Influence of Sensory Innervation on Epithelial Renewal and Wound Healing

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1 Introduction

The main function classically attributed to peripheral somatosensory system is to receive, transduce, and channel external or internal information toward central regions of the nervous system. However, there are numerous examples throughout the body of mammals which indicate that some neurons from sensory ganglia are not restricted to generate afferent impulses. This population of neurons is characterized by its capacity to release neuropeptides from their peripheral terminals. It is postulated that through this neurosecretory character, peptidergic neurons of dorsal root ganglia influence diverse processes in their targets (efferent function). This notion is also supported by the presence of receptor sites and degrading enzymes for neuropeptides in all tissues innervated by peptidergic neurons. Nevertheless, it is often assumed that efferent functions of sensory ganglia are only relevant in clearly pathological events (e.g., neurogenic inflammation). Indeed, it is the fact that sensory nerves participate in pathological events that explains a resurgence of the study and an effort to characterize the effects and mechanisms that govern the interaction between sensory nerves and their peripheral targets such as the skin.

The synthesis and transport of neuropeptides to the peripheral terminals of dorsal root ganglion (DRG) neurons have been documented in various species [1–4]. Thus, efferent functions of DRG neurons may represent a conserved mechanism for tissue renewal and functional maintenance during normal physiological conditions. There are systematic observations about the deleterious effects related to sensory denervation which provokes major changes of gene regulation on its targets [5, 6]. Moreover, the generation of antibodies to label fine terminals at the periphery has revealed that peptidergic terminals are in almost every part of the mammalian body, including the skin, muscle, bone, immune organs, teeth, blood vessels, and viscera. In these regions it has been observed both the existence of synaptic-like contacts between peptidergic endings and some target cells and the expression of neuropeptide receptors by different cell types [7–10]. Overall the anatomical and functional studies suggest that peptidergic innervation plays an active and continuous role on epithelial renewal, wound repair, glandular secretion, and mineralized tissue formation that is just beginning to be understood.

In this chapter, we will discuss several aspects of sensory innervation and the proposed mechanisms by which sensory terminals influence epithelial homeostasis. A brief survey of the main anatomical and neurochemical characteristics of

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Recent Clinical Techniques, Results, and Research in Wounds (2018)
https://doi.org/10.1007/15695_2018_130, © Springer International Publishing AG
Published Online: 11 July 2018

the nerve terminals that innervate the skin will be made. All of this will be discussed under the context of the noceffector concept proposed by Kruger [11] which states that peptidergic neurons of DRG devote most of its biological existence to have an effector or trophic influence on its target.

2 Cytology and Neurochemistry of Dorsal Root Ganglion Neurons: An Overview

Broadly, two main classes of neurons have been described in sensory ganglia based on cell body size, cytoplasmic appearance, axonal diameter, and axonal myelin content. Due to light or dark appearance of its cytoplasm in electron and light microscopy studies, DRG neurons are subdivided into large light (also named A cells) and small dark neurons (also named B cells) [12, 13]. Furthermore, it has been determined by immunohistochemical studies that light appearance is given by a rich content of the 150 and 200 kDa neurofilament subunits [14]. Likewise, dark neurons have a cytoskeleton primarily constituted by the intermediate filament protein called peripherin [15, 16]. Besides these cytological features, it is known that electrical properties such as conduction velocity correlate with soma size and fiber diameter [17]. Thus, large light neurons (soma diameter > 35 mm) correspond to neurons with myelinated fibers [14, 18, 19]. These large-caliber and myelinated axons are the well-known A-fibers which are divided into three subgroups, namely, A, B, and C, from fastest to slowest. In addition, the small dark neurons (<20 mm) give rise to C-fibers which are unmyelinated fibers and, consequently, the thinnest and slowest fibers in sensory nerves [17, 20]. This relationship between anatomical parameters and functional properties does not necessarily apply to medium-sized neurons (20–35 mm). For instance, some A cells skewed toward the large population are neurofilament-negative, and, conversely, neurons skewed toward the small population are neurofilament-positive [18, 21]. Rather than a clear subdivision of neuronal populations, there is a perplexing scenario of subpopulations with overlapping phenotypic and functional properties.

Besides its afferent (i.e., sensory) role, C- and Aδ-fiber neurons are mainly implicated in tissue management [22–24]. Although neuropeptide content is associated with pain modulation, it has been recently documented that a fraction of peptidergic neurons does not process exclusively nociceptive stimuli [2, 23, 25–27]. Moreover, efforts to define a biochemical profile to predict receptive modalities have not been successful at all. Some DRG neurons have the intrinsic genetic program to express neuropeptides, and others acquire a peptidergic phenotype only after they have contacted a target in late embrionary stages [28–30]. Apparently, peptidergic phenotype is related to localization of peripheral terminals in the target tissue rather than to a sensory modality [2, 23, 25]. Indeed, it has been postulated that peptidergic neurons constitute a nocifensor system that probably lacks a sensory function [31]. In fact, there is still much debate about the existence of two separate populations for afferent and efferent role in dorsal root ganglion. For the sake of convenience, we will refer for those cranial and DRG neurons having an efferent role only as peptidergic neurons or noceffectors, assuming that if a neuron presents vesicles with peptides in the peripheral terminals, it conveys a specific message that helps maintain tissue homeostasis, regardless if this neuron transmits a sensory stimuli or whether it is a noxious/non-noxious stimuli [11].

In elegant studies using genetic axonal tracers, the peptidergic and non-peptidergic populations in mice are shown to be topographically segregated. For instance, in mouse epidermis, non-peptidergic fibers terminate in the stratum granulosum, while most of the peptidergic fibers terminate in the stratum spinosum [32]. Similarly, this segregation continues in the spinal cord and in ascending pathways. Peptidergic neurons project to spinal lamina I and the outer region of lamina II (II_o), and these spinal neurons project heavily to the brain stem (parabrachial nuclei) and thalamus, while non-peptidergic neurons

connect with second-order interneurons in the internal region of lamina II (II_{inner}). These interneurons project to lamina V which then project to several limbic and striatal regions [32, 33]. The spatially segregated pathways suggest that these groups of neurons have at least different sensory processing capacities. If this anatomical separation could also be relevant for efferent functions of sensory neurons remains to be determined.

Neuropeptide content in DRG neurons has been reported in various vertebrates as rodents, primates, felines, birds, and reptiles [1–4]. The proportion of peptidergic neurons varies depending on the species, and inside a species varies according to the spinal cord level [34]. Regardless of the animal species, the peptidergic population is consistently composed by a subpopulation of C-fibers neurons and in smaller fraction by a subpopulation of A-fiber neurons [2, 3, 25, 35]. The major peptides synthesized by DRG neurons are substance P (SP) and calcitonin gene-related peptide (CGRP). In addition, DRG neurons also synthesize other peptides such as somatostatin, neuropeptide Y, galanin, vasoactive intestinal polypeptide, pituitary adenylate cyclase-activating polypeptide-38, and opioids.

3 CGRP and Substance P in Dorsal Root Ganglia: Synthesis, Release, and Receptors

3.1 CGRP

The calcitonin gene peptide superfamily consists of four members with potent vasoactive properties that include calcitonin, CGRP, adrenomedullin, and amylin [36, 37]. CGRP exists in two isoforms encoded by different genes, α and β in rat and I and II in human. While the rat isoforms differ in one amino acid residue, in humans they differ in three [38, 39]. The most noticeable site of synthesis of α-CGRP in the peripheral nervous system is the DRG, whereas β-CGRP is preferentially expressed by enteric neurons. The translation of I-CGRP mRNA generates a 121 and 128 amino acid precursor in rats and humans, respectively. The first 25 amino acids of this precursor correspond to the signal peptide, a sequence that assists the targeting of the messenger to the endoplasmic reticulum. The next 103 residues correspond to the proCGRP [40]. The final 37 amino acid peptide is created by proteolytic cleavage of flanking peptides in proCGRP [41].

CGRP receptor belongs to the G-protein-coupled receptor superfamily. A molecular, biological approach has revealed that CGRP receptor is a heterodimer composed of the calcitonin receptor-like receptor protein (CRLR or CLR) and receptor activity-modifying protein 1 (RAMP-1) [37]. The latter is required to transport CRLR to the plasma membrane and to control a specific pattern of glycosylation that determines the affinity for CGRP [42]. The CGRP receptor is associated with the formation of cAMP through the activation of adenylyl cyclase. The biological effects of CGRP end with a proteolytic cleavage by proteases as neutral endopeptidase, insulin-degrading enzyme, and endothelin-converting enzyme-1 [43, 44].

3.2 Substance P

SP is a member of the tachykinin family that includes peptides with a conserved FXGLM-NH2 C-terminal sequence. The mRNAs that encode SP, neurokinin A, neuropeptide K, and neuropeptide G are derived from the preprotachykinin 1 gene. In DRG neurons, alternative RNA splicing of the primary transcript results in the generation of four mRNAs called α-, β-, γ-, and δ-TAC1 [45, 46]. SP precursor sequences are encoded by all four TAC1 mRNAs, but what directs the alternative splicing in the range of tissues where tachykinins are expressed is still unknown [47–49]. Putatively, the posttranslational processing of all these precursors gives rise to the active form of substance P that consists of 11 amino acid residues [50, 51].

The effects of tachykinins are mediated through a group of three G-protein-coupled metabotropic receptors: neurokinin-1 (NK1), neurokinin-2 (NK2), and neurokinin-3 (NK3). Substance P binds preferentially to NK1 receptor

[52, 53]. The activation of tachykinin receptor leads to inositol phosphate accumulation [54]. NK1 receptor stimulation in tracheal smooth muscle causes Ca^{2+} release from intracellular stores through the activation of both inositol triphosphate and ryanodine receptors. In muscle cells, the Ca^{2+} release from the sarcoplasmic reticulum in response to NK1 activation is coupled to Ca^{2+} influx through channels located in the plasma membrane [55]. Once released, SP is inactivated by the action of the neutral endopeptidase and the angiotensin-converting enzyme [56].

4 Release of SP and CGRP from Somatosensory Nerves

CGRP and SP are strongly expressed in normal DRG neurons, which suggests that they are ready to use whenever it is needed. A great portion of the neurons with capacity of peptide release are recognized for being capsaicin sensitive. Capsaicin is the pungent ingredient in hot chili peppers of the *Capsicum* genus, and it has been a valuable pharmacological and clinical tool, because it has allowed studying both afferent and efferent functions of DRG neurons [57]. The notion that C- and Aδ-fibers have a neurosecretory function dates from the early years of the twentieth century. Experiments by Bayliss [58] assigned an efferent role for the nerve fibers that emerge from posterior roots. They noticed that, when central ends of these fibers were excited at lumbar level, the impulse generated (i.e., antidromic process) provoked vascular dilatation at their peripheral ends in the hind limbs of various species. Nowadays it is known that antidromic stimulation of C and Aδ produces vasodilatation and increases plasma extravasation [59, 60]. Immunohistochemical and pharmacological experiments had revealed that CGRP induces arterial vasodilatation, whereas SP provokes an increase in vascular permeability [61, 62]. Overall, these vascular changes and concomitant activation of mast cells, lymphocytes, and neutrophils lead to what is called neurogenic inflammation. Thus, the main efforts to understand peptide release from peripheral terminals of peptidergic DRG neurons have been centered on factors involved in inflammation. In this regard, capsaicin is widely known for its capacity to induce neurogenic inflammation by releasing SP and CGRP from peripheral terminals. It is believed that capsaicin releases neuropeptides exclusively via activation of the vanilloid receptor 1 (TRPV1), but other members of TRPV family might be involved [63]. TRPV1 is a nonselective cation channel that allows entry of calcium and, besides capsaicin, is also gated by nociceptive stimuli such as low pH and heat [64, 65].Classical exocytosis occurs when Ca^{2+} influx into the terminals and initiates exocytotic mechanisms that release neuropeptides and/or other neurotransmitters [66]. The addition of capsaicin to nerve, skin, and mucosal explants induces peptide release, but it is prevented if explants are incubated in Ca-free medium containing EGTA [67–69]. The notion that this effect is partially mediated by TRPV1 is supported by the fact that a competitive antagonist of TRPV1, namely, capsazepine, diminished CGRP concentrations in eluates quantified by immunoassay or radioimmunoassays [68, 69]. Ruthenium red, a noncompetitive channel blocker of TRPV1, attenuates neuropeptide release in response to capsaicin [67]. In addition, acidic stimulation promotes CGRP release in the nerves and skin through TRPV1-dependent mechanism [70]. Noxious heat (40–50 °C) evokes CGRP release in a calcium-dependent manner, as shown that both incubating in calcium-free medium and skin loaded with (BAPTA) diminished CGRP release [71, 72]. However, it has been shown that neither capsazepine nor Ruthenium Red abolished completely peptide release from nerve and skin explants [71, 73]. It is proposed that other heat-activated channels of TRPV subfamily (V1–V4) might be involved in neuropeptide release from peripheral terminals [71, 73]. This is supported by the fact that neonatal capsaicin denervation does not eliminate all peptidergic fibers in different targets. Likewise, TRPV1 is not expressed by all peptidergic neurons, and its presence in fibers varies with the type of

target [74]. It is noteworthy that TRPV members are coexpressed in DRG neurons and potentially different members may heteromultimerize, contributing to functional heterogeneity and a more complex pharmacology [75–77]. In considering TRPV channels as key elements for regulating peptide release from peripheral terminals, it must be taken into account that these channels are sensitized by vanilloids, temperature, and proinflammatory mediators, which results in distinct biophysical and regulatory properties [78]. TRP participation in peptide release on both patho- and physiological conditions awaits further investigation to define its precise contribution.

Regarding factors coming from a target, there are some inflammatory mediators capable to evoke or sensitize SP and CGRP release in certain tissues and conditions. For instance, bradykinin alone can induce neuropeptide release in the rat trachea and skin and in the heart of guinea pig [72, 79, 80]. Bradykinin evokes a significantly CGRP release only in the trachea, whereas in the skin, it only stimulates release of SP [72, 80]. The effects of bradykinin seem to be mediated through the activation of B_2 receptor which activates phospholipase C, resulting in formation of diacylglycerol and activation of protein kinase C [72, 80, 81]. The sole action of histamine, serotonin, prostaglandin E2, or proinflammatory cytokines seems not to be sufficient to promote exocytosis in peripheral terminals [69, 79, 80, 82, 83]. The action of these mediators is favored by conditions such as acid pH or noxious heator in combination with other inflammatory mediators. The interaction of serotonin and histamine sensitizes bradykinin effect on CGRP and SP release [72, 80]. Near inflammation zones and tumors, leukocytes and thrombocytes produced proinflammatory cytokines. In this regard, stimulation of rat skin from hind paw with IL-1b and TNF-a augmented heat-induced release of CGRP in a dose-dependent manner [82]. As in the case of bradykinin, cytokines activate receptors coupled with kinases which may sensitize heat-activated ion channels by phosphorylation and lead to a major release of peptides [84]. It has also been observed that noceffector activity is also exerted to inhibitory modulation. Plasma extravasation in rat skin, bronchoconstriction of guinea pig and human, and contraction of the left atrium of guinea pig heart are blocked by the presence of nociceptin, an opioid-related peptide [85–88]. These processes require neuropeptides release from noceffector terminals. Indeed, release of substance P and CGRP from rat isolated trachea in response to electrical field stimulation was diminished by nociception [89]. It has been proposed that nociceptin stimulates the G-protein-coupled orphan receptor ORL_1 to activate an inward-rectifier K^+ channel. The latter reduces neuropeptide release from noceffector endings via a membrane hyperpolarization which probably counteracts TRPV1 gating [86]. Likewise, μ-/κ-/δ-opioid receptor agonist inhibited electrical-induced release from noceffector endings in several preparations, although not all agonists are effective in all sites tested [83, 90–93]. The actual effect of endogenous opioids and its physiological relevance for efferent functions remains to be elucidated. Apart from these factors that can be found in most tissues, apparently there are some tissue-specific signals capable to evoke peptide release. That is the case of the conversion of trans-urocanic acid to cis-urocanic acid by ultraviolet radiation in the stratum corneum of the skin. In rodents cis-urocanic acid may increment microvascular blood flow of hind paw and diminished contact hypersensitivity by means of releasing SP and CGRP [94].

As could be inferred for the depleting effects of capsaicin in neuropeptide contents in different preparations in vitro, long-term synthesis of neuropeptides is intimately related with the amount of these neuropeptides that are available for release from the noceffector endings. Several reports indicate that neuropeptide exocytosis can be achieved by two means: local factors that stimulate direct or indirectly TRP channels and antidromically stimulations of peripheral endings which rely in axonal conduction by activation of voltage-dependent calcium channels. Since much of the research has dealt with inflammatory conditions, little is known if the same factors could modulate synthesis and release of

neuropeptides in noninjury conditions. Although capsaicin has helped to elucidate the pharmacology of noceffector terminals, it remains unclear which are the endogenous ligands that have similar effects as capsaicin and the dynamics of production and sources of such TRPV1 agonist in normal and pathophysiological conditions. Only a few molecules such as anandamide, arachidonate, and diacylglycerol have been shown to activate TRPV1 in a capsaicin-like manner [95, 96]. An intriguing issue that deserves further study is the role of antidromic process in vivo. It is known that a suprathreshold stimulus depolarizes primary afferents in the spinal cord (i.e., dorsal root reflex), which could trigger efferent action of noceffector [97]. Furthermore, dorsal rhizotomy, periaqueductal gray matter stimulation, and blockage in the spinal cord of GABAA, non-NMDA, or 5-HT3 receptors interfere with development of neurogenic cutaneous inflammation [98, 99]. This data implies that local mechanisms and/or central nervous mechanisms could modulate exocytotic release at periphery. The understanding of these mechanisms may clarify how noceffectors coordinate normal processes, such as hair growth, bone metabolism, gland secretion, and vascular tone.

5 Efferent Effects of Peptides Released by Somatosensory Nerves on Skin Physiology

Noceffectors establish synaptic-like contacts with Langerhans cells, melanocytes, and mast cells in the skin [7–9]. In other targets like the smooth muscle, epithelium, viscera, lymphoid organs, blood vessels, teeth, and bone, where noceffectors lack specialized contacts, they appear to establish a paracrine way of communication [10, 11, 100]. Cellular elements located in the aforementioned targets not only possess receptors for the peptides released by noceffectors from their C- and Aδ-fiber terminals but also express peptidases that terminate with the biological effects of such peptides [28, 29]. The anatomical and physiological evidence so far summarized suggests that, besides its ability to send information to the spinal cord (afferent role), the anatomical and functional organizations of ganglion sensory neurons render them capable of releasing the content of its vesicles and transmit a specific message to their peripheral targets (efferent role).

The skin receives innervation that originates from DRG and trigeminal ganglion. Nerve plexus of large caliber arrive at the deepest part of the dermis. As nerves ascend through the skin, they ramify in thinner plexuses. At the border between the dermis and the epidermis, individual fibers cross the basal membrane and terminate as free nerve endings in either stratum spinosum or stratum granulosum (Fig. 1). Free nerve endings also innervate structures immersed in the dermis like hair follicles, blood vessels, and sebaceous glands [101–103]. Many of these free nerve endings present immunoreactivity for SP and CGRP, and its distribution within the skin is conserved between individuals of the same species. The presence of SP and CGRP receptors in keratinocytes, fibroblasts, melanocytes, endothelial cells, and immune cells has been elucidated by immunohistochemical studies and functional assays [8, 104, 105].

A long-standing issue in the field of dermatology is related to the observation that cutaneous denervation is followed by trophic changes which are manifested as alterations in skin, nails, and subcutaneous tissues [106, 107]. Not until recent investigation, anecdotal observations have been replaced for a careful quantification of efferent activity of peptidergic DRG neurons. Recently, it has been established that skin noceffector is involved in modulating expression of genes of cytoskeleton, extracellular matrix, transcription factors, proteases, receptors, intracellular transducers, and adhesion molecules [5]. Taken altogether, these findings indicate that noceffector activity influences several kinds of cellular elements in its targets. Therefore, it is conceivable that malfunction of noceffectors may be a causal factor in some dermatological diseases.

In rodents and humans, epidermis becomes thinner after nerve injury [108, 109]. Sciatic nerve transection in rodents diminishes keratinocyte incorporation of analogs of

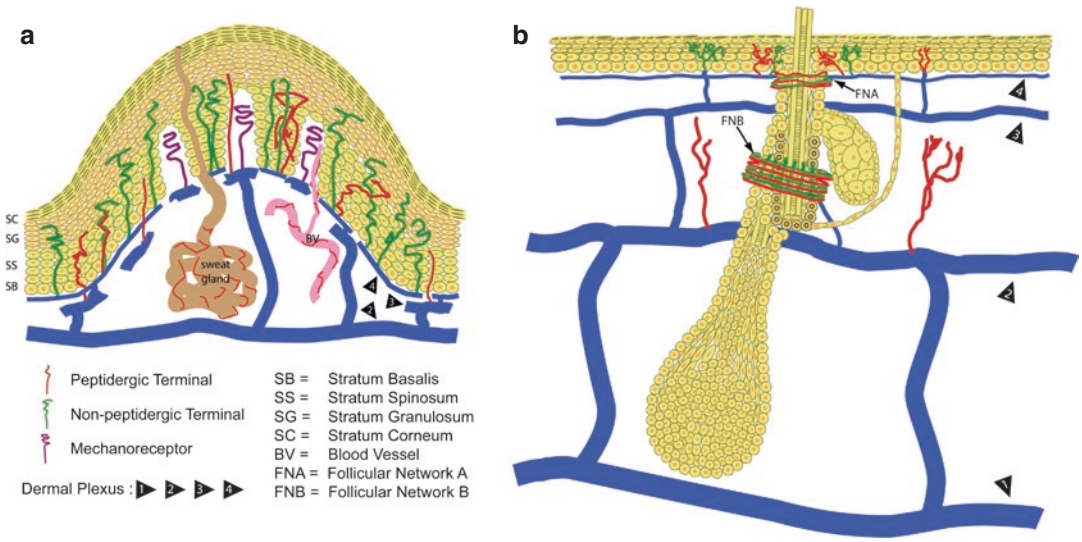

Fig. 1 General arrangement of sensory innervation in mammalian skin. In (**a**) glabrous skin and (**b**) hairy skin axons from dorsal root ganglia are grouped in the dermis as large plexuses. As axons reach the superficial layers of the skin, they travel in smaller plexuses. Sebaceous glands, blood vessels, and epidermis are innervated by ramified terminal fibers

thymidine up to 40% which suggest a reduction of keratinocyte proliferation [109–111]. Both epidermal thickness and proliferation are restored if reinnervation is permitted [108, 110, 111]. Due to alterations in motor innervation also affect keratinocyte proliferation, it is argued that lack of movement rather than neuropeptide secretion from noceffectors is the cause of skin atrophy. Dorsal rhizotomy or ganglionectomy, procedures that conserve normal gait, also produces epidermal thinning. Sensory denervation of dorsal skin, which does not support body weight, produces epidermal thinning [112, 113]. An insight into the mechanism of this phenomenon comes from in vitro and in vivo studies. In cultures of keratinocytes, fibroblasts, and endothelial cells, substance P promotes cell proliferation [114, 115], while CGRP promotes the proliferation of melanocytes and endothelial cells [8].

The hair follicles receive peptidergic innervation which shows immunoreactivity for substance P and CGRP (Fig. 1). Normal hair cycle is accompanied by substantial morphological, cellular, and biochemical changes in many skin compartments, such as changes in the thickness of the epidermis and dermis, reorganization of the skin vasculature and the extracellular matrix, as well as variations in the number and functional activity of major skin cell populations [116]. This tissue remodeling is associated with tightly regulated sprouting and regression of peptidergic fibers. The number of CGRP and SP fibers increases from telogen to anagen in the dermis and subcutis [117, 118]. Peptidergic nerve fibers are concentrated around and above the bulge region where one major population of epithelial hair follicle stem cells resides. Thus, it is conceivable that noceffectors participate actively in hair cycle modulation and concomitant tissue remodeling. SP-releasing microcapsules implanted at resting growth phase of hair (telogen) stimulate growth phase (anagen) in mice skin [119], while treatment with substance P in anagen induces a premature regression of hair follicles (catagen) [120]. CGRP subcutaneous implants failed to promote transition of telogen to anagen [118]. Further investigations are required to define the precise role of the combination of nerve-derived signals in hair cycle.

Overall, the evidence indicates that noceffectors interact with almost all cell populations in the skin. By means of this interaction, the

optimum functioning of major physiological processes that maintain the skin in a healthy state is preserved. Neuropeptide release is involved in modulating epidermal renewal, hair growth, blood flow, and immunological priming. Accordingly, it is not surprising that alteration in the synthesis and release of neuropeptides may result in disturbance of skin homeostasis. That could be the case of some variants of dermatological diseases like atopic dermatitis, psoriasis, urticaria, or vitiligo whose etiology is unknown and sometimes attributed to a neurological origin. A common denominator in these diseases is an elevated number of nerve fibers in the dermis and epidermis containing SP and CGRP compared with healthy skin [121–123]. In addition, more frequent contacts of nerve fibers with mast cells and blood vessels are observed [124, 125]. Until now little is known if peripheral nerve fiber sprouting responds to a diminishing in peptide release which in turn evokes secretion of neurotrophic signals from a target organ. For instance, keratinocytes in psoriatic lesions have reduced expression of the transcription factor Jun B with concomitant augmented levels of mRNA of two chemotactic proteins, S100A8 and S100A9, which are involved in the onset of psoriasis [126]. Remarkably, sensory denervation leads to an upregulation of S100A8 and S1009 genes [5]. Likewise, psoriatic lesions contain an increased number of keratinocytes expressing NGF, whose synthesis is promoted by neuropeptide release [105, 127].

