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Evaluating dermal myelinated nerve fibers in skin biopsy

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Abstract

Although there has been extensive research on small, unmyelinated fibers in the skin, little research has investigated dermal myelinated fibers in comparison. Glabrous, non-hairy skin contains mechanoreceptors that afford a vantage point for observation of myelinated fibers that have previously been seen only with invasively obtained nerve biopsies. This review discusses current morphometric and molecular expression data of normative and pathogenic glabrous skin obtained by various processing and analysis methods for cutaneous myelinated fibers. Recent publications have shed light on the role of glabrous skin biopsy in identifying signs of peripheral neuropathy and as a potential biomarker of distal myelin and mechanoreceptor integrity. The clinical relevance of a better understanding of the role of dermal myelinated nerve terminations in peripheral neuropathy will be addressed in light of recent publications in the growing field of skin biopsy.

Keywords

glabrous skin biopsy; myelinated nerve fibers; molecular architecture; Meissner corpuscles; peripheral neuropathy

Introduction

In the peripheral nervous system (PNS), nerves may be classified as small caliber unmyelinated fibers with axons encircled by a single layer of non-compact Schwann cell membrane or different caliber myelinated fibers with axons encased by multiple layers of compacted Schwann cell membrane to form compact myelin. Molecules on myelinated nerve fiber membranes are highly compartmentalized to form molecular architectures within 4 discrete compartments: the node of Ranvier, the paranode, the juxtaparanode, and the internode, each of which contains a specialized, non-overlapping set of protein constituents.[1, 2] Voltage-gated sodium channels are highly concentrated at the node of Ranvier adjacent to the paranodal region, which is comprised of loops of Schwann cell membrane and interacting axolemmal protein (Contactin-associated protein, Caspr) and Schwann cell protein (Connexin 32). Both CASPR and Connexin 32 participate in Schwann cell-axon interactions and electrically isolate the nodal region. The juxtaparanodal region, the portion of myelin and the interacting axolemma adjacent to the paranode, contains voltage-gated potassium channels (Kv1.1 and 1.2) on the axolemma. The internodes between 2 nodes contain the myelin structural proteins myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22), and myelin basic protein (MBP), which participate in

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forming the tightly-compacted myelin sheath, thereby reducing the electrical capacitance of the internode.

Diseases of the PNS (peripheral neuropathies) may predominantly perturb either myelin (de-/dysmyelinating neuropathies) or axons (axonal neuropathies). These disorders often disrupt the molecular architecture of the myelinated nerve fiber axolemma including, but not limited to, delocalization or abnormal subtype expression of voltage-gated ion channels,[3, 4]under-/over-expression of compact myelin proteins,[5] and septate junction protein abnormalities with myelin decompaction[2]. In the past, morphological and molecular changes of myelin and axons in humans have been observed through sural nerve biopsies[6] or occasionally through nerves taken at autopsy.[7] Knowledge from these studies has significantly advanced our understanding of normal physiological functions of the PNS and the pathogenesis of peripheral nerve diseases while aiding in their diagnosis.

Recently, skin biopsy has emerged as an alternative approach to assess pathological and molecular information in the PNS. Skin biopsy is a minimally invasive procedure in which a small 2-5 mm disposable punch is used to painlessly excise an anesthetized section of skin. It has become a valuable tool in the diagnosis and monitoring of small fiber sensory neuropathy, which preferentially affects unmyelinated fibers and is most commonly sampled from the hairy skin of the leg.[8] In comparison, cutaneous myelinated fibers have been much less explored, although numerous neuropathies are known to involve such distal myelinated nerve fibers. Recent studies have begun evaluating dermal myelinated fