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Cutaneous innervation in impaired diabetic wound healing

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Abstract

Type 2 diabetes is associated with several potential comorbidities, among them impaired wound healing, chronic ulcerations, and the requirement for lower extremity amputation. Disease-associated abnormal cellular responses, infection, immunological and microvascular dysfunction, and peripheral neuropathy are implicated in the pathogenesis of the wound healing impairment and the diabetic foot ulcer. The skin houses a dense network of sensory nerve afferents and nerve-derived modulators, which communicate with epidermal keratinocytes and dermal fibroblasts bidirectionally to effect normal wound healing after trauma. However, the mechanisms through which cutaneous innervation modulates wound healing are poorly understood, especially in humans. Better understanding of these mechanisms may provide the basis for targeted treatments for chronic diabetic wounds. This review provides an overview of wound healing pathophysiology with a focus on neural involvement in normal and diabetic wound healing, as well as future therapeutic perspectives to address the unmet needs of diabetic patients with chronic wounds.

INTRODUCTION

In the United States, more than 30 million individuals above the age of 18 years have type 2 diabetes (T2D)[1] and more than 34% of the US adult population are prediabetic (an estimated 88 million adults).[2] From 2012 to 2017, the costs of diabetes increased by 26% to $327 billion USD,[1] and the global economic burden is projected to exceed $627 billion USD by 2035.[3] Smoking,[4] a sedentary lifestyle,[5] and obesity[6] are recognized risk factors for developing diabetes-related complications, including life-threatening heart attacks and strokes.

Chronic, nonhealing wounds, particularly ulcerations of the foot, are the leading cause of nontraumatic lower extremity amputations in the United States at a frequency of approximately 200,000 annually.[7,8] Disease-associated abnormal cellular responses, infection, immunological and microvascular dysfunction, and peripheral neuropathy are implicated in the pathogenesis of the wound healing impairment and diabetic foot ulcer.[9,10] Current treatment options are limited, particularly pathogenesis-based therapeutic approaches for preventing or healing diabetic ulcers.

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MECHANISM OF NORMAL WOUND HEALING

The skin acts as a protective barrier, preventing desiccation and representing the body's key site of protection against the environment. Receptors on keratinocytes and epidermal sensory nerve afferents detect extrinsic stimuli and respond to prevent and react to an injury. When a wound occurs in a healthy individual, communication among keratinocytes, nerves, and other cells leads to a series of distinct, yet overlapping phases as part of the "cascade of healing" (Fig 1). Hemostasis, an immediate response, involves the constriction of injured blood vessels and activation of platelets to form a fibrin clot,[11] which serves as a scaffold for incoming inflammatory cells.[12] Proinflammatory cytokines, among them interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α,[13] and interferon-gamma (IFN-γ),[14] recruit neutrophils into the wound bed, followed by monocytes, which become tissue-activated macrophages 48–96 hours postinjury.[15] These cytokines and growth factors stimulate epithelial cells, endothelial cells, and fibroblasts to proliferate and migrate into the wound area. Fibroblasts fill the injured area and differentiate into myofibroblasts, first synthesizing collagen III, but also collagen I, proteoglycans, and matrix metalloproteinases (MMPs),[16] to form and remodel the extracellular matrix. Angiogenesis occurs in this developing granulation tissue matrix due to the presence of low oxygen,[17] low pH, and high lactate levels.[18] Keratinocytes begin the re-epithelization process through successful migration across the matrix, with proliferation and then differentiation into a functional epidermis. Migration ends when keratinocytes from opposing edges meet. A thin epithelial layer is established as keratinocytes form new adhesions to the underlying matrix.[19] Through keratinocyte proliferation and differentiation, the multilayer epidermis is ultimately formed. Growth factors, such as epidermal growth factor (EGF),[20] keratinocyte growth factor (KGF),[21] insulin-like growth factor (IGF)-1,[22] and transforming growth factor (TGF)-α,[23] regulate keratinocyte activity.[24] Although re-epithelization is a clinical indicator of wound healing, granulation tissue reinforcement completes the reparative process. The remodeling phase of wound healing, also known as the maturation phase, involves strengthening the scarred area by crosslinking and improving collagen fiber alignment, with apoptosis of cells from the healing process that are no longer needed.

WOUND HEALING ABNORMALITIES IN DIABETES: TISSUE AND CELLULAR FACTORS

Diabetes affects inflammation,[25] matrix deposition,[26] and angiogenesis[27] of the wound healing cascade as a result of numerous factors (Fig 1; Table I). Many studies of diabetic wounds have investigated the impaired blood flow leading to poor oxygenation and nutrient delivery, chronic exposure to hyperglycemia, immune cell dysregulation, and propensity for bacterial colonization and infection. Importantly, chronic induction of proinflammatory cytokines, such as TNF-α[28] and IL-1β,[29,30] stalls the inflammatory phase and disrupts wound healing. Defects have been described in neutrophil function, leukocyte chemotaxis, macrophage phagocytosis, and bactericidal capacity in diabetic wounds, leading to inadequate bacterial clearance.

Normal healing is characterized by a transition in the ratio of macrophage phenotypes M1 and M2, reflecting the shift from inflammatory to proliferative functions. Classically activated M1 macrophages secrete high amounts of proinflammatory cytokines (eg, IL-1, IL-6, IL-12, TNF-α)[31] and oxidative metabolites (eg, nitric oxide and superoxide) to facilitate pathogen killing activity and wound debridement early after wounding. Varied stimuli, including IL-4 and IL-13 signaling,[32] evoke M2 macrophage activation, and aid in the resolution of inflammation. The phenotypic switch to an M2 macrophage is marked by upregulation of classical M2 cytokines and growth factors, such as IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β1, and platelet-derived growth factor (PDGF), to encourage granulation tissue formation, angiogenesis, and cellular proliferation.[32,33]

In diabetes, the ratio of M1 (proinflammatory) to M2 (anti-inflammatory) macrophages is increased, impeding the proliferative phase in wound healing.[34] Diabetes also increases neutrophil extracellular traps (NETs), aggregates of de-condensed chromatin formed by neutrophils to neutralize organisms, which suggests another deleterious effect of the enhanced inflammatory response.[35,36] Hyperglycemia accelerates the formation of advanced glycation end product (AGE),[37] which are thought to contribute to impaired healing by increasing oxidative stress,[38] changing the expression and function of proteins that are critical to wound repair,[39,40] enhancing the inflammatory response by activation of transcription factors,[41,42] and potentially leading to exaggerated cellular apoptosis.[43]

In addition to increased levels of glycosylated proteins, a measure of poor diabetic control,[44,45] glycosphingolipids, and particularly ganglioside GM3, are increased in diabetic tissues, including skin,[46–48] and have been implicated in impaired healing. Increases in GM3 suppress growth factor-induced responses, including insulin, IGF-1,[49] and EGF receptor signaling, leading to delayed skin cell migration and inhibition of cell proliferation. Suppression of expression of GM3 synthase (GM3S), the enzyme required for synthesis of GM3, using genetic (knockout or topically applied siRNA nanoconstructs) or biochemical (glucosylceramide synthesis small molecule inhibition) approaches accelerates healing in wounded mouse models of diet-induced type 2 diabetes.[46,48] Inhibition of GM3S in cultured keratinocytes reverses the glucose- and TNF-α-induced slowing of cell migration through increasing insulin- and IGF-1-induced IGF-1 receptor phosphorylation and activating Rac1.[46–48] These data suggest GM3 depletion as a pathogenesis-based direction in therapy.

Diabetic cells retain “metabolic memory,” including at the level of histone modification,[50,51] genome-wide DNA methylation,[51] and microRNA expression patterns.[52,53] Hyperglycemia leads to an altered miRNA signature in wound healing, which may be linked to the dysregulated inflammation in diabetes[54] and has been positively correlated to the severity of diabetic foot ulcers.[55] Dysregulated expression of miRNAs influences the cellular transcriptome and may impair wound healing in diabetes. For example, miR-27–3p overexpression in diabetic foot ulcer-derived fibroblasts (DFUFs) and diabetic mice,[56] as well as downregulation of miR-129 and −355 in human diabetic wounds,[57] hinders healing by impairing fibroblast function and inhibiting MMP-9 expression, respectively. In a comparative analysis of miRNA expression profiles in human DFUFs vs normal foot-

derived fibroblasts (NFF), aberrant expression of miR–21–5p, miR–34a–5p, and miR–145–5p was linked to DFUF dysfunction in proliferation, migration, and differentiation.[58]

CONTRIBUTIONS OF PERIPHERAL NERVE DYSFUNCTION TO POOR DIABETIC WOUND HEALING

Both sensory and autonomic nerves populate skin.[59] Autonomic nerves are restricted to the dermis and regulate lymphatic function, blood circulation, and appendageal function.[60] In contrast, cutaneous sensory nerves predominate and are widely distributed in skin, including extending into the upper epidermis to interface with the environment. The trigeminal ganglia comprise many of the sensory neurons innervating the head. Conversely, the dorsal root ganglia largely innervate the rest of the body. Neuronal afferents that traverse the dermis to the epidermis originate in dorsal root ganglia in the spinal cord and only their dendritic extensions populate skin, with sensations transmitted from the peripheral nerve terminals to the body in the spinal cord. Distal foot skin represents the longest extension of a dorsal root ganglion in the body.

Early sensory nerve classification was strictly based on the size, speed of impulse conduction, and function (including neuropeptide secretion and type of sensation recognized). The thinly myelinated Aδ low-threshold mechanoreceptors are nerve fibers that carry thermal, mechanoreceptive (pressure), and acute nociceptive (pain) signals. The small, unmyelinated C fibers (~70%) transmit information related to pain, temperature, and itch,[61] sending slower and more sustained impulses than Aδ fibers.[62,63] C fibers are classically divided into 2 subsets, peptidergic (PEP) and nonpeptidergic (NP). Peptidergic C fibers produce neuropeptides such as substance P (SP) and calcitonin gene–related peptide (CGRP), and express the tropomyosin receptor kinase A (TrkA). In contrast, nonpeptidergic C fibers bind to isolectin B4 (IB4) and express the ATP-binding purinergic receptor P2 × 3. However, markers for the PEP and NP C fibers are not absolute, for example, 1 subset of NP C fibers expresses *Calca,* the gene that encodes for neuropeptide CGRP (Table II). Indeed, use of single cell transcriptomics has demonstrated a previously unappreciated level of heterogeneity among DRG neurons, indicating that their functions may be very precisely tuned according to phenotype. Moreover, this type of analysis has highlighted important differences in the properties of DRG neurons based on sex and species, illustrating the translational challenges that face novel therapies for pain and itch based on regulation of sensory neuron function. Aδ and C fibers are considered polymodal due to their ability to sense various different stimuli.[65] TH-expressing C fiber low-threshold mechanoreceptors (C-LTMRs), which are unmyelinated and express tyrosine hydroxylase and dopamine/L-DOPA, are also likely involved in pain sensation but not well understood.[66]

Diabetic neuropathy occurs in almost 90% of diabetic foot ulcers.[67] Neuropathies selectively target C and Aδ fibers, with degeneration linked to impaired healing in both type 1 and type 2 diabetes.[68] Sensory neuropathy can lead to neuropathic pain and/or loss of sensation, increasing the risk of injury and foot ulceration by 8- to 18-fold and lower extremity amputation by 2- to 15-fold.[69] Length-dependent “dying back” of axons, primarily involving the distal portions of the longest myelinated and unmyelinated sensory axons, results in

nerve dysfunction.[70] Given the predominant degeneration of the small C and Aδ fibers, a common initial presentation is symmetric loss of temperature, light touch sensation, or painful prickling sensations in a “stocking and glove” distribution.

Diagnosis of small fiber neuropathy is based on the presence of specific sensory deficits, discovered through examination and validated by structural and functional assessments. Structural assessment relies on skin biopsy[71–75] for nerve fiber assessment that combines quantification of intraepidermal nerve fiber density (IENFD) and dermal nerve bundles. Functional assessment involves quantitative sensory testing, pain-related and laser induced potential recording, and single axon recording using microneurography.[76] Noninvasive measures, such as quantifying axon-reflex mediated vasodilation (LDIflare technique)[77–79] and measuring nerve conduction velocity,[80,81] have also been investigated to evaluate abnormal sensory nerves. These measures have shown the reduction in cutaneous innervation in biopsies of diabetic human subjects based on reduced immunoreactivity to PGP9.5 (detecting sensory neurons) and a variety of neuropeptides (particularly calcitonin gene-related peptide (CGRP), substance P (SP), and neuropeptide Y) (Table III).[71,72] Characteristic findings in human diabetic skin include fewer and more fragmented nerves throughout the dermis[82,83] and reduction in nerve afferents in the epidermis[84–88] and papillary dermis,[89] even in the absence of clinically-detectable sensory neuropathy.[90] Diabetic subjects can show markedly reduced amplitudes and neural conduction velocities associated with nerve fiber loss.[91] Similar changes in the anatomy of cutaneous sensory afferents have also been observed in rodent models of diabetes, in which different subpopulations of DRG neurons, such as the sodium channels NaV1.8 or the G-protein coupled receptor MrgD (member D of the Mas-related G-protein coupled receptors or Mrgprs), can be precisely identified by making use of the genetic expression of fluorescent markers.

Reduction in VEGFR-expressing dermal blood vessels[82,92] and presence of a low-grade inflammatory cell infiltration[93] have been associated with the innervation abnormalities in diabetes.[90] Despite these known abnormalities, the role of cutaneous innervation and specific neuronal subsets in normal healing is more poorly understood than the role of other tissue types, such as the vasculature, keratinocytes, fibroblasts, and immune cells.[94–106]

POOR WOUND HEALING AND REDUCED INNERVATION: RESULTS FROM EXPERIMENTAL DENERVATION

Skin nociceptive effectors modulate gene expression of extracellular matrix (ECM), transcription factors, cytoskeleton, proteases, receptors, intracellular transducers, and adhesion molecules,[107] suggesting a role in wound healing. Studies in the chick embryo suggest a positive reciprocal association between nerves and wound repair.[108] Chemical and surgical denervation studies support a role for cutaneous innervation in wound healing, with reduction in small nerve fibers by at least 70% leading to features of poor wound repair.[109] Reduction of sensory nerves by subcutaneous capsaicin treatment in nondiabetic mice and rats delayed re-epithelialization, reduced epidermal stem cell migration, and suppressed angiogenesis and VEGF expression.[110–112] In both nondiabetic and diabetic

mouse models, chemical ablation of sympathetic nerves using intraperitoneal injection of 6-hydroxydopamine (6-OHDA) delayed wound re-epithelization and reduced inflammation,[113] but in leptin-deficient diabetic mice also increased wound contraction.[114]

Surgical denervation in nondiabetic mice, rats, and rabbits led to markedly delayed closure of wounds on the ear pinnae and dorsum and have shown delayed wound contraction,[115] altered keratinocyte proliferation,[116] delayed re-epithelialization,[117] and reduced granulation tissue.[118] Transplantation into denervated wounds of skin-derived precursors (SKPs),[119] a population of neural crest-related stem cells within the dermis that participate in cutaneous nerve regeneration,[120] leads to wound cell proliferation, increased nerve fiber density, and higher neuropeptide levels (nerve growth factor [NGF], SP, and CGRP) in mice.