6 Role of Peptidergic Nerves on Epithelial Renewal and Wound Repair

To get a better understanding on how sensory nerves influence epithelium physiology, we performed a series studies using neonatal capsaicin treatment. This chemical denervation model allowed us to reduce the amount of peptidergic terminals in the skin and to determine whether the reduction of peptidergic terminals affects epithelial homeostasis both in noninjury conditions and during wound repair [128, 129]. We employed design-based stereological methods to assure an unbiased quantification of biological structures (Fig. 2). Most of the dermatological research has relied on qualitative or 2D sampling which may overestimate or underestimate the magnitude of a certain cellular responses. For example, the data of cell number is usually expressed as a ratio quantity (i.e., cell/unit area) which can be misinterpreted if the reference space is not the same between experimental conditions. In contrast, stereological estimations of the number, length volume, or area of biological objects are performed by a systematic random sampling without any assumption of spatial distribution, size, shape, and object orientation. Stereological probes facilitate the comparison of experimental conditions by expressing the data of measured parameters as an absolute quantity (i.e., millions of cells). Rather than to offer a guide on how to design a stereological study, the main intention of this section is to show how this methodology was used to study the role of innervation during wound healing [130–132].

Although it is well-known that neonatal capsaicin treatment eliminates a great number of DRG neurons with C- and Aδ-axons, there was no quantitative data about the repercussion of capsaicin treatment on the development of epidermal innervation. For this purpose, we quantified the amount of intraepidermal nerve fibers (IENF) immunoreactive for protein gene product 9.5 (PGP^+) and calcitonin gene-related peptide ($CGRP^+$) in the glabrous skin of the rat [129]. In control animals, the total estimated length of PGP^+ and $CGRP^+$ fibers remained relatively constant at 1, 3, and 6 months. These findings suggest that nerve supply generated during development is only redistributed as animal ages. Moreover, we also observed changes on IENF morphology which indicate that nerve fibers undergo continual remodeling over time (Fig. 3). Accordingly, the arborization and location of sensory endings in the mouse cornea showed substantial changes over

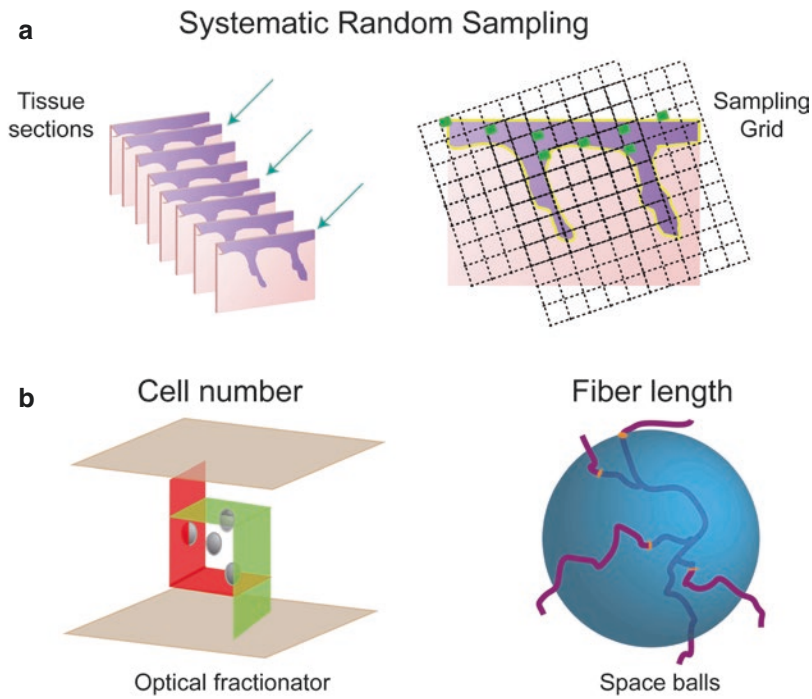

Fig. 2 Stereological quantification of cell number and fiber length. Reliable and unbiased estimates of volume, number, area, and length of biological objects are obtained by design-based stereology methods. (**a**) Sections and counting sites are determined by a systematic random sampling which assures a representative sampling through the analyzed area. (**b**) The estimation of the total number of cells is performed by counting the cells inside a virtual box or optical dissector. The fiber length is obtained by counting the intersections of the nerve fibers with a stereological probe called space balls. Both procedures require thick tissue sections (>20 mm)

a 1-month period [133]. Capsaicin treatment reduced the total length of PGP^+ fibers on average by 80%, and that of $CGRP^+$ fibers was reduced by 55%. While IENF showed an intricate morphology in control rats, the nerve endings in the epidermis of treated animals had a straight thick morphology and were poorly ramified. Despite the reduction of the nerve supply to the epidermis, the keratinocyte proliferation was not altered in capsaicin-treated rats. Interestingly, the quantitative analysis of IENF on capsaicin-treated rats revealed that peptidergic fibers were the predominant type of fibers in the epidermis as was also confirmed by a double-immunofluorescence staining for CGRP and beta III tubulin (Fig. 3). Thus, we hypothesized that the remaining peptidergic innervation is sufficient to maintain adequate epithelial renewal in noninjury conditions, but in conditions of high cell demand, denervated epithelia are not able to generate the number of cells required for epithelial expansion.

Until recently, all efforts to show a beneficial action of sensory nerves during skin wound repair have been limited to document the impact of denervation upon the time of wound closure [134–138]. Since the discovery of adult stem cells in different parts of the body, it became clear that the innervation is an essential part of the stem cell niche. Little is known about the exact interaction between neurons and progenitor cells. By using the neonatal capsaicin denervation, we explored whether sensory innervation was involved in the modulation of the epithelial progenitors that participate in reepithelialization of the hairy skin [128]. The hair follicle is an

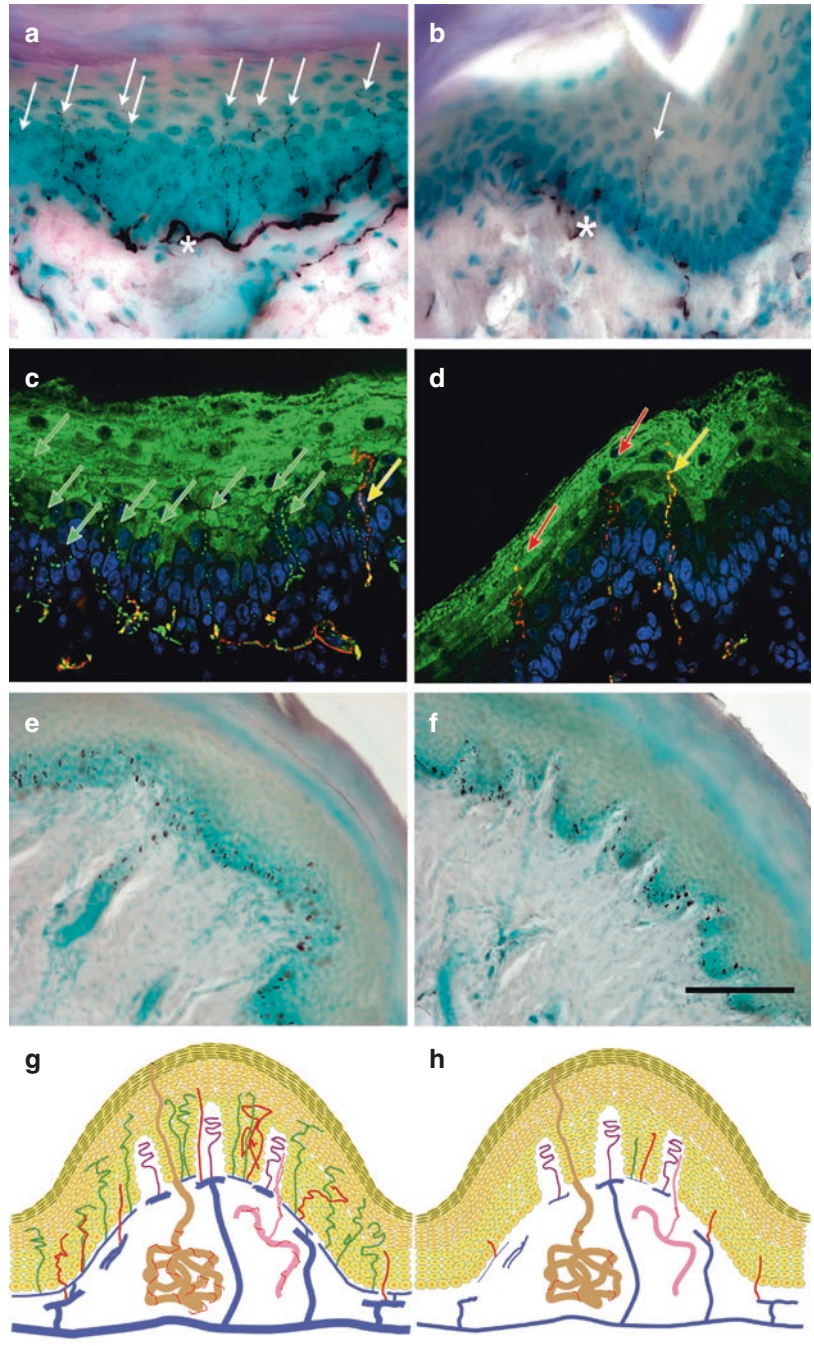

Fig. 3 Effects of neonatal capsaicin treatment on skin innervation. (**a**) Glabrous skin sections were immunostained for protein gene product 9.5. (**b**) Capsaicin treatment decreased the number of intraepidermal nerve fibers and fiber complexity. While in (**c**) control rats the most abundant type of nerve fibers was of non-peptidergic type (green arrows: immunoreactivity for beta III tubulin), (**d**) the epidermis of treated rats showed almost exclusively peptidergic fibers (red and yellow arrows: immunoreactivity for CGRP). Despite the reduction of intraepidermal nerve fibers in capsaicin-treated rats (F), the keratinocyte proliferation was similar in (**e**) control and (**f**) treated rats. (**g**) Sensory innervation in the epidermis of glabrous skin of control rats. (**h**) Summary of the changes induced by neonatal capsaicin treatment. Scale bar = 150 mm

excellent model to study the signals and mechanisms that may govern the neural modulation of stem cells. Based on the anatomical location of sensory fibers in the bulge region of the hair follicle, we evaluated the possibility that nerve-derived signals may influence the activation or migration of epithelial progenitors (Fig. 4). During the first 47 h post-wound, the epidermal proliferation was reduced in the capsaicin-treated rats, while the proliferation in the hair follicles was the same as in control rats. To determine if the low number of

