of the precursor protein

angiotensinogen (ATG) by the protease renin to form biologically inactive ANGI, which is converted to active ANGII by angiotensin converting enzyme, mast cell chymase and members of the cathepsin family of enzymes; collectively, these proteins comprise the renin-angiotensin system (RAS). Prior studies have shown that inflammatory cells express RAS proteins and can generate ANGII^{28, 31, 58} which is known to promote axon sprouting and regeneration^{14, 17, 21, 22, 47}. In unmyelinated sensory neurons of the dorsal root ganglion (DRG), ANGII promotes outgrowth by activating ANGII receptor type 2 (AT2)^{2, 3, 14}, which is present in rat¹⁴ and human⁴² DRG.

We postulated that vestibular inflammatory cells generate ANGII to act upon AT2 receptors on mechanosensory nociceptor axons to induce sprouting, thereby contributing to hyperinnervation and possibly hypersensitivity in PVD. Accordingly, the objectives of the present study were to determine if there is an exaggerated inflammatory RAS in painful regions of the vulva, and if this results in increased release of ANGII which acts on sensory neuronal AT2 receptors to promote axon sprouting. If so, this may provide a new therapeutic strategy for pharmacological management of PVD, as drugs that block AT2 also prevent inflammatory hypersensitivity in vivo¹⁵.

Materials and methods

Tissue procurement and processing for histological analysis from human subjects

Premenopausal women aged 18–45 years old with dyspareunia and meeting Friedrich's criteria²⁰ for PVD and electing superficial vestibulectomy were recruited at the Program in Vulvar Health at OHSU. They had severe dyspareunia, defined as pain with every episode of vaginal penetration resulting in reduction in frequency or avoidance of sexual intercourse. The examination criterion for severe PVD was exquisite tenderness in portions of the vulvar vestibule with recoil from light rolling touch with a moistened Q-tip. Additionally, the tenderness could be extinguished by a 2–3 minute application of 4% lidocaine topical solution. Women were free from hormonal medications, topical or oral neuromodulators, steroids, pain medications or ACE inhibitors, and patients had no other cause for localized pain. To standardize hormonal influences, surgeries were scheduled during the follicular phase of the menstrual cycle just prior to ovulation (d10–14), and serum progesterone was assayed to confirm preovulatory status (<3ng/ml). The study protocol was approved by the institutional review board of OHSU (#8519) and all subjects provided informed consent.

Tissue was obtained from 14 patients undergoing localized vestibulectomy for treatment of severe primary (11) or secondary (3) provoked vestibulodynia. Tissue for analysis represented the most tender areas of the vestibule as defined by gently rolling a moistened cotton swab over the vestibular tissue (cotton swab test). Patients were asked to verbally rate their pain while the examining provider (CML, MFG) observed their behavior with examination. The pain scale was 0–3 with 0 = normal painless touch; 1 (mild) = irritation or annoyance but not painful; 2 (moderate) = sharp or burning pain, and 3 (severe) = extreme burning pain with recoiling. The most tender areas corresponded to 4 or 8 o'clock just outside the hymen ring (urethra at 12 o'clock) and all samples were from patients with a rating of 3. In addition, a 4mm punch biopsy was taken from an area of the vestibule judged to show clinically normal sensitivity (nontender) as assessed by the cotton swab test at either

the right or left anterior vestibule lateral and anterior to the urethral meatus. We also obtained from 7 asymptomatic age-matched control subjects a 4mm punch biopsy from a nontender area of the vestibule corresponding to the most tender areas of patients with PVD (4 o'clock or 8 o'clock, next to a Bartholin's duct orifice, Control). In some cases, the amount of tissue available precluded analysis of all variables described in this study but this did not compromise study power. Tissue was fixed immediately by immersion in Zamboni's solution for 24 hours at 4°C, transferred to 0.01MPBS, and shipped at 4°C overnight to KUMC. Tissue was washed daily for 7 days with PBS, followed by cryoprotection in 20% sucrose, snap-frozen in tissue freezing medium (EMS, Hatfield, PA), and stored at -80°C. Ten-micron serial cryosections were cut perpendicular to epithelium, thaw-mounted onto coated slides (Fisher Scientific, Pittsburgh, PA), air-dried and stored at -80°C.

Sections were rinsed in PBS containing 0.3% Triton X-100 (Sigma, St. Louis, MO), blocked with normal goat serum (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, and incubated overnight at room temperature or 4°C with antiserum against protein gene product 9.5 (PGP9.5), transient receptor potential vanilloid 4 (TRPV4), neurofilament H (NF-H), TCR α/β , CD79 α , CD68, chymase, angiotensinogen (AGT), or renin (Table 1). Primary antibody binding was visualized with fluorochrome-conjugated secondary antibodies. Double staining was performed using PGP9.5 with TRPV4; AGT with TCR α/β , CD79 α , CD68 or chymase; and renin with TCR α/β , CD68, CD79 or chymase. All incubations were carried out in a humidified chamber and slides were coverslipped with Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL). Antibody specificities were confirmed by primary antibody omissions, preadsorption with excess antigen, and western blot. All analyses were conducted blindly on coded, randomized samples.

Quantitative Analyses of innervation and cells

Preliminary examination of our specimens revealed that most visible innervation was present in the subepithelial region of the tissue specimen^{10, 60}. To estimate vestibular innervation, 3 sections each separated by ~0.2mm were sampled for each vestibular specimen. A sampling region (432×346 μ m) juxtaposed with the epithelial/subepithelial interface was captured for analysis using a Nikon Eclipse TE300 and Nikon Fluor 20X objective with an Optronix MagnaFire camera. For each section, images for analysis were obtained from the tissue center and 1mm on either side of the central field selected under bright field illumination. The apparent percentage area of subepithelium occupied by fluorescing immunoreactive axons was quantified by superimposing a stereological grid (AnalySis v.3.2, Soft Imaging System GmbH, Münster, Germany) with intersects at 20 μ m intervals. Numbers of intersection points overlying stained axons were counted and divided by total points over all subepidermal tissue within the sample field, as described previously¹⁵. Measurements of TRPV4 innervation were obtained from samples double stained with PGP9.5 and only structures immunostained by both antibodies were counted. Values from the 9 sampled regions for each vestibular tissue were averaged to provide a single value for that subject.

Numbers of inflammatory cells were determined from the same sections and regions used for innervation analysis. Images for analysis (AnalySis v.3.2) were captured using a Nikon

Eclipse 80i microscope with a Fluor 40X objective and DSFi1 camera. Standardized counts were obtained from a sample frame (342×257µm) positioned immediately below the epithelium, and all cells lying completely or partially within this field were included. Cells immunoreactive for different cell markers were counted individually to provide indices of the numbers of inflammatory cell subtypes (T-cells, B-cells, macrophages or mast cells) per unit area of section. These sections were also immunostained for the RAS proteins, angiotensinogen (AGT) or renin, and numbers of cells per unit area expressing these proteins were also determined. To determine which cell types contribute to the local vestibular RAS, we calculated percentages of AGT- or renin-ir cells that also expressed one of the inflammatory cell subtype markers. To determine if the level of RAS protein expression within individual inflammatory cell subtypes varied under different conditions, we also calculated the percentage of individual cell subtypes expressing either AGT or renin. Data are presented as average numbers of positive cells per mm² of sub-epidermal tissue or as percentages of a given cell population.