Experimental intracutaneous excision axotomy in human subjects with diabetes and neuropathy leads to slower healing than in healthy controls.[121] Punch biopsies of the distal thigh skin were performed, followed by concentric overlapping biopsies at various time points. Compared to healthy controls, the diabetic wounds had reduced re-epithelialization and granulation tissue, poor vascularization, and diminished dermal innervation and Schwann cells in the axotomy site.[121] Blood vessel growth into the excision site preceded dermal nerve fiber regeneration in both diabetics and nondiabetics, suggesting that blood vessels act as a framework for later axon and Schwann cell growth.[121] Diabetic subjects with epidermal denervation through capsaicin treatment also showed delayed rates of reinnervation when compared to healthy controls.[121]

Prevention of nerve degeneration improves healing.

Neuropathy has been noted in many of the rodent models of diabetes and poor wound healing (Table IV). The degree of obesity and severity of diabetes vary in these models.[89,109,122] In general, obese mice with more severe diabetes show a more severe wound healing impairment. Regardless of the extent of diabetes and obesity, however, the reversal of neuropathy has been shown to be convergent with improvement in wound healing. Knockout of GM3 synthase (with resultant ganglioside GM3 reduction) reversed both the neuropathy (characterized by loss of sensory neurons and increased sensitivity to pain with von Frey testing) and the wound healing defect in mice fed a high-fat diet, regardless of the extent of obesity or diabetes.[123] Diet-induced obese diabetic mice with selective chemokine receptor CXCR4 deletion from Nav1.8-positive dorsal root ganglia (DRG) neurons failed to develop of mechanical allodynia and small fiber degeneration, despite diet-induced obesity and diabetes.[124] Antagonism of CXCR4 by AMD 3100, a small molecule inhibitor, improved wound healing in db/db mice. These observations suggest some potential therapeutic directions for diabetic neuropathy and improved healing.

Cutaneous afferents are not static, but undergo remodeling based on environmental cues. Deep wounding has been linked to active retraction of preexisting axons from the wound region in streptozotocin-induced (type 1) diabetic mice, as evidenced by reduced expression of the growth-related axon plasticity marker, GAP43.[125] In contrast, superficial perturbation may increase remodeling. Hair clipping in transgenic mice with fluorescent axons led to epidermal proliferation, increases in the expression of follicular stem cell markers, and axon remodeling.[126] Schwann cells in peripheral nerves also possess exceptional plasticity. Injury

to peripheral nerves has been shown to activate peripheral glia by reprogramming them into "repair cells", which prompts glial cell dedifferentiation, proliferation, and dissemination into the wound bed to promote healing.[127] Better understanding of these responses is important for uncovering the role of nerve plasticity in normal and diabetic wound repair.

A COMPLEX NETWORK OF SENSORY NERVES IN MOUSE SKIN

As indicated above, our understanding of the subtypes of sensory nerves in skin is rapidly evolving through transcriptomic[128–130] and proteomic big data analysis of murine (and more recently monkey and human) DRGs. More than a dozen morphologically, physiologically, and genetically distinct primary somatosensory neuron subtypes have been described based on studies that utilize single cell RNA-sequencing techniques and microarrays.[128,131–134] For example, studies of the molecular properties and receptor and ion channel expression of DRG neurons have led to identification of the neuropeptide Y (NPY) receptor,[135] MRGPRs,[136] voltage gated Na+ (Na_v) channels,[137,138] transient receptor potential (TRP) channels,[138–140], ATP receptors (such as purinoceptor P2 × 3 and P2 × 4),[141] and tyrosine kinase receptors (TRKs)[142] (Table II). The TRP family of receptor ion channels are major signal detectors and transducers in nociceptive neurons and, with Nav1.8 [143] and MRGPRD,[144] are thought to play a major role in transmitting the sensation of chronic skin pain.

More recently, the number of DRG neuron subtypes in mice and human has expanded into at least 14 subtypes, although the ultimate degree of heterogeneity may well be greater. These subtypes are based on coupling single cell RNA sequencing (scRNA-seq) and single-cell polymerase chain reaction (PCR) confirmation[128,145] with in vivo whole-cell patch-clamp recording of randomly selected DRG neurons. Moreover, another interesting benefit of a single cell transcriptional approach is the possibility of discovering transcriptional plasticity associated with pathological states, which may increasingly guide our choice of therapeutic targets. Determining the functional consequences of different types of transcriptomal patterning is of great interest (Table V).[146] The rapid progress in determining sensory neuronal subsets involved in pain promises to open the door to delineating their roles in diabetic neuropathy and wound healing. Transcriptome profiling of DRG neurons has now been performed in rodents with and without pathological conditions, such as chronic pain induced by inflammation or injury[133,147,148] and diabetes.[149] In rats, 66 RNA transcripts related to inflammation, hyperalgesia/analgesia, cell growth, and cell survival were differentially expressed between diabetics and controls. Diabetics showed not only an increase in pain-related genes, but in regenerative-related genes, suggesting an attempt to switch to a regenerative program.[149]

Early studies suggest differences between human and mouse sensory neuronal subsets.

Importantly, early studies of scRNA-seq of human DRGs have shown differences in the subsets of mouse (see Table VI) vs human peripheral afferents.[150] An integrative analysis with RNA-seq data of human and mouse DRGs revealed broad conservation of nociceptor-enriched genes (eg, *P2XR3* [P2 × 3 receptor], *SCN10A* [Na_v1.8], *SCN11A* [Na_v1.9], *NTRK1* [TrkA], and *MRGPRD* [MRGPRD]) across mouse and human DRGs,[151] although

the relative expression of different subsets and co-expression of markers on various subsets differed between mice and humans.[150] For example, *in situ* hybridization by multiplex RNAscopy showed overlap of CGRP and P2 × 3R neuronal subpopulations present in human lumbar DRGs, but not in mouse DRGs.[152] Differences in the mRNA expression patterns of human vs mouse DRGs for transient receptor potential channels, cholinergic receptors, potassium channels, and sodium channels was also found.[152] Overall, there is only partial concordance of preclinical results related to wound healing in vitro (57%) and in small laboratory mammals (53%) with clinical results in humans.[153] These differences between human and mouse DRG subsets and their function may explain the poor correlation in results of preclinical rodent vs human clinical trials in response to therapeutics for diabetic healing.

Given these molecular and electrophysiological differences between rodent and human DRG sensory neuron and cutaneous afferent subtypes, it will be crucial to validate the molecular mechanisms underlying impaired wound healing in diabetes and the potential therapeutic targets using human samples (eg, human 3D skin equivalent models of diabetes[154] and diabetic wound biopsies). Nevertheless, transcriptional heterogeneity among neuronal clusters contributes to the functional specificity and responses to cutaneous stimuli of specific neuronal subtypes in both mice and humans. Given the diversity of neurons, elucidating key neuronal subpopulations in the context of diabetic neuropathy would advance our understanding of impaired diabetic wound healing.

BIDIRECTIONAL COMMUNICATION BETWEEN NEURONS AND OTHER CELLS IN HEALTHY AND DIABETIC SKIN

More recently, attention has focused on neuropeptides, such as CGRP and SP (one set of neurotransmitters typically released by many DRG neurons), and neuromodulators (acting on neurons) as messengers for bidirectional communication between skin cells and nerve afferents, including in studying wound repair. In skin, neuropeptides released by peripheral sensory nerves bind to receptors on a variety of skin cells, among them keratinocytes,[155–157] dermal vascular endothelial cells,[158,159] dermal dendritic cells,[160,161] Langerhans cells,[162–165] mast cells,[166,167] and fibroblasts[168] (Table III). Neuropeptide-specific receptor expression in both neuronal and skin cells suggest a close functional interaction between neurons and skin.[169] In addition, epidermal cells (keratinocytes, Langerhans cells, and Merkel cells) can also express neuroactive molecules and participate in neurogenic inflammation.[170] For example, ATP, neurotrophins, and cytokines[171,172] are secreted by keratinocytes and are capable of modulating sensory neurons. Keratinocytes have been shown to communicate with nonpeptidergic (ie, MrgD+/IB4-binding C fibers) and deeper projecting peptidergic C fibers, as well as Aδ fiber nociceptors.[131,132,173–175] For example, ATP release by keratinocytes activates P2 × 4 receptors on sensory neurons to relay touch perception from skin, but *P2 × 4* knockdown in mice also dampens the firing rate of deeper afferents.[173] Furthermore, skin cells are capable of releasing axon guidance cues through netrins and semaphorins, which may direct nerve fiber growth.[176,177]

NEUROPEPTIDES AS MEDIATORS IN CUTANEOUS WOUND HEALING

Although barely detectable in unstimulated skin, neuron-derived neuropeptides are significantly increased by wounding[98] and direct chemical and electrical stimulation.[178–182] In fact, increases in specific neuropeptides are linked temporally to the inflammation, proliferation, and migration phases of wound healing (Fig 1). Several neuropeptides, among them CGRP, SP, corticotropin releasing factor (CRF), α-melanocorticotropin releasing hormone (α-MSH), neurotensin (NT), neurokinin A (NKA), and neuropeptide Y (NPY), mediate important wound healing functions (Table III). These neuropeptides have also been shown to influence vasodilation[183,184] and inflammatory responses,[185] which are critical to normal healing in animal models.

Chronic nonhealing wounds and skin from subjects with diabetes and disease-associated peripheral neuropathy have increased expression and activation of neutral endopeptidase (NEP),[186] a cell surface metalloprotease that degrades SP. Consistent with the observation in humans, over-expressed NEP in mutant diabetic mice diminishes the proinflammatory effects of SP that promote healing.[187] Similarly, genetically modified nondiabetic mice without neuropeptide Y receptor (NPY-2Ra)[188] or CGRP[112] have a significant delay in cutaneous wound healing and decreased neovascularization. In diabetic mouse models, neuropeptide application to wounds, including neurotensin (NT)[189] and SP,[190] improves healing, variably reducing the inflammatory cell infiltrate,[191] increasing angiogenesis,[192] and increased fibroblast proliferation and collagen deposition.[193] In nondiabetic mice, intraperitoneal injection of α-MSH[194] before skin wounding antagonizes inflammation, accelerates wound healing, and improves collagen fiber organization. A more holistic investigation into which neuropeptides are predominantly impacted by diabetes (especially human) is needed.

ANIMAL MODELS OF NEUROPATHY AND DIABETIC WOUND HEALING

Rodents are commonly used models for wound healing studies, but have been criticized because of the propensity of rodent skin to heal by contraction, rather than primarily by re-epithelialization, and the marked difference in epidermal thickness (ie, mice have 2–3 layers vs the 7–10 layers of human epidermis).[195,196] Nevertheless, several murine models of diabetes and associated neuropathy (Table IV) have been splinted to prevent wound contracture and encourage healing by re-epithelialization.[197,198] Rodent models traditionally utilize wounds on the back rather than the typical human location on the foot, although an open full-thickness excision wound on the footpad of T2D rodents has recently been used.[199] Despite their limitations, rodent models are the most feasible and cost-effective systems for studying genetic or biochemical alterations in sensory nerves or nerve subsets and their impact on wound healing in diabetes.[110,200]

Larger animals, however, can also serve as models of diabetes for studies with neuropeptide supplementation or tracking changes in cutaneous nerves. Rabbit ears as the site for wound experiments have the advantage being cartilaginous (naturally splinted) to limit contraction.[201,202] The alloxan-induced type 1 diabetic rabbit model was used to show dysregulation in cytokine and neuropeptide gene expression in diabetic wounds.[95,97]

Similarly, a rabbit model of diabetic neuroischemic wound healing illustrated how a combination of a high M1/M2 ratio, failure to mount postinjury cytokine response, and diminished neuropeptide expression, contribute to wound-healing impairment in diabetes.[95]

Porcine skin is thought to be most similar to human skin with respect to anatomy (including neuroanatomy), immune cells, and collagen biochemistry, although the dermal microvascular density is less than in human skin.[203,204] In contrast to the relatively poor concordance of rodent with human wound healing study results, pig models were 78% concordant with human studies regarding wound healing therapies.[203] In the porcine neuropathic pain model, as in human neuropathic pain,[205] cutaneous (especially epidermal) small caliber C and Aδ afferents are decreased, keratinocyte expression of $Na_v1.7$, the endothelin A receptor, and CGRP are increased (all expected to lead to nociceptor excitatory algesia), and expression of the keratinocyte endothelin B receptor, which mediates inhibitory analgesic mechanisms, is decreased.[205]

The Ossabaw pig is a relatively new model of T2D[206] and has been used to study wound healing impairment. Ossabaw swine are obesity-prone. When fed a high-fat diet, they develop at least 5 of the 6 criteria of the metabolic syndrome, including primary insulin resistance, obesity with significant visceral adipose expansion, hypertriglyceridemia and increased LDL: HDL cholesterol, mild hypertension, and coronary artery disease.[206,207] Wounds in high fat diet Ossabaw pigs have exaggerated and persistent inflammation, lower abundance of endothelial cells in the granulation tissue (impaired vascularization), reduced fibroblast markers, and disorganized granulation tissue.[208] Ocular neuronal and vascular alterations in the early time course of diabetic retinopathy pathogenesis were observed by electron microscopy in young Ossabaw pigs,[209] suggesting that the diabetic Ossabaw pig model may be used for examining neurologic influences and treatment responses in diabetic wound healing that could more easily translate to humans with diabetes and chronic wounds.