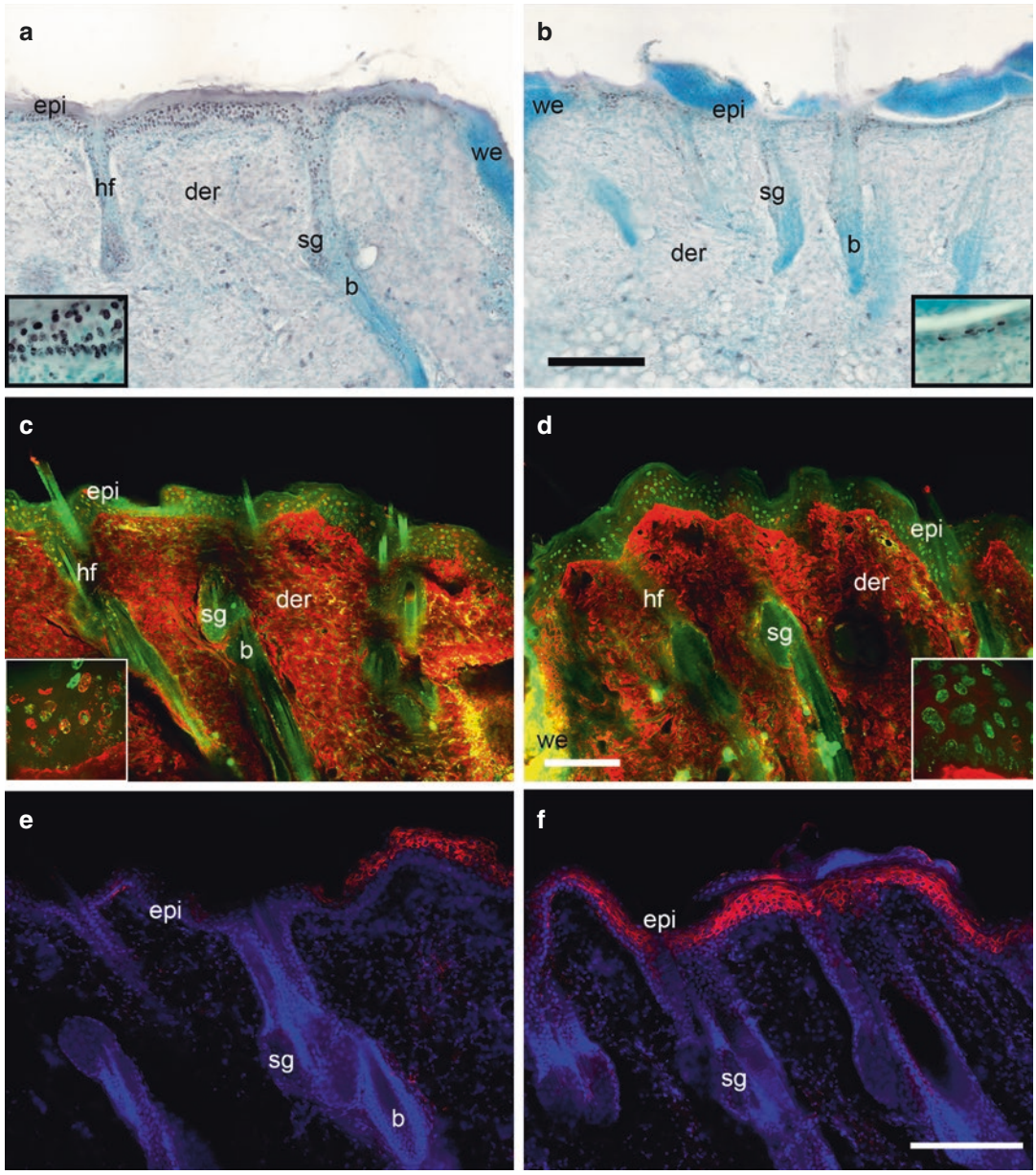

Fig. 4 Effects of capsaicin treatment on wound healing. After 47h after wounding, the epidermis of (**a**) control rats was thicker and showed more $BrdU^+$ nuclei than (**b**) capsaicin-treated rats. At 61h after wounding, the epidermis of (**c**) control rats presented more IdU/CldU labeled nuclei than (**d**) treated rats, which suggest that denervation is related to less migration of stem cell progeny from the hair follicle. Noteworthy, at 61 h after wounding, we observed an increased area of epidermis expressing keratin 6 in (**f**) capsaicin-treated rats than in (**e**) controls. *epi* epidermis, *der* dermis, *sg* sebaceous gland, *b* bulge, *we* wound edge, *hf* hair follicle. Scale bar = 200 mm. Modified from [128]

bromodeoxyuridine-positive cells ($BrdU^+$) in the epidermis of treated rats resulted from a reduced mobilization of transit amplifying cells from the hair follicle, we performed pulse and chase experiments with halogenated thymidine analogs (iododeoxyuridine, IdU; chlorodeoxyuridine,

CldU). This procedure is based on the principle that cells in the hair follicle proliferate faster than the cells in the epidermis allowing to track the fate of the double-labeled cells in different skin compartments. Remarkably, the proportion of $IdU^+/CldU^+$ cells in the epidermis increased over time only in the control group. This finding suggests that the deficiency of sensory nerves hampers the traffic of cells from the follicle toward the epidermis. Although it has been shown that cells from the hair follicle are dispensable for reepithelialization, the migration of these cells accelerates the reestablishment of the epidermis [139, 140]. In capsaicin-treated animals, the efflux of hair follicle cells is diminished which correlates with an extended time for wound closure. Moreover, treated rats showed an extended recruitment of epithelial precursors as indicated by the broader area of epidermis expressing keratin 6, a marker of epidermal activation. Our results revealed that epithelial precursors must migrate more distance to reach the border of the wound in denervated rats. Taken together, our findings may explain the delay in reepithelialization observed in several models of denervation. From a clinical perspective, it would be desirable to understand the mechanism and signals behind the activation of distinct regions of the epithelium to better contend with chronic wounds. In this regard, it is noteworthy that the stem cell niche of the bulge showed the presence of receptors for substance P and CGRP (Fig. 5).

Conclusions

This chapter summarizes the evidence that sensory neurons of dorsal root ganglia are not restricted to transmit information toward the central nervous system. These neurons are thought to be crucial participants in the maintenance of tissue integrity and functionality. Nevertheless, we are just glimpsing the potential of neurosecretory function of the so-called sensory neurons for body health. Perhaps the notion that these neurons are merely transducers of noxious information has delayed advancement toward the understanding of efferent functions. Moreover, it is frequently assumed that peripheral release of neuropeptides is restricted to a noxious condition just because at spinal cord level, neuropeptides serves as cotransmitters of painful transmission. This view, however, responds in great extent to technical limitations for recording peripheral activity. Therefore, this field awaits for future improvement in procedures to investigate peripheral release in more physiological terms. This issue is extremely important because the available preparations only permit to study local factors that regulate noceffector activity and overlook systemic factors which may be more important during normal conditions. Although tissue homeostasis does not rely entirely on noceffectors, they seem to be an essential component because different cell populations express receptors and degradatory enzymes for neuropeptides. Accordingly, alterations in the communication between noceffectors and peripheral targets could lead to a variety of functional modifications in the innervated target. Although, at first glance, it could be considered that neuropeptide effects on different organs are non-related with each other, we think that such effects must be the manifestation whereby the brain interacts with the body regulating central issues for its homeostasis both in health and disease.

Regarding wound healing, dorsal root ganglion neurons are involved in processes such as reepithelialization, angiogenesis, and inflammation. Here we described a mechanism based on neural regulation of epithelial SC physiology. Peptidergic neurons seem to promote the mobilization of stem cell progeny from the hair follicle and to modulate the activation of epidermal progenitors (Fig. 6). The myriad of nerve-derived signals is not limited to neuropeptides. Both sympathetic and sensory neurons could act in concert to regulate diverse aspects of adult stem cells [141, 142]. Next research efforts should reveal the molecular pathways that the nervous system modulate to understand how neurons regulate the activation and differentiation of SC in different niches of the body and its possible implications for tumor formation.