DRG neurite outgrowth in vestibular tissue-conditioned medium

For tissue neurite outgrowth assays, a 6mm punch biopsy was obtained from 4 controls and from nontender and tender areas of 5 patients (4 rated 3, one rated 2 by cotton swab test). These were cut into 4 smaller pieces, transferred to L-15 medium (ThermoFisher Scientific, Grand Island, NY), and immediately shipped at 4°C to KUMC overnight. Upon arrival, each sample was rinsed in sterile PBS, cut into smaller pieces (~0.5mm³) and placed in 3ml Neurobasal medium containing B27 (ThermoFisher Scientific, Grand Island, NY) and 5% FBS (Sigma, St Louis, MO). To generate vestibular-conditioned medium which contained diffusible proteins released by the tissue, pieces were cultured for 48 hours at 37°C in an incubator with 5% CO₂. Conditioned medium was harvested, filtered through 0.22µm syringe filter and stored at -80°C.

T13-S2 DRGs were isolated from neonatal rats (<3 d) using protocols approved by the KUMC animal care and use committee (ACUP #2016–2318) and in compliance with all federal, state and local guidelines. DRGs were digested with collagenase and dispase at 37°C for 45min, gently triturated with fire-bored Pasteur pipet, filtered through 40µm cell strainer and spun down at 300g for 7min. The cell pellet was resuspended in Neurobasal A medium (ThermoFisher Scientific, Grand Island, NY) supplemented with 10%FBS, B27, L-glutamine and primocin (InvivoGen, San Diego, CA) and plated on poly-D-lysine and laminin coated 24 well plates (Corning, Corning, NY). Cultures were maintained in 5% CO₂ at 37°C for 24h. Medium was then changed to vestibular-conditioned medium diluted with equal volume of fresh medium above plus uridine and FrdU ^{6, 7, 14}.

Cells were cultured for 24h with medium from control, nontender or tender tissue either alone, or containing 1µM PD123319 or 10µg/ml angiotensin II neutralizing antibody (GeneTex GTX37789, Irvine, CA). The ANGII antibody was generated in rabbit using native human/rat ANGII (DRVYIHPF) as the immunogen. The rabbit IgG purified with an antibody purification kit (Abcam, Cambridge, UK) according to the manufacturer's protocol in order to remove sodium azide. Cultures were fixed with 4% paraformaldehyde, rinsed with PBS, and blocked with normal donkey serum (Millipore, Temecula, CA) for 1h at room

temperature. Neurites and cell bodies were visualized by immunostaining with a chicken anti-peripherin antibody overnight at 4°C, followed by a Cy3 conjugated donkey anti chicken.

Neurite outgrowth was measured in 12 randomly selected fields (total combined area = 1.8mm²) captured using a Nikon Eclipse TE300 with Optronics camera at 20X. A stereological grid (AnalySis v.3.2) was superimposed over each image, and the number of line intersections overlying stained neurites was divided by total intersections within the field and multiplied by total field area. This was then divided by the number of neurons with at least one neurite in the field, averaged for each well, and expressed as neurite area (μm²) per neuron as reported previously^{6, 14, 34, 55}.

Statistical analysis

Numbers of samples included for analysis was determined using Glass' delta to estimate effect size. Values are presented as mean ± SEM. Statistical comparisons were made using one-way ANOVA with posthoc comparisons by the Student-Newman-Kuels test or using multiple t-test with Bonferroni-Holm correction (Sigmaplot 12.5, Systat Software Inc., San Jose, CA). Statistical significance (two-tailed P value) was accepted at P = 0.05.

Results

Innervation is increased in tender areas of the vestibule

We first confirmed that the tender tissue from patients with PVD displayed hyperinnervation relative to nontender tissue and asymptomatic control tissue. Immunostaining for the pan-neuronal marker PGP9.5 in tissues from control biopsies revealed moderately abundant axons throughout the dermal-epidermal junction and sub-epidermal regions (Fig. 1A), and this was similar to the pattern observed in nontender areas of patients with PVD (Fig. 1B). Nerve fiber density in vestibular tissue from tender areas appeared to be greater than in nontender areas or control tissue (Fig. 1C). Quantitative analysis revealed that innervation density was comparable in control and nontender areas, but increased in tender areas by 67% and 69% (Fig. 1D; P=0.021 and 0.027), respectively.

While analysis of PGP9.5-ir confirms hyperinnervation reported by others^{8, 24, 57, 60}, the contributing fiber types are unknown. Prior studies showed that thermally sensitive CGRP-ir fibers do not contribute substantively to hyperinnervation⁵⁷, although expression of TRPV1, a voltage-gated calcium channel mediating thermal nociception, may be increased⁵⁶. Because PVD is characterized primarily by increased mechanical sensitivity, we assessed the contribution of mechano-nociceptive neurons. TRPV4, a member of the transient receptor potential family of ligand-gated ion channels^{35, 53}, is important in transducing osmotic and mechanical stimuli in sensory nociceptor neurons^{36, 54} and has been shown to contribute to mechanical hyperalgesia in a model of small-fiber painful peripheral neuropathy¹. In control vestibular tissue, TRPV4-ir was localized on fine nerve fibers in regions immediately beneath epidermis or dermal papilla (Fig. 1E), and a similar pattern was observed in nontender tissue (Fig. 1F). In tender tissues, TRPV4-ir fibers appeared more abundant (Fig.

1G), and quantitative analysis (Fig. 1H) showed that innervation density was increased by 139% and 115% relative to control and nontender tissues ($P=0.011$ and 0.002), respectively.

Mechanical sensation is also mediated by low threshold, medium-diameter A β myelinated fibers which can be detected by immunostaining for Neurofilament H (NF-H)²⁹. Fibers stained by this marker were of larger diameter and distributed relatively deeply in the dermis in control tissue (Fig. 1I). There was no obvious difference in innervation density in controls relative to nontender (Fig. 1J) or tender (Fig. 1K) tissues, as confirmed by quantitative analysis (Fig. 1L).

Inflammatory cell density is increased in tender areas of the vestibule

To clarify whether different types of inflammatory cells are present and increased in PVD, we immunostained sections to visualize selective markers including T-cell Receptor α/β (TCR) for T-cells³⁰, the surface protein CD79 α for B-cells^{27, 38}, CD68 as macrophage marker³⁹, and chymase to identify mast cells⁵¹.

TCR-ir T-cells were observed predominantly in upper dermis adjacent to the epidermal border, with smaller numbers scattered within epidermis in both control (Fig. 2A) and nontender tissues (Fig. 2B). T-cells appeared more abundant in painful areas (Fig. 2C), and quantitative analysis (Fig. 2D) showed they were encountered 75% and 220% more frequently in tender as compared to control ($P=0.007$) or nontender ($P<0.001$) tissue. T-cells tended to be more abundant in controls as compared to nontender areas, although the difference did not achieve statistical significance ($P=0.085$).

CD68-ir macrophages were observed throughout dermis in control specimens (Fig. 2E), and distributions and frequency were similar in nontender tissues (Fig. 2F). Tender vestibular tissue, however, contained higher densities of macrophages than nontender or control tissue (Fig. 2G). While macrophage numbers were comparable in control and nontender zones (Fig. 2H), they were increased by approximately 75% in tender area as compared to control ($P=0.048$) or nontender zones ($P=0.020$).

CD79 α -ir B cells were present in lower abundance than T-cells and were also mostly localized to the upper dermis in both controls (Fig. 2I) and in nontender areas (Fig. 2J). In tender areas, B cells were much more abundant (Fig. 2K), increasing by 8- ($P=0.004$) and 19.8-fold ($P=0.008$) relative to control and nontender areas, respectively (Fig. 2L).

Mast cells exist in a quiescent state where their contents remain intact, but can be activated to release their contents in the process known as degranulation. Using mast cell chymase as a marker, we found that about 1/3 showed evidence of degranulation in control tissue (Fig. 2M and inset). In nontender tissues, numbers of both quiescent and degranulated mast cells appeared similar to controls (Fig. 2N). We did not observe marked differences in tender tissue (Fig. 2O) although quantitative analysis showed a small increase in degranulated cells in the tender area (Fig. 2P) relative to the nontender tissue ($P=0.046$).

Overall, the total numbers of inflammatory cells were markedly elevated in the tender areas of the vestibule. The largest increases were in T-cells, which increased approximately 2–3-fold, and B-cells which increased 8–13-fold relative to control and nontender tissue.

Local renin-angiotensin system in vestibule

We have shown previously that hypersensitivity and hyperinnervation occur in association with appearance of renin and angiotensinogen-containing inflammatory cells after irritant injection in the rat foot pad ¹⁵. To determine if human vestibular tissue contains ANGII-synthesizing proteins, we immunostained sections for these critical RAS proteins.

Angiotensinogen (AGT): Vestibular sections were immunostained for the precursor protein AGT, a globular protein from which angiotensin I is cleaved. In control tissue, AGT-ir cells were mainly observed within the upper dermis (Fig. 3A). Nontender tissue also contained AGT-ir cells in roughly the same location as control tissue but at lower frequency (Fig. 3B). In contrast, tender areas showed markedly increased numbers of AGT-ir cells (Fig. 3C). Quantitative analysis (Fig 3D) confirmed that AGT-ir cell density in tender tissues was greater than control ($P=0.011$) or nontender tissue ($P=0.002$).

To determine which cells contribute to the vestibular AGT-expression, we immunostained sections for the T-cell marker TCR. As noted above, T-cells were present in control (Fig. 3E) and nontender tissues (Fig. 3F), but were considerably more abundant in tender areas (Fig. 3G). Double staining showed that AGT-ir was present in a significant proportion of T-cells in control tissue (Fig. 3H), nontender (Fig. 3I) and tender tissues (Fig. 3J), and the number of AGT-ir T-cells was increased in the tender tissues relative to nontender tissues, but not controls (Fig. 3K). T-cells represented approximately one half of all AGT-ir cells in control and nontender tissue, but in tender tissues this was reduced somewhat relative to controls ($P=0.038$, Table 2). Nonetheless, only about one in ten T-cells actually expressed AGT-ir and this did not vary among the different conditions (Table 2).

We assessed whether CD79 α -ir B-cells also contribute to local AGT production. As noted above, numbers of AGT-ir cells were modest in control (Fig. 4A) and nontender tissue (Fig. 4B), but elevated in the tender area (Fig. 4C, D). CD79 α -ir were also present in all sections but increased in the tender tissue (Fig. 4E, F, G). Co-staining showed that AGT-ir co-localized with CD79 α -ir in control (Fig. 4H) and nontender tissue (Fig. 4I), but co-stained cells were observed with much greater frequency in tender tissue (Fig. 4J). Numbers of AGT +CD79 α -ir cells were increased approximately 10-fold in tender as compared to nontender ($P=0.008$) and control tissues ($P=0.004$, Fig 4K). In control and nontender tissues, B-cells represented just under half of all AGT-ir cells, and this proportion increased to over half in tender tissue (Table 2, $P=0.006$). A majority of B-cells displayed AGT-ir, and this did not change significantly under the different conditions (Table 3).

Vestibular tissues were also co-stained for AGT in combination with CD68 for macrophages and chymase for mast cells. In neither case was co-localization evident, indicating that neither macrophages nor mast cells contribute substantively to AGT production in the normal or tender tissues (Tables 2, 3).

Renin: Renin cleaves AGT to produce angiotensin I, a 10 amino acid residue lacking biological activity, but which is readily converted to biologically active ANGII by multiple peptidases including chymase ⁴⁶, angiotensin converting enzyme (ACE), and cathepsin G which are broadly distributed in tissues. To determine renin's presence in vestibular tissues,

we co-stained sections for renin and inflammatory cell markers. In control vestibular tissue, renin-ir cells were distributed throughout the dermis (Fig. 5A), and distributions and numbers were similar in tissue from nontender areas (Fig. 5B). In tender areas, renin-ir cells were substantially more abundant (Fig. 5C), being increased by 98% and 80% relative to control and nontender areas (Fig. 5D, $P < 0.001$ for both comparisons).

To assess the contribution of T-cells, renin-immunostained sections were co-stained for TCR α/β . T-cells were present in control (Fig. 5E), nontender (Fig. 5F) and tender tissues (Fig. 5G) as noted previously. Relatively small numbers of renin-ir cells displaying TCR α/β were present in control (Fig. 5H) and nontender (Fig. 5I) tissues, while tender tissues showed significantly more renin-ir/TCR-ir cells than controls (Fig. 5J, K, $P = 0.002$). T-cells represented approximately 40% of all renin-ir cells, and this did not vary by condition (Table 2). Only about 10–20% of T-cells in all conditions displayed renin-ir (Table 3).

We evaluated renin expression in macrophages from fields co-stained for renin (Fig. 6A–D) and for CD68). CD68 immunostaining confirmed the presence of macrophages in control (Fig. 6E) and nontender tissues (Fig. 6F), as well as their augmented abundance in tender tissues (Fig. 6G). Examination of sections co-stained for renin and CD68 showed similar numbers of renin-positive macrophages in control (Fig. 6H) and nontender (Fig. 6I) areas, but greater abundance in tissue from tender areas (Fig. 6J), with increases of 83% and 82% (Fig. 6K, $P = 0.012$ and $P = 0.005$) respectively. Renin was expressed in most macrophages in all conditions, with $80 \pm 3\%$ in control, $82 \pm 5\%$ in nontender, and $84 \pm 4\%$ in tender areas (Table 3). In addition, macrophages comprised the overwhelming majority of all renin-expressing vestibular cells under all conditions (Table 2).

We also examined renin expression in B-cells and mast cells. We found no evidence of co-localization of renin with the B-cell marker CD79, indicating the B-cells are not a substantial contributor to the renin cell emigrees in the tender vestibule (Tables 2, 3). Chymase-ir mast cells were essentially devoid of renin-ir in control, nontender (Fig. 7A) and tender areas (Fig. 7B), indicating that mast cells are not significant contributors to renin expression in the vestibule (Tables 1, 2).

Tender vestibular tissue promotes DRG neurite outgrowth through ANGII activation of AT2

To determine whether vestibular tissue provides cues to sensory neurons that promote sprouting in PVD, we cultured specimens from control, nontender, and tender areas of patients for 48 hours in culture medium, and then used this conditioned medium to assess impact of tissue-derived diffusible factors on rat DRG sensory axon growth. Neurons cultured in the presence of control tissue-conditioned medium appeared healthy and elaborated axons (Fig. 8A) similar to that observed previously^{6, 7, 14}. Nontender tissue-conditioned medium supported neuronal survival and axon outgrowth similar to that of control tissue (Fig. 8B). In contrast, axon outgrowth was more profuse in medium conditioned with tender tissue (Fig. 8C, D).

To determine mechanisms of vestibular tissue-induced axon outgrowth, we conducted cultures in the presence of the AT2 antagonist, PD 123319. AT2 blockade is effective in preventing DRG neuronal outgrowth in response to both endogenous and exogenously

derived ANGII in culture¹⁴. Inclusion of the AT2 antagonist in medium from nontender tissue cultures did not affect neurite outgrowth (Fig. 8E). However, PD123319 prevented the increased outgrowth seen in tender tissue-conditioned medium (Fig. 8F), reducing it to levels comparable to nontender tissue and controls (Fig. 8D).

The ability of PD 123319 to block neurite outgrowth provides evidence that activation of the AT2 receptor is necessary for enhanced neurite outgrowth that occurs in the presence of tender vestibular tissue, and suggests that ANGII or a closely related agonistic molecule is released by the tender tissue. To provide evidence as to the nature of this substance, we conducted experiments in which we added an ANGII function blocking antibody to the culture medium. Addition of the ANGII antibody did not alter outgrowth by DRG neuronal cultures in nontender tissue-conditioned medium (Fig. 8G). However, addition of the antibody prevented the increased outgrowth normally induced by conditioning of the medium with tender tissue (Fig. 8H, D).

Discussion

In patients with PVD, substantial differences in tissue composition occur in tender zones relative to nontender or control tissues, as summarized in Fig. 9. We found an overall increase in innervation consistent with prior reports^{8, 10, 32, 57, 60}. Importantly, our findings show that this hyperinnervation is derived in part by axons with a mechanical nociceptor phenotype. Sensory mechanical transduction is generally considered to be mediated by 2 distinct neural subpopulations: low threshold myelinated mechanoreceptors that respond to light touch and typically have specialized nerve endings, and higher-threshold unmyelinated C-fibers giving rise to free nerve endings that detect noxious mechanical stimuli. Using NF-H as a marker, we found no differences in innervation density of myelinated fibers among the different tissues, indicating that low-threshold mechanoreceptor axons apparently do not contribute to hyperinnervation in PVD. Similarly, neither heat-sensitive nociceptors⁵⁷ nor noradrenergic sympathetic fibers (unpublished) were found to occur in elevated numbers in tender zones in PVD. In contrast, fibers containing TRPV4, which is present in mechanonociceptor fibers, did show a significant increase in density in tender tissues. Therefore, tender zone hyperinnervation is due at least in part to proliferation of fibers known to detect mechanical pain, which may contribute to the profound hypersensitivity to touch that occurs in PVD.

Painful vestibular areas were also characterized by striking increases in numbers of inflammatory cells. Concordant with prior findings, numbers of T-cells, B-cells and macrophages were elevated^{12, 33}. Interestingly, while some groups have reported substantial increases in mast cell presence within painful vestibular zones^{10, 13}, our findings did not support a proliferation of this cell type. Nonetheless, it does appear that mast cell activity is affected, as the proportion of mast cells undergoing degranulation was higher in the tender areas as compared to nontender areas.

Inflammatory cells are known to release a variety of neuroactive substances, including ANGII. Our observations indicate that these cells normally display elements of the RAS including the essential precursor protein AGT, and renin which is requisite for the formation

of the ANGII precursor, ANGI. Many peptidases are capable of converting ANGI to ANGII, including chymase⁴⁹ which we show was abundant in resident mast cells; others that are likely to participate in ANGII generation are angiotensin converting enzyme I and cathepsin G⁴⁰, both of which are widely distributed in inflamed tissue^{11, 25}. Hence, under control and nontender conditions, elements of a vestibular RAS are present but in rather modest numbers (Fig. 9).

Tender tissues, in contrast, show a markedly robust RAS. The increase seems to be due largely to proliferation and/or emigration of cells carrying the RAS proteins rather than induction of RAS properties in resident cells. Hence, the percentages of inflammatory cells expressing AGT and renin did not increase in the tender as compared to control and nontender tissues. Thus, unlike other conditions where inflammatory cell RAS components are reported to be upregulated³⁷, the establishment of the local inflammatory RAS in tender vestibular tissue is attributable to the presence of greater numbers of inflammatory cells.

The establishment of the RAS in tender vestibular zones involves a complex orchestration of inflammatory cell dynamics (Fig. 9). Under normal conditions, the predominant inflammatory cell type present is the T-cell, with fewer macrophages and minimal B-cells. However, in the tender zones, while T-cells and macrophages are increased, there is a dramatic increase in numbers of B-cells; while T-cells are the predominant source of AGT in normal tissues, B-cells play a more prominent role under tender conditions. In contrast, renin is presented primarily by macrophages with T-cells playing a lesser role. It is noteworthy that combined percentages for renin expression in T-cells and macrophages exceeds 100%. This is probably attributable to the expression of TCR α/β by small numbers of macrophages⁵. Mast cells are an important source of the ANGI-cleaving protease, chymase, but lacked other RAS components. This indicates that, unlike cardiac mast cells which express renin⁴⁵, vestibular mast cells lack this protease but presumably contribute to the formation of ANGII through the cleavage of ANGI by chymase.

Collectively, these findings indicate that all necessary components of an ANGII-synthesizing RAS are present locally within the vestibule and are elevated in tender zones. While ANGII is well accepted as a classical cardiovascular hormone, it also has multiple effects on other systems including the nervous system. RAS components have long been known to be present in the brain⁵⁰, and ANGII has been shown to promote axon regeneration^{21, 47} and neurite sprouting^{17, 22}. More recent studies have shown that rat DRGs contain an endogenous RAS that serves as an autocrine regulator of axon growth via the AT2 receptor¹⁴. This has been confirmed subsequently in humans and rats, and immunohistochemistry demonstrated ANGII-like immunoreactivity within DRG neurons^{2, 42}. Hence, an active RAS is present in sensory neurons and mediates sensory axon sprouting via the AT2 receptor.

AT2 is a G-protein coupled receptor whose activation by ANGII or other ligands results in accumulation of cAMP² and activation of the p38 and p42/44 MAPK pathway⁵². Blockade of the AT2 receptor prevents the influx of calcium associated with capsaicin-induced depolarization of heat-sensitive DRG neurons². Thus, AT2 activation can result in both sensory axon sprouting and heightened sensitivity to molecules that excite these neurons.