IN VITRO MODELS OF WOUND HEALING

As an additional means to consider the impact of diabetic conditions in human models, researchers have studied cocultures of human skin cells and/or neurons[210] to mimic in vivo conditions.[211–214] Primary human keratinocytes cocultured with rat cutaneous primary afferent DRGs have up-regulated NGF production, and show both directed neurite outgrowth and enhanced keratinocyte proliferation, further emphasizing the dynamic interaction of sensory neurons and keratinocytes.[215] A 3D coculture system of injured human skin explants with either rat sensory neurons or neuropeptides enabled the study of sensory neuron and neuropeptide influences on wound healing processes. The cocultures with rat sensory neurons promoted keratinocyte and fibroblast proliferation, stimulated collagen expression, and increased the enzymatic activity of matrix metalloproteins; addition of the neuropeptides led to human dermal fibroblasts proliferation, adherence, differentiation into myofibroblast, and increased matrix metalloprotein enzymatic activities in the early phases of wound healing.[99] The quality of most 3D models is compromised, however, by having nonhuman components, with all-human cell models preferred.

Primary human dorsal root ganglia (DRG) from cadavers[216] or sensory neurons from induced pluripotent stem cells[217] have also been cultured with cutaneous immune cells, keratinocytes, or fibroblasts.[218–220] A coculture model of human keratinocytes and neural crest cell-derived sensory neurons demonstrated functional cross-talk between cell types through Ca^{2+} imaging experiments.[221,222] A tissue-engineered skin model of peripheral nerve regeneration by incorporating collagen sponge populated with human endothelial cells and/or human fibroblasts was used to assess the influence of endothelial and epidermal cells on neurite growth. Spontaneous formation of numerous thick myelin sheaths surrounding motor fibers after long-–term culture was observed.[223]

In vitro human 3-dimensional (3D) tissue models (human skin equivalents) have also been engineered to resemble normal human skin in their morphology, proliferation, differentiation, and transcriptional patterns and responses.[224] These models have been adapted to study the keratinocyte-fibroblast interactions in diabetes during wounding using 3D models with human diabetic foot ulcer fibroblasts embedded into the bed underlying normal keratinocytes.[154] Healing is delayed, with reduced keratinocyte migration to re-epithelialize the wound and impaired extracellular matrix deposition compared to 3D cultures with foot fibroblasts from healthy controls.[154,225] Incorporating neurons (and vasculature) into this model could be useful in understanding the influence of nerves in diabetic healing. Indeed, a tissue-engineered wound healing model made of: i) a perforated epidermal compartment with green fluorescent human keratinocytes; ii) a dermal compartment; and iii) sensory neurons demonstrated the impact of sensory neurons on wound closure via secretion of neuropeptide SP.[96] Microfluidic cell culture systems[226,227] also provide a platform for probing functional properties of neurons and investigating neuronal-non-neuronal cell crosstalk.

FUTURE THERAPEUTIC PERSPECTIVES

Beyond cell cultures and 3D skin equivalents, the development of 3D printed skin equivalents offers the ability to incorporate skin features that traditional cell cultures lack, such as blood vessels and glands. A recently developed vascularized 3D printed skin model composed of epidermis, dermis, and hypodermis reflected the complexity of the human skin, including epidermal stemness and stratification.[228] Similarly, fabrication of synthetic biocompatible vascular networks in combination with electrospinning and 3D printing techniques enabled the study of cutaneous angiogenesis in a more physiologically relevant environment[229] with endothelial cell migration and tube formation in vitro. The development of more dynamic in vitro approaches through tissue engineering allows closer modeling of native human cell behaviors and may be a potential avenue for human neuronal investigations.

CONCLUSIONS

Current treatment options for individuals with diabetic foot ulcerations are limited, resulting in amputations and a large unmet need for improved management, ideally based on understanding disease pathogenesis. Much of the basic research that addresses nerves in wound healing is associative, but nevertheless supports the notion that cutaneous sensory

innervation, local neuropeptide release, and other mediating factors affect healing, including in diabetic models.

At this time, therapies that can reduce the pain of diabetic neuropathy, such as gabapentin, pregabalin, duloxetine, and amitriptyline, do not reverse the neuropathy itself and have not been noted to ameliorate the wound healing issues.[230] Better understanding of the specific roles of nerve subtypes within DRGs in wound healing will be critical and may well suggest novel therapeutic targets. While not without their limitations, emerging in vivo and in vitro large animal and human models provide an opportunity to further investigate the molecular and cellular features of wound repair and advance our understanding of neural involvement in wound healing pathology. Translation of these observations related to reversal of the neuropathy and better healing in animal models could lead to a disease-modifying approach.

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Abbreviations:

α-MSH	alpha-melanocorticotropin releasing hormone
CGRP	calcitonin gene–related peptide
C-LTMR	C fiber low-threshold mechanoreceptor
CRF	corticotropin releasing factor
DRG	dorsal root ganglia
EGF	epidermal growth factor
GM3	monosialo-dihexosylganglioside
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor-1
IL	interleukin
MMPs	matrix metalloproteinases
MRGPRs	Mas-related G-protein coupled receptors
NGF	nerve growth factor
NPY	neuropeptide Y

NT	neurotensin
SP	substance P
TNF-α	tumor necrosis factor-α
TRKs	tyrosine kinase receptors
TRP	transient receptor potential
T2D	type 2 diabetes
VEGF	vascular endothelial growth factor

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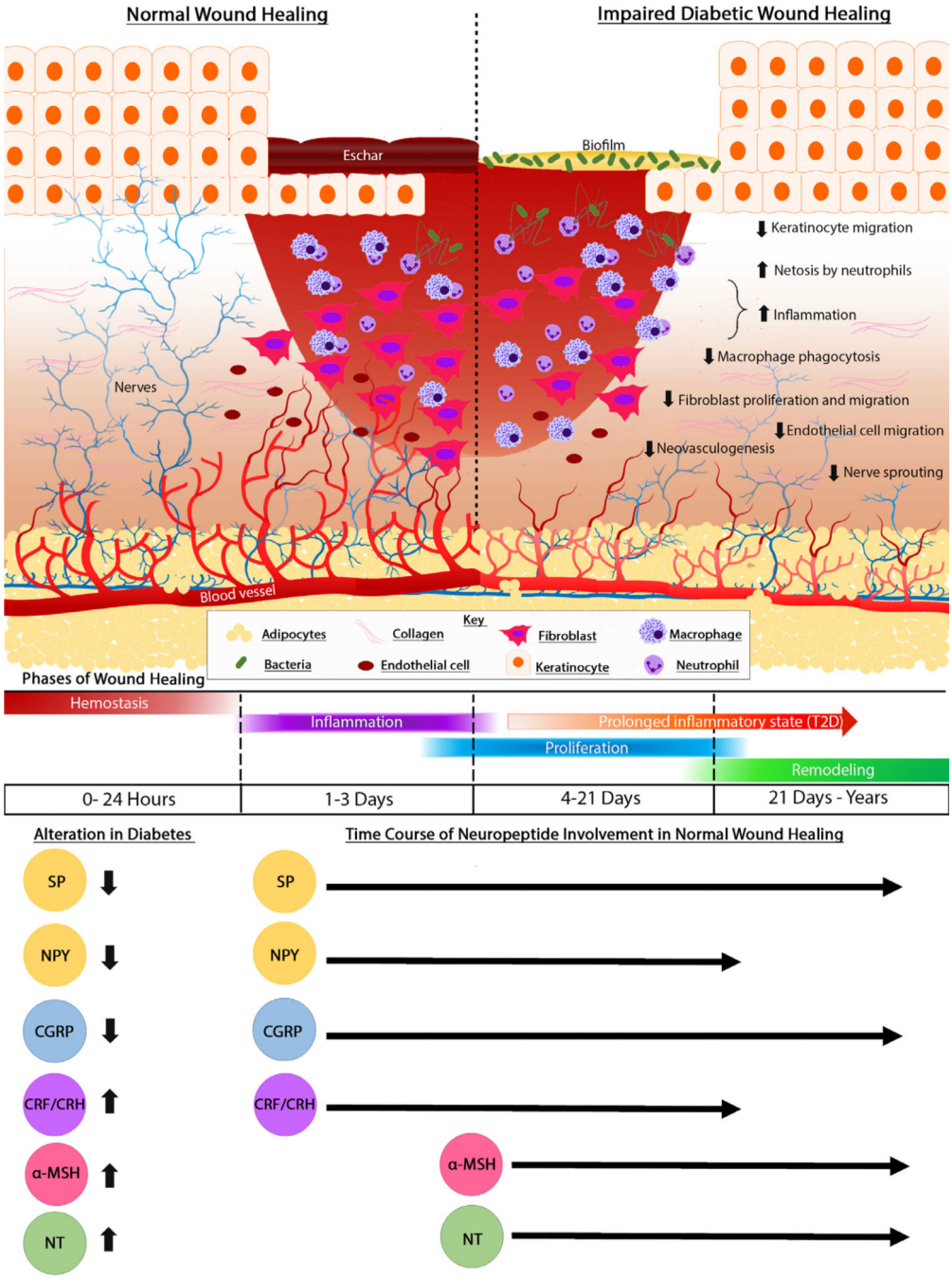