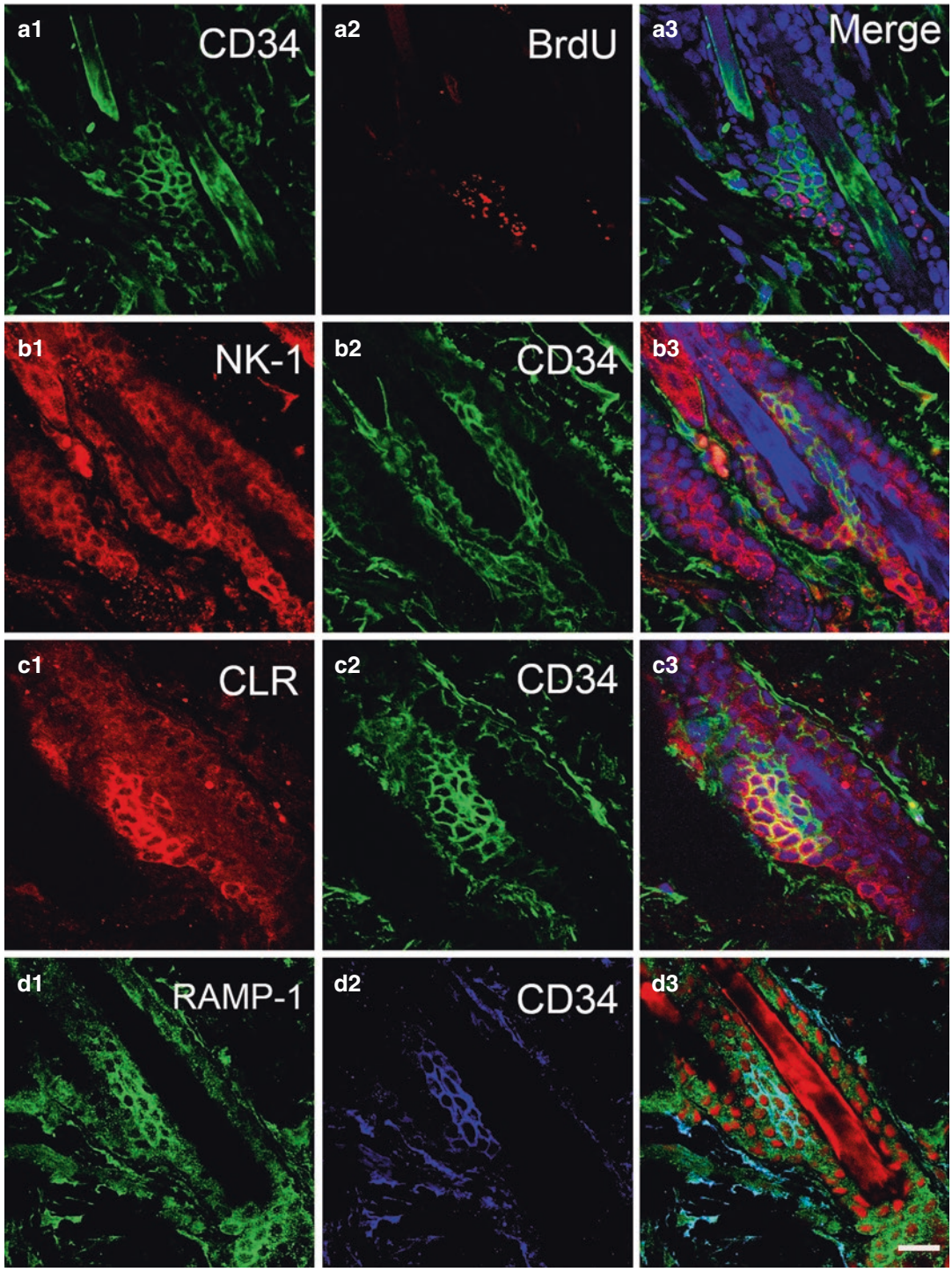

Fig. 5 Neuropeptide receptors in the bulge region of the hair follicles. (**a**) Label-retaining cells were found in the bulge region of the rat hair follicles after 8 weeks of BrdU pulses in a region displaying expression of CD34. (**b**) By confocal microscopy, we found that (**b**) substance P receptor (NK-1) and (**c**, **d**) CGRP receptor (CLR and RAMP-1) were expressed by stem cells from the hair follicle. Scale bar = 20 mm. Modified from [128]

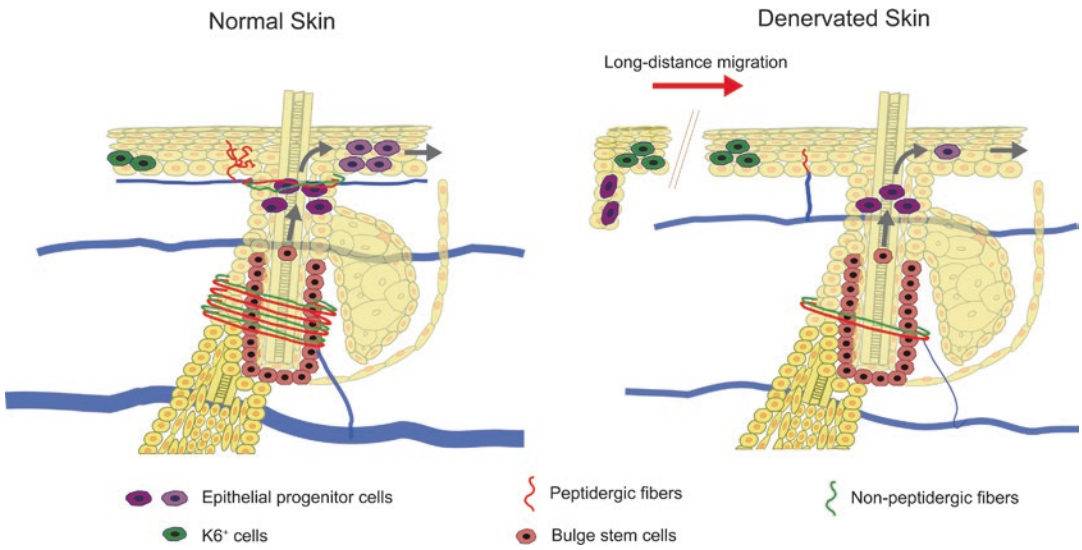

Fig. 6 Wound model explaining the effector function of sensory nerves. (Left) In normal skin, epithelial progenitor cells are activated both in the epidermis and the hair follicles which migrate toward the wound edge to promote reepithelialization. (Right) In partially denervated skin, there is less migration from stem cell progeny from the hair follicles toward the epidermis. Late on time, there is a recruitment of epidermal progenitors far from the wound edge. The lateness of this event and the longer distance of migration by epithelial progenitors to reach the wound edge may explain the delay in wound closure commonly observed in different denervation models

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