The relevance of the AT₂ receptor to analgesia and pain is underscored by the relief from painful post-herpetic neuralgia noted in clinical trials using the AT₂ antagonist EMA401⁴⁸, which is closely related to PD123319.

Animal model studies indicate that AT₂ blockade may also be useful for inflammatory pain. Inflammation induced by injection of the irritant complete Freund's adjuvant in the hind paw of rats results in hyperinnervation and hypersensitivity¹⁶. This is associated with the emigration of inflammatory cells and the establishment of a local RAS within the hind paw. Chronic administration of the AT₂ blocker PD123319 is adequate to completely block both the mechanical hypersensitivity and the hyperinnervation caused by inflammation, consistent with increased sprouting and excitability due to locally produced ANGII acting on the DRG axonal AT₂ receptors¹⁵.

Taken in the context of these earlier studies, our current findings that tender vestibular tissues from patients severely affected by PVD contain increased numbers of inflammatory cells expressing all essential RAS elements, and produce immunoreactive ANGII sufficient to induce AT₂-mediated DRG neuronal sprouting, provides causal evidence that this system is involved in vestibular hyperinnervation and, most probably, vestibular mechanical hypersensitivity. These findings further lead us to propose that AT₂ blockade may be effective in providing a pharmacological approach to managing PVD, in addition to therapies that may act to reduce the influx of RAS-containing inflammatory cells into the vestibular tissue of susceptible individuals.

Acknowledgements

Supported by NIH R01HD049615 (PGS), with core facility support by U54 HD 090216 and P30 HD002528. The authors have no conflicts of interest to declare.

References

1. Alessandri-Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J Neurosci*. 24:4444–4452, 2004 [PubMed: 15128858]
2. Anand U, Facer P, Yiangou Y, Sinisi M, Fox M, McCarthy T, Bountra C, Korchev YE, Anand P. Angiotensin II type 2 receptor (AT₂ R) localization and antagonist-mediated inhibition of capsaicin responses and neurite outgrowth in human and rat sensory neurons. *Eur J Pain*. 17:1012–1026, 2013 [PubMed: 23255326]
3. Anand U, Yiangou Y, Sinisi M, Fox M, MacQuillan A, Quick T, Korchev YE, Bountra C, McCarthy T, Anand P. Mechanisms underlying clinical efficacy of Angiotensin II type 2 receptor (AT₂R) antagonist EMA401 in neuropathic pain: clinical tissue and in vitro studies. *Molecular pain*. 11:38, 2015 [PubMed: 26111701]
4. Bazin S, Bouchard C, Brisson J, Morin C, Meisels A, Fortier M. Vulvar vestibulitis syndrome: an exploratory case-control study. *Obstet Gynecol*. 83:47–50, 1994 [PubMed: 8272306]
5. Beham AW, Puellmann K, Laird R, Fuchs T, Streich R, Breysach C, Raddatz D, Oniga S, Peccerella T, Findeisen P, Kzhyshkowska J, Gratchev A, Schweyer S, Saunders B, Wessels JT, Mobius W, Keane J, Becker H, Ganser A, Neumaier M, Kaminski WE. A TNF-regulated recombinatorial macrophage immune receptor implicated in granuloma formation in tuberculosis. *PLoS Pathog*. 7:e1002375, 2011 [PubMed: 22114556]
6. Bhattacharjee A, Rumi M, Staecker H, Smith PG. Bone Morphogenetic Protein 4 Mediates Estrogen-Regulated Sensory Axon Plasticity in the Adult Female Reproductive Tract. *J Neurosci*. 16:1704–1712, 2013

7. Blacklock AD, Johnson MS, Krizsan-Agbas D, Smith PG. Estrogen increases sensory nociceptor neuritogenesis in vitro by a direct, nerve growth factor-independent mechanism. *Eur J Neurosci.* 21:2320–2328, 2005 [PubMed: 15932591]
8. Bohm-Starke N, Hilliges M, Falconer C, Rylander E. Increased intraepithelial innervation in women with vulvar vestibulitis syndrome. *Gynecol Obstet Invest.* 46:256, 1998 [PubMed: 9813445]
9. Bohm-Starke N, Rylander E. Surgery for localized, provoked vestibulodynia: a long-term followup study. *J Reprod Med.* 53:83–89, 2008 [PubMed: 18357798]
10. Bornstein J, Goldschmid N, Sabo E. Hyperinnervation and mast cell activation may be used as histopathologic diagnostic criteria for vulvar vestibulitis. *Gynecol Obstet Invest.* 58:171–178, 2004 [PubMed: 15249746]
11. Burster T, Macmillan H, Hou T, Boehm BO, Mellins ED Cathepsin G: roles in antigen presentation and beyond. *Mol Immunol.* 47:658–665, 2010 [PubMed: 19910052]
12. Chadha S, Gianotten WL, Drogendijk AC, Weijmar Schultz WC, Blindeman LA, van der Meijden WI. Histopathologic features of vulvar vestibulitis. *International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists.* 17:7–11, 1998 [PubMed: 9475185]
13. Chaim W, Meriwether C, Gonik B, Qureshi F, Sobel JD. Vulvar vestibulitis subjects undergoing surgical intervention: a descriptive analysis and histopathological correlates. *European journal of obstetrics, gynecology, and reproductive biology.* 68:165–168, 1996
14. Chakrabarty A, Blacklock A, Svojanovsky S, Smith PG. Estrogen elicits dorsal root ganglion axon sprouting via a renin-angiotensin system. *Endocrinology.* 149:3452–3460, 2008 [PubMed: 18388195]
15. Chakrabarty A, Liao Z, Smith PG. Angiotensin II receptor type 2 activation is required for cutaneous sensory hyperinnervation and hypersensitivity in a rat hind paw model of inflammatory pain. *J Pain.* 14:1053–1065, 2013 [PubMed: 23726047]
16. Chakrabarty A, McCarron KE, Smith PG. Hypersensitivity and hyperinnervation of the rat hind paw following carrageenan-induced inflammation. *Neurosci Lett.* 495:67–71, 2011 [PubMed: 21439352]
17. Cote F, Do TH, Laflamme L, Gallo JM, Gallo-Payet N. Activation of the AT(2) receptor of angiotensin II induces neurite outgrowth and cell migration in microexplant cultures of the cerebellum. *J Biol Chem.* 274:31686–31692, 1999 [PubMed: 10531378]
18. Eva LJ, Rolfe KJ, MacLean AB, Reid WM, Fong AC, Crow J, Perrett CW. Is localized, provoked vulvodynia an inflammatory condition? *J Reprod Med.* 52:379–384, 2007 [PubMed: 17583235]
19. Farmer MA, Taylor AM, Bailey AL, Tuttle AH, Macintyre LC, Milagrosa ZE, Crissman HP, Bennett GJ, Ribeiro-da-Silva A, Binik YM, Mogil JS. Repeated vulvovaginal fungal infections cause persistent pain in a mouse model of vulvodynia. *Sci Transl Med.* 3:101–191, 2011
20. Friedrich EG, Jr. Vulvar vestibulitis syndrome. *J Reprod Med.* 32:110–114, 1987 [PubMed: 3560069]
21. Gallinat S, Yu M, Dorst A, Unger T, Herdegen T. Sciatic nerve transection evokes lasting upregulation of angiotensin AT2 and AT1 receptor mRNA in adult rat dorsal root ganglia and sciatic nerves. *Brain research. Molecular brain research.* 57:111–122, 1998 [PubMed: 9630555]
22. Gendron L, Cote F, Payet MD, Gallo-Payet N. Nitric oxide and cyclic GMP are involved in angiotensin II AT(2) receptor effects on neurite outgrowth in NG108–15 cells. *Neuroendocrinology.* 75:70–81, 2002 [PubMed: 11810036]
23. Goetsch MF. Surgery combined with muscle therapy for dyspareunia from vulvar vestibulitis: an observational study. *J Reprod Med.* 52:597–603, 2007 [PubMed: 17847757]
24. Goetsch MF, Morgan TK, Korcheva VB, Li H, Peters D, Leclair CM. Histologic and receptor analysis of primary and secondary vestibulodynia and controls: a prospective study. *Am J Obstet Gynecol.* 202:614 e611–618, 2010 [PubMed: 20430353]
25. Gonzalez-Villalobos RA, Shen XZ, Bernstein EA, Janjulia T, Taylor B, Giani JF, Blackwell WL, Shah KH, Shi PD, Fuchs S, Bernstein KE. Rediscovering ACE: novel insights into the many roles of the angiotensin-converting enzyme. *Journal of molecular medicine.* 91:1143–1154, 2013 [PubMed: 23686164]

26. Harlow BL, Stewart EG. A population-based assessment of chronic unexplained vulvar pain: have we underestimated the prevalence of vulvodynia? *J Am Med Womens Assoc.* 58:82–88, 2003
27. Herren B, Burrows PD. B cell-restricted human mb-1 gene: expression, function, and lineage infidelity. *Immunol Res.* 26:35–43, 2002 [PubMed: 12403343]
28. Hoch NE, Guzik TJ, Chen W, Deans T, Maalouf SA, Gratze P, Weyand C, Harrison DG. Regulation of T-cell function by endogenously produced angiotensin II. *Am J Physiol Regul Integr Comp Physiol.* 296:R208–216, 2009 [PubMed: 19073907]
29. Hsieh ST, Kidd GJ, Crawford TO, Xu Z, Lin WM, Trapp BD, Cleveland DW, Griffin JW. Regional modulation of neurofilament organization by myelination in normal axons. *J Neurosci.* 14:6392–6401, 1994 [PubMed: 7965044]
30. Hunig T, Wallny HJ, Hartley JK, Lawetzky A, Tiefenthaler G. A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. *The Journal of experimental medicine.* 169:73–86, 1989 [PubMed: 2783336]
31. Jurewicz M, McDermott DH, Sechler JM, Tinckam K, Takakura A, Carpenter CB, Milford E, Abdi R. Human T and natural killer cells possess a functional renin-angiotensin system: further mechanisms of angiotensin II-induced inflammation. *J Am Soc Nephrol.* 18:1093–1102, 2007 [PubMed: 17329576]
32. Leclair CM, Goetsch MF, Korcheva VB, Anderson R, Peters D, Morgan TK. Differences in primary compared with secondary vestibulodynia by immunohistochemistry. *Obstet Gynecol.* 117:1307–1313, 2011 [PubMed: 21606740]
33. Leclair CM, Leeborg NJ, Jacobson-Dunlop E, Goetsch MF, Morgan TK. CD4-positive T-cell recruitment in primary-provoked localized vulvodynia: potential insights into disease triggers. *Journal of lower genital tract disease.* 18:195–201, 2014 [PubMed: 24633162]
34. Liao Z, Smith PG. Persistent genital hyperinnervation following progesterone administration to adolescent female rats. *Biol Reprod.* 91:144, 2014 [PubMed: 25359899]
35. Liedtke W, Choe Y, Marti-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, Heller S. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell.* 103:525–535, 2000 [PubMed: 11081638]
36. Liedtke W, Friedman JM. Abnormal osmotic regulation in *trpv4*–/– mice. *Proc Natl Acad Sci U S A.* 100:13698–13703, 2003 [PubMed: 14581612]
37. Ma H, Ye B, Wei Q, Zhu XD. Genome wide gene expression analysis of macrophages from ankylosing spondylitis patients under interferon-gamma treatment. *European review for medical and pharmacological sciences.* 17:2798–2803, 2013 [PubMed: 24174363]
38. Mason DY, Cordell JL, Tse AG, van Dongen JJ, van Noesel CJ, Micklem K, Pulford KA, Valensi F, Comans-Bitter WM, Borst J, Gatter KC. The IgM-associated protein mb-1 as a marker of normal and neoplastic B cells. *J Immunol.* 147:2474–2482, 1991 [PubMed: 1747162]
39. Micklem K, Rigney E, Cordell J, Simmons D, Stross P, Turley H, Seed B, Mason D. A human macrophage-associated antigen (CD68) detected by six different monoclonal antibodies. *Br J Haematol.* 73:6–11, 1989 [PubMed: 2803980]
40. Owen CA, Campbell EJ. Angiotensin II generation at the cell surface of activated neutrophils: novel cathepsin G-mediated catalytic activity that is resistant to inhibition. *J Immunol.* 160:1436–1443, 1998 [PubMed: 9570564]
41. Papoutsis D, Haefner HK, Crum CP, Opiari AW, Jr, Reed BD. Vestibular Mast Cell Density in Vulvodynia: A Case-Controlled Study. *Journal of lower genital tract disease.* 20:275–279, 2016 [PubMed: 27224531]
42. Patil J, Schwab A, Nussberger J, Schaffner T, Saavedra JM, Imboden H. Intraneuronal angiotensinergic system in rat and human dorsal root ganglia. *Regul Pept.* 162:90–98, 2010 [PubMed: 20346377]
43. Pukall CF, Binik YM, Khalife S, Amsel R, Abbott FV. Vestibular tactile and pain thresholds in women with vulvar vestibulitis syndrome. *Pain.* 96:163–175, 2002 [PubMed: 11932072]
44. Reed BD, Haefner HK, Punch MR, Roth RS, Gorenflo DW, Gillespie BW. Psychosocial and sexual functioning in women with vulvodynia and chronic pelvic pain. A comparative evaluation. *J Reprod Med.* 45:624–632, 2000 [PubMed: 10986680]

45. Reid AC, Silver RB, Levi R. Renin: at the heart of the mast cell. *Immunol Rev.* 217:123–140, 2007 [PubMed: 17498056]
46. Reilly CF, Tewksbury DA, Schechter NM, Travis J. Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. *J Biol Chem.* 257:8619–8622, 1982 [PubMed: 6807977]
47. Reinecke K, Lucius R, Reinecke A, Rickert U, Herdegen T, Unger T. Angiotensin II accelerates functional recovery in the rat sciatic nerve in vivo: role of the AT2 receptor and the transcription factor NF-kappaB. *Faseb J.* 17:2094–2096, 2003 [PubMed: 14500552]
48. Rice AS, Dworkin RH, McCarthy TD, Anand P, Bountra C, McCloud PI, Hill J, Cutter G, Kitson G, Desem N, Raff M, for the EMAsg. EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: a randomised, double-blind, placebo-controlled phase 2 clinical trial. *Lancet.* 2014
49. Roszkowska-Chojacka MM, Walkowska A, Gawrys O, Baranowska I, Kalisz M, Litwiniuk A, Martynska L, Kompanowska-Jezierska E. Effects of chymostatin, a chymase inhibitor, on blood pressure, plasma and tissue angiotensin II, renal haemodynamics and renal excretion in two models of hypertension in the rat. *Experimental physiology.* 100:1093–1105, 2015 [PubMed: 26011164]
50. Saavedra JM. Brain angiotensin II: new developments, unanswered questions and therapeutic opportunities. *Cellular and molecular neurobiology.* 25:485–512, 2005 [PubMed: 16075377]
51. Sahashi K, Ibi T, Zhang G. Immunohistochemical localization of chymase; a mast cell marker and clinical significance in diseased human skeletal muscle. *Rinsho Shinkeigaku.* 37:615–620, 1997 [PubMed: 9396359]
52. Smith MT, Wyse BD, Edwards SR. Small Molecule Angiotensin II Type 2 Receptor (AT R) Antagonists as Novel Analgesics for Neuropathic Pain: Comparative Pharmacokinetics, Radioligand Binding, and Efficacy in Rats. *Pain Med.* 2013
53. Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nature cell biology.* 2:695–702, 2000 [PubMed: 11025659]
54. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. *The Journal of biological chemistry.* 278:22664–22668, 2003 [PubMed: 12692122]
55. Tague SE, Clarke GL, Winter MK, McCarson KE, Wright DE, Smith PG. Vitamin D deficiency promotes skeletal muscle hypersensitivity and sensory hyperinnervation. *J Neurosci.* 31:13728–13738, 2011 [PubMed: 21957236]
56. Tympanidis P, Casula MA, Yiangou Y, Terenghi G, Dowd P, Anand P. Increased vanilloid receptor VR1 innervation in vulvodynia. *Eur J Pain.* 8:129–133, 2004 [PubMed: 14987622]
57. Tympanidis P, Terenghi G, Dowd P. Increased innervation of the vulval vestibule in patients with vulvodynia. *Br J Dermatol.* 148:1021–1027, 2003 [PubMed: 12786836]
58. Weinstock JV, Blum AM. Granuloma macrophages in murine schistosomiasis mansoni generate components of the angiotensin system. *Cell Immunol.* 89:39–45, 1984 [PubMed: 6386191]
59. Wesselmann U, Bonham A, Foster D. Vulvodynia: Current state of the biological science. *Pain.* 2014
60. Westrom LV, Willen R. Vestibular nerve fiber proliferation in vulvar vestibulitis syndrome. *Obstet Gynecol.* 91:572, 1998 [PubMed: 9540943]
61. Witkin SS, Gerber S, Ledger WJ. Differential characterization of women with vulvar vestibulitis syndrome. *Am J Obstet Gynecol.* 187:589–594, 2002 [PubMed: 12237632]
62. Xie Y, Shi L, Xiong X, Wu E, Veasley C, Dade C. Economic burden and quality of life of vulvodynia in the United States. *Curr Med Res Opin.* 28:601–608, 2012 [PubMed: 22356119]

Highlights

- Provoked vulvodynia is characterized by excessive mechano-nociceptor axon numbers.
- Tender tissues have abundant inflammatory cells forming a renin-angiotensin system.
- Locally produced angiotensin II acts on AT2 receptors to induce axon sprouting.

Figure 1.

Innervation of the human vestibule. Tissue sections were immunostained for the pan-neuronal marker PGP9.5 (A, B, C), the mechanosensory marker TRPV4 (E, F, G) and the myelinated fiber marker neurofilament H (NF-H). Sections were obtained from vestibular tissue punch biopsies of non-symptomatic controls (A, E, I), punch biopsies of nontender vestibular regions in patients with provoked vestibulodynia (B, F, J), and from an excised tender region of vestibulodynia patients (C, G, K). In PGP9.5-immunostained tissues, tender region (N=10) nerve density (D) was greater than that of controls (^a $P=0.021$; N=7) and nontender tissues (^b $P=0.027$; N=10). TRPV4 axon density (H) in the tender area (N=5) was greater than control (^c $P=0.011$; N=5) or nontender regions (^d $P=0.002$; N=5). Density of axons labeled for NF-H (L) did not differ among control (N=5), nontender (N=5) and tender regions (N=5). Scale bar in K = 100 μ m for all micrographs.

Figure 2.

Inflammatory cells in human vestibule. Cells were immunostained with markers for Tcells (TCR α/β , A-C), macrophages (CD68, E-G), B-cells (CD79, I-K) and mast cells (chymase, M-O) in tissue sections (epidermis at top of panels) from control (A, E, I, M), nontender (B, F, J, N) and tender (C, G, K, O) regions. D: Numbers of T-cells were elevated in tender regions (n=10) relative to nontender (^a $P < 0.001$, n=10) and control (^b $P = 0.007$, n=7) regions. H: CD68positive macrophages were elevated in tender (n=5) relative to nontender (^c $P = 0.020$, n=5) and control (^d $P = 0.048$, n=5) regions. L: Numbers of CD79-positive B-cells were elevated in tender regions (n=6) relative to nontender (^e $P = 0.008$, n=5) and control (^f $P = 0.004$, n=5) regions. P: Numbers of chymase-positive degranulated mast cells were elevated in tender regions (n=6) relative to nontender (^g $P = 0.046$, n=6) regions but not controls (n=6). Scale bar for A-K= 100 μ m, for M-O= 50 μ m.

Figure 3.

Vestibular T-cells express Angiotensinogen (AGT). AGT-immunoreactive (AGT-ir) cells were present in dermis of control (A), nontender (B) and tender (C) vestibular tissue. Numbers of AGT-positive cells (D) were increased in tender regions (N=10) relative to control (^a $P=0.011$, N=6) and nontender regions (^b $P=0.002$, N=10). T-cells identified by immunostaining for TCR α/β were also present in dermis of control (E), nontender (F) and tender regions (G). Co-staining showed colocalization of AGT and TCR α/β in subsets of cells in control (H), nontender (I) and tender (J) tissues, and numbers of AGT-expressing T-cells (K) were increased in tender relative to nontender (^c $P=0.002$) but not controls. Scale bar in J=100 μ m for all micrographs.

Figure 4.

Vestibular B-cells express angiotensinogen (AGT). Panels show AGT-ir cells in control vestibule (A), nontender vestibule (B) and tender vestibule (C), with numbers (D) in the tender region (N=5) significantly greater than control (^a $P=0.004$, N=6) and nontender regions (^b $P=0.008$, N=5). B-cells displaying CD79 α were present in control vestibule (E), nontender vestibule (F) and tender vestibule (G). Costaining showed B-cells expressing AGT in control (H), nontender (I) and tender (J) regions, and number of AGT-ir B-cells (K) was increased in tender regions relative to control (^c $P=0.004$) and nontender tissue (^d $P=0.008$). Scale bar=100 μ m.