Fig 1.

Cellular mechanisms involved in normal wound healing and their impairment in chronic diabetic wounds. **Top**: In contrast to healing in normal skin (left), healing in diabetic skin (right) is impaired. Chronic diabetic wounds have an epidermis that migrates poorly, an influx of dysfunctional inflammatory cells, and surface biofilm. In addition to impaired proliferation and migration of fibroblasts and endothelial cells in diabetic wounds, sensory innervation is deficient, with a reduction in intraepidermal nerve fiber density. **Bottom**: Normal wound repair involves a temporal sequence of overlapping phases: hemostasis,

inflammation, cell proliferation and migration, and remodeling. Unlike normal wounds, chronic wounds are stalled in the inflammatory phase. Neuropeptides have crucial roles at each stage of wound repair and are dysregulated in diabetes. While tachykinins substance P (SP) and neuropeptide Y (NPY), as well as calcitonin gene-related peptide (CGRP) are downregulated during diabetic wound healing, corticotropin releasing factor (CRF), α-melanocorticotropin releasing hormone (α-MSH), and neurotensin (NT) are upregulated, contributing to delayed healing.

Table I.

Changes in diabetes that affect stages of healing in animal models and patients with diabetes[26,27,34,36,231–247]

Alterations in inflammatory phase	Alterations in proliferative phase	Alterations in remodeling phase
↑ Levels of proinflammatory cytokines (IL-6, MCP1, TNF-α)	↓ Fibroblast proliferation and migration	↑ MMPs
↓ Phagocytosis	↓ Keratinocyte differentiation and migration	↓ Collagen and elastin content
↑ NETosis	↓ Functional levels of growth factors (PDGF, IGF-1, VEGF)	ECM glycation and ↑ crosslinking
↓ Numbers of CD4+ T cells	Impaired angiogenesis	↓ Levels of TIMPs (MMP inhibitors)
↑ Oxidative stress	Endothelial cell dysfunction	↑ Presence of nonsolubilized and fragmented ECM fibrils
Impaired macrophage polarization (proinflammatory to proreparative phenotype switch)	Pericyte dysfunction	↓ Wound contraction and wound strengthening
Impaired neutrophil function	Mast cell dysfunction	Fibroblast senescence

Abbreviations: ECM, extracellular matrix; *IGF-1,* insulin-like growth factor-1; *IL-6,* interleukin-6; *MCP1,* monocyte chemoattractant protein-1; *MMPs,* matrix metalloproteinases; *NET,* neutrophil extracellular traps; *PDGF,* platelet-derived growth factor; *TIMPs,* tissue inhibitors of metalloproteinases; *TNF-α,* tumor necrosis factor alpha; *VEGF,* vascular endothelial growth factor.

Table II.

Classification of sensory neurons from adult mouse DRGs and markers[128]

Neuron size	**Small neurons**						**Large neurons**				
Principal neuron types **Classical markers**	**PEP** ***Tac1, Calca***		**NP** ***IB4, P2 × 3, Plxnc1***			**TH** ***Th***	**NF** ***Nefh, Ldhd***				
Subpopulations	**PEP1**	**PEP2**	**NP1**	**NP2**	**NP3**	**-**	**NF1**	**NF2**	**NF3**	**NF4**	**NF5**
Myelinated/unmyelinated	Unmyelinated	Myelinated	Unmyelinated			Unmyelinated	Myelinated				
Gene markers/products	TRPV1	Nav1.8/9	TRPA1	TRPV1	TRPV1	TRPA1	TrkB^{high}	TrkB^{low}	TrkC^{high}	TrkC^{low}	TrkC^{low}
	Nav1.8/9	TrkA	TRPC3	TRPA1	TRPA1	NAV1.8/9	NEFH	RET	RET	ASIC1	ASIC1
	TrkA	CGRP	MRGPRD	TRPC3	TRPC3	VGLUT3		NEFH	NEFH	CNTNAP2	CNTNAP2
	CGRP	Fam19A1	P2 × 3	Nav1.8/9	Nav1.8/9	Piezo2				NEFH	NEFH
	PLXNC1^{low}	NEFH		MRGPRA3	P2 × 3						
Gene expression/subtype	*Plxnc1*	*Fam19a1*$^{+}$	*P2 × 3*^{hgh}	*Ntrk1*$^{+}$	*Sst*$^{+}$	*Th*$^{+}$	*Ntrk2*^{high} *Necab2*	*Ntrk2*^{low}	*Ntrk3*^{high}	*Ntrk3*^{low}	
	Tac1^{+}	*Calca*$^{+}$		*Calca*$^{+}$	*P2 × 3*^{low}	*Plxnc1*$^{-}$		*Calb1*	*Pv*$^{-}$	*Pv*$^{+}$	
	Calca^{+}	*Nefh*$^{+}$				*Ntrk1*$^{-}$			*Fam19a1*$^{+}$	*Fam19a1*$^{-}$	
						Nefh^{-}				*Cntnap2*$^{+}$	
										Spp1^{+}	
Modality-specific function	Peptidergic		Nonpeptidergic			C-LTMRs	AS-LTMRs	Aβ-LTMRs (RA, SA)		Proprioceptors	

Abbreviations: ASIC1, acid sensing ion channel subunit 3; *Aβ-LTMRs (RA, SA),* alpha beta low threshold mechanoreceptor (rapidly adapting or slowly adapting); *Aδ-LTMRs*, alpha-delta low-threshold mechanoreceptor; *Calb1*, calbindin 1; *Calca*, calcitonin-related polypeptide-*α*; *CGRP*, calcitonin gene-related peptide; *C-LTMRs*, C-low threshold mechanoreceptors; *Cntnap2*$^{+}$, contactin associated protein 2-positive; *Fam19A1*, family with sequence similarity 19 member A1; *IB4*, isolectin-B4; *Ldhd*, lactate dehydrogenase B; *MRGPR*, Mas-related G-protein coupled receptor member; *Nav1.8/9*, voltage-gated sodium (NaV) channels 1.8/1.9; *Necab2*, N-terminal EF-hand calcium binding protein 2; *Nefh*, neurofilament heavy; *NF*, neurofilament-positive; *NP*, nonpeptidergic; *PEP*, peptidergic; *Piezo2*, piezo type mechanosensitive ion channel component 2; *Plxnc1*, plexin C1; *Pv*, parvalbumin; *P2 × 3*, P2X purinoceptor 3; *RET*, RET proto-oncogene; *Spp1*$^{+}$, secreted phosphoprotein 1; *Sst*$^{+}$, somatostatin-positive; *Tac1*, tachykinin precursor 1; *TH/Th*, tyrosine hydroxylase; *Trpa1*, transient receptor potential ankyrin 1; *Trpc3*, transient receptor potential cation channel subfamily C member 3; *Trpv*, transient receptor potential cation channel; *Trk*, tyrosine receptor kinase; *VGLUT3*, vesicular glutamate transporter 3.

Table III.

Neuropeptides and their functions in wound healing[60,96,99,102,103,105,112,158,188,189,248–298]

Neuropeptide/ dysregulation in diabetes	Neuropeptide receptor(s)	Target cell-types in skin	Cytokines in wound healing	Biological function in skin	Involvement in wound healing phase
Substance P (SP) ↓	NK-1R	Endothelial cell, Keratinocyte, Fibroblast, Mast cell, Monocyte & Macrophages, Granulocytes, Lymphocytes	TNF-α, IL-1β, IL-2, IL-6, IL-8, TGF-β	Vasodilation; Vascular permeability; Cell proliferation and migration; Leukocyte attraction and adhesion; Polymorphonuclear cell infiltration; Plasma extravasation; Angiogenesis; Neurite Outgrowth; Immune cell proliferation and ↑ chemotaxis; ↑ NGF; Collagen remodeling	Inflammatory Proliferative Remodeling
Calcitonin gene-related peptide (CGRP) ↓	CL-R/RAMP1	Endothelial cell, Keratinocyte, Mast cell, Monocyte & Macrophage, Melanocyte, Granulocyte, Lymphocyte	TNF-α, IL-1β, IL-1α, IL-8, IL-2, IL-6, VEGF	Vasodilation; Vascular permeability; Cell proliferation; Immunomodulation; Angiogenesis; Acts in combination with SP; ↑ NGF; Collagen remodeling	Inflammatory Proliferative Remodeling
Neuropeptide Y (NPY) ↓	Y1–Y6	Endothelial cell, Mast cell, Monocyte & Macrophage, Granulocyte, Lymphocyte	TNF-α and IL-2	Migration of macrophages; Cell proliferation; vasoconstriction; angiogenesis	Inflammatory Proliferative
Neurotensin (NT) ↑	NTR1–R3	Keratinocyte, Fibroblast, Mast cell, Dendritic cell	IL-6, TNF-α, IL-8	↑ EGF; Angiogenesis; Anti-inflammatory; Mast cell degranulation; Cell migration	Proliferative Remodeling
α-Melanocyte stimulating hormone (α-MSH) ↑	MC1R–5R	Keratinocyte, Mast cell, Fibroblast, Endothelial cell, Monocyte & Macrophage, Melanocytes	TNF-α, IL-β, IL-8, IL-10, IFN-γ	Anti-inflammatory; Angiogenesis; Immunomodulation	Proliferative Remodeling
Corticotropin releasing factor (CRF) ↑	CRFR1–R2	Keratinocyte, Fibroblast, Monocyte	TNF-α, IL-1β, IL-1α, IL-2, IL-6, IL-4, IL-10, IL-8, IFN-α, IFN-γ, MIP-1α, KGF-1	Proinflammatory cytokine release; Angiogenesis; Mast cell activation and degranulation; Cell migration and proliferation; Immunomodulation	Inflammatory Proliferative

Abbreviations: CL-R/RAMP1, calcitonin receptor-like receptor, receptor activity-modifying protein 1; *CRFR*, corticotropin releasing factor receptor; *EGF*, epidermal growth factor; *IFN-α*, interferon-α; *IFN-γ*, interferon-γ; *IL*, interleukin; *KGF-1*, keratinocyte growth factor-1; *MIP-1α*, macrophage inflammatory protein-1α; *NGF*, nerve growth factor; *NK-1R*, neurokinin 1 receptor; *NTR*, neurotensin receptor; *MCR*, melanocortin receptor; *SP*, substance P; *TGF-β*, transforming growth factor-β; *TNF-α*, tumor necrosis factor-α; *VEGF*, vascular endothelial growth factor.

Table IV.

Type 2 diabetes models with delayed healing and known evidence of neuropathy

Model	Comments	References
Mouse models		
B6.Cg-Lep^{ob}/J (ob/ob)	Very obese, high glucose and insulin levels	282–285
Monogenic *JAX stock #000632*		
BTBR-ob/ob	Very obese, high glucose and insulin levels	286–289
Monogenic	Retinopathy	
JAX stock #004824	Nephropathy	
BKS.Cg-Dock7^{m} +/+ Lepr^{db}/J (db/db)	Very obese, high glucose and insulin levels	290–293
Monogenic	Myocardial disease	
JAX stock #000642		
C57BL/6J diet-induced obese	Less obesity than genetic models and moderate increase in glucose and insulin	294–299
JAX stock # 380050	Hypertension Endothelial dysfunction	
Rat models		
Zucker diabetic fatty (ZDF)	Obese, high glucose and insulin levels	300–305
Monogenic	Hydronephrosis and hypertension	
Otsuka Long-Evans Tokushima fatty	Late onset obesity and increases in glucose and insulin levels (moderate range)	303,304
Polygenic		
Zucker diabetic Sprague-Dawley (ZDSD/Pco)	Obesity and early onset high glucose and insulin	305,306
Polygenic	Osteoporosis and nephropathy	307–309
Goto-Kakizaki	Not obese	
Polygenic	High glucose and insulin	
High-fat diet-low dose streptozotocin (STZ)-treated	Obesity, high glucose and insulin Retinopathy Renal dysfunction Cardiomyopathy	310–312

Table V.