Figure 5.

Vestibular macrophages express renin. Renin-ir is present in cells in control (A), nontender (B) and tender regions of the vestibule (C). Numbers of renin-ir cells (D) increased in the tender regions (N=5) as compared to control (^a $P < 0.001$, N=5) and nontender regions (^b $P < 0.001$, N=5). Cells displaying the CD68-ir macrophage marker are present in control (E), nontender (F) and tender regions of the vestibule (G). Cells colocalizing both markers were present in control (H), nontender (I) and tender vestibule (J), and numbers of co-labeled cells (K) were increased in tender regions relative to controls (^c $P = 0.012$) and nontender regions (^d $P = 0.005$). Scale bar=100 μ m.

Figure 6.

Vestibular T-cells express renin. Renin-ir cells are present in control (A), nontender (B) and tender regions of the vestibule (C), and cell numbers (D) were increased in tender regions (N=5) compared to control (^a, $P=0.002$, N=5) and nontender regions (^b, $P<0.032$, N=5). Cells displaying the T-cell marker, TCR α/β are present in control (E), nontender (F) and tender (G) regions of the vestibule. Cells expressing both markers were present in control (H), nontender (I) and tender vestibule (J), and numbers of co-labeled cells (K) were increased in tender regions relative to controls (^c, $P=0.002$). Scale bar=100 μ m.

Figure 7.

Vestibular mast cells express chymase but rarely express renin. Mast cells are identified by the presence of immunoreactive chymase, which can convert angiotensin I to angiotensin II. Mast cells were present in nontender vestibule (A) where little colocalization with chymase (arrow) with renin was apparent. Chymase-ir mast cells were also present in tender regions (B), but similarly showed minimal expression of renin-ir. Scale bar=100µm.

Figure 8.

Conditioned medium from human vestibular tissue promotes AGNII/AT2-enhanced neurite outgrowth from dissociated rate dorsal root ganglion (DRG) neurons. Media conditioned with control tissue (A, CON) gives rise to healthy neurite outgrowth by dissociated neonatal small-diameter neurons detected by peripherin-ir. Nontender tissue (B, NT) conditioning promotes similar outgrowth. Outgrowth is more profuse when medium has been conditioned with tender tissue (C, T), and is significantly greater than control or nontender conditioned medium (D, $P < 0.001$). Addition of the AT2 antagonist PD123,319 (PD) to the medium did not affect outgrowth of nontender-conditioned cultures (E), but reduced outgrowth induced by tender conditioning (F, $P < 0.001$) to levels comparable to control and nontender tissue (D). Addition of an antibody to ANGII (Ab) to the medium also did not affect outgrowth after nontender conditioning (G) but completely reversed outgrowth induced by tender tissue conditioning (H, D, $P < 0.001$). $N = 4$ for control cultures and 5 for all other cultures. Scale bar = 100 μm.

Figure 9.

Schematic diagram summarizing changes in innervation and RAS inflammatory cells in PVD. In asymptomatic control patient tissues, and in nontender areas of patients with PVD, numbers of axons are low. Inflammatory cells are present, and low numbers of T-cells, B-cells and macrophages express RAS proteins. In tender regions of PVD patients, mechanonociceptive hyperinnervation is evident in conjunction with proliferation of RAS-expressing inflammatory cells. These include massive increases in T-cells, a small proportion expressing AGT and/or renin, a massive proliferation of AGT-expressing B-cells, and a dramatic increase in renin-expressing macrophages. Mast cells did not display RAS markers and did not change in numbers.

Table 1.

Antibodies used for immunofluorescence staining.

	Name (host species)	Concentration (temperature/ duration)	Source
Primary antibodies	PGP9.5 (Rb)	1:4000 (RT/ON)	AbD Serotec (Kidlington, UK)
	PGP9.5 (Ms)	1:200 (4°C/ON)	Abcam (Cambridge, UK)
	TRPV4 (Rb)	1:300 (4°C/ON)	Abcam (Cambridge, UK)
	NF-H (Rb)	1:3000 (RT/ON)	Sigma (St. Louis, USA)
	TCR α/β (Ms)	1:100 (RT/ON)	AbD Serotec (Kidlington, UK)
	CD79 α (Ms)	1:75 (RT/ON)	DAKO (Glostrup, Denmark)
	CD68 (Ms)	1:50 (RT/ON)	Millipore (Temecula, CA, USA)
	Chymase (Ms)	1:300 (RT/ON)	Millipore (Temecula, CA, USA)
	AGT(Rb)	1:1000 (RT/ON)	Millipore (Temecula, CA, USA)
	Renin (Rb)	1:4000 (RT/ON)	Gift from Dr. T. Inagami, Vanderbilt University, Nashville, TN
	Peripherin (Ck)	1:600 (4°C/ON)	Millipore (Temecula, CA, USA)
Secondary antibodies	Cy3 anti-Rb (Gt)	1:400 (RT/1h)	Jackson ImmunoResearch (West Grove, PA, USA)
	Cy3 anti-Ms (Gt)	1:400 (RT/1h)	Jackson ImmunoResearch (West Grove, PA, USA)
	Alexa 488 anti-Ms (Gt)	1:800 (RT/1h)	ThermoFisher (Waltham, MA USA)
	Alexa 488 anti Ck (Donkey)	1:1000 (RT/1h)	ThermoFisher (Waltham, MA USA)

Rb = rabbit, Ms = mouse, Gt = goat, Ck=chicken, ON = overnight, RT = room temperature.

Table 2.

Inflammatory cells constituting the vestibular RAS (percentage by cell type). Cells immunostained for angiotensinogen or renin were counted, and percentages expressing immunoreactivity for different inflammatory cell markers (in parentheses) determined in co-stained samples.

Angiotensinogen	Control	Nontender	Tender	P value
T-cells (TCR)	66±7	54±7	43±3*	*0.038 vs control
Macrophages (CD68)	0	0	0	-
B-cells (CD79)	44±4	33±4	54±3*	*0.006 vs nontender
Mast cells (chymase)	0	0	0	-
Renin				
T-cells (TCR)	37±2	41±9	40±5	ns
Macrophages (CD68)	80±3	73±9	74±5	ns
B-cells (CD79)	0	0	0	-
Mast cells (chymase)	0	0	0	-

Table 3.

Percentages of inflammatory cells displaying RAS proteins. Numbers of cells expressing different inflammatory cell markers (in parentheses) were counted, and the percentage of cells expressing either angiotensinogen or renin immunoreactivity were counted in co-stained specimens.

Angiotensinogen	Control	Nontender	Tender	P value
T-cells (TCR)	13±3	8±2	11±2	ns
Macrophages (CD68)	0	0	0	-
B-cells (CD79)	77±6	69±14	56±3	ns
Mast cells (chymase)	0	0	0	-
Renin				
T-cells (TCR)	9±2	20±5	11±2	ns
Macrophages (CD68)	80±3	82±5	84±4	ns
B-cells (CD79)	0	0	0	-
Mast cells (chymase)	0	0	0	-