Classification of sensory neurons from adult mouse DRGs based on scRNA-seq clusters[129] and compared with previous marker-based classification

Neuron cluster based on transcriptional analysis and predicted function	Markers	Subtypes	Subtype markers	Classification of DRGs based on size, myelination, and markers——Small neurons——Large neurons						Highly expressed TR channel types per cluster subtype
				PEPPeptidergic (myelinated+unmyelinated)	NPNonpeptidergic (unmyelinated)		TH (unmyelinated)C-LTMRsTh		NF(myelinated)	
				Tac1	*Calca*	*IB4*	*P2 × 3r*	*Plxnc1*	*Nefh*	*Ldhb*	
Small fiberneurons											
C1	Gal	C1–1	*Asic3*^{high}								*Trpv1*^{high}
MHN	*Adcyapl*		*Cldn9*								*Trpm8*^{high}
		C1–2	*Zcchcl2* *Sstr2*								*Trpm3*^{high}
C2	*Nppb*	02–1	*Pvalb*$^{+}$								*Trpv2*^{high}
MHN (MI, IS)	*Il31ra*		*S100b*$^{-}$								
		C2–2	*Pva1b*$^{-}$ *S100b*$^{+}$								*Trpm3*^{high}
C3	*Th*		–								*Trpv2*^{high}
C-LTMR	*Zfp521*										*Trpv4*^{high}
C4	*Mrgpra3*	C4–1	*Mrgprb4*$^{-}$ *Ptpn6*$^{-}$								
MHN (IS)	*Rspo1*	C4–2	*Mrgprb4*$^{+}$ *Ptpn6*$^{+}$								
C5	*Mrgprd* (highly expressed)	C5–1	*Gfra3*$^{+}$ *Sstr2*$^{+}$								*Trpm3*^{high}
MHN (IS)	*Prkcq, Lpar3*	C5–2	*Gfra3*$^{+}$ *Sstr2*$^{+}$								*Trpv2*^{high}
C6	*Mrgprd* (moderately	C6–1	*Ntrk1*$^{+}$ *Prkcq*^{low}								

Neuron cluster based on transcriptional analysis and predicted function	Markers	Subtypes	Subtype markers	Classification of DRGs based on size, myelination, and markers——Small neurons——Large neurons						Highly expressed TR channel types per cluster subtype
				PEPPeptidergic (myelinated+unmyelinated)	NPNonpeptidergic (unmyelinated)		TH (unmyelinated)C-LTMRsTh		NF(myelinated)	
				Tac1	*Calca*	*IB4*	*P2 × 3r*	*Plxnc1*	*Nefh*	*Ldhb*	
	expressed) *S100b*										
MHN		C6–2	*Ntrk1* *Prkcq*^{high}								*Trpv2*^{high}
Large fiber neurons											
C7	*Nxph1, Wnt7a, S100b*	–	–								
MHN (MS)											
C8	*Trappc31, Cgn11, S100b*	C8–1	*Ntrk3*^{high}								*Trpm8*^{high}
MR			*Htr1d*^{high}								
		C8–2	*Ntrk1*^{high} *Htr3a*^{high}								*Trpm8*^{high}
C9	*Baiap2l1, Cadps2, S100b*	C9–1	*Asic3*$^{-}$ *Cgnl1*^{low}								
MN		C9–2	*Asic3*$^{+}$ *Cgnl1*^{high}								
C10	*Gal,*	–	–								*Trpv2*^{high}
MR or P	*Rspo1*										
		–	–								*Trpv1*^{high} *Trpa1*^{high} *Trpm3*^{high}

Gray-filled squares indicate highly expressed markers per cluster.

Abbreviations: Adcyap1, pituitary adenylate cyclase-activating polypeptide 1; *Asic3*, acid sensing ion channel subunit 3; *Baiap2l1*, brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1; *Cadps2*, calcium-dependent secretion activator 2; *Calca*, calcitonin-related polypeptide-*α*; *Cgnl1*, cingulin-like 1; *C-LTMR*, C fiber low-threshold mechanoreceptor; *Cldn9*, claudin 9; *Gal*, galanin; *Gfr*a3, GDNF family receptor-*α*3; *Htr1d*, 5-hydroxytryptamine receptor 1D; *Il31ra*, interleukin-31 receptor A; *IS*, itch-sensitive; *Ldhb*, lactate dehydrogenase B; *Lpar3*, lysophosphatidic acid receptor 3; *MHN*, mechanoheat nociceptor; *MI*, mechanically insensitive; *MN*, mechanical nociceptor; *MR*, mechanoreceptor; *Mrgpra3*, Mas-related G-protein coupled receptor member A3; *Mrgprb4*, Mas-related G-protein coupled receptor member B4; *Mrgprd*, Mas-related G-protein coupled receptor member D; *MS*, mechanically sensitive; *Nefh*, neurofilament heavy; *NF*, neurofilament-positive; *NP*, nonpeptidergic; *Nppb*, natriuretic peptide B; *Ntrk1*, neurotrophic receptor tyrosine kinase 1; *Nxph1*, neurexophilin 1; *P*, proprioceptor; *Plxnc1*, plexin C1; *Ptpn6*, protein tyrosine phosphatase non receptor type 6; *Prkcq*, protein

kinase c theta; *Pvalb*, parvalbumin; *P2 × 3r*, purinergic receptor P2 × 3; *PEP*, peptidergic; *Rspo1*, R-spondin 1; *Sstr2*, somatostatin receptor 2; *S100b*, S100 calcium binding protein B; *Tac1*, tachykinin precursor 1; *TH/Th*, tyrosine hydroxylase; *Trappc3l*, trafficking protein particle complex subunit 3-like protein; *Trpm*, transient receptor potential melastatin; *Trpv*, transient receptor potential cation channel; *Wnt7a*, Wnt family member 7A; *Zcchc12*, zinc finger CCHC domain-containing protein 12; *Zfp521*, zinc finger protein 521.

Table VI.

Spatial transcriptomics to define human DRG neuronal subtype clusters[313]

Neuron dass	**Aβ and Aδ fibers**				**C fibers**					
Subtype	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
Markers	*NTRK3* ^{HI} *NTRK2* ^{LOW}	*NTRK3* *NTRK2* ^{LOW}	*NTRK2* ^{HI} *NTRK3* ^{LOW}	*NTRK3* *SCN10A*	*TRPM8* *TRPV1* *SCN10A*	*LPAR3*	*PENK*	*TRPA1*	*CHRNA3*	*SCN11A* *SCN11A* *NPPB* *GFRA2* *IL31RA*
Putative Functlonal Classification	*Aβ-SA* LTIMR	*Aβ-RA* LTMR	*Aδ* LTMR	Putative *Aβ* nociceptor	Cold nociceptor	LPAR3+ nociceptor	PENK+ nociceptor	TRPA1+ nociceptor	Putative silent nociceptor	Putative itch nociceptor
Preferentlal upregulatlon of genes by sex	Females	Females	Females	None	Males	Females	Females	Females	Males	Males

Abbreviations: Aβ-RA, alpha-beta rapidly adapting fibers; *Aβ-SA*, alpha beta sLOWly adapting fibers; *Aδ LTMR*, alpha delta LOW threshold mechanoreceptor; *CHRNA3*, cholinergic receptor nicotinic alpha 3; *GFRA2*, GDNF family receptor alpha 3; *IL31RA*, interleukin-31 receptor A; *LPAR3*, lysophosphatidic acid receptor 3; *NPPB*, natriuretic peptide B; *NTRK*, neurotrophic receptor tyrosine kinase; *PENK*, proenkephalin; *SCN10A*, sodium voltage-gated channel alpha subunit 10/ also NaV 1.8; *SCN11A*, sodium voltage-gated channel alpha subunit 11/ also *NaV 1.9*; TRPA1, transient receptor potential ankyrin 1; *TRPM8*, transient receptor potential cation channel subfamily melastatin member 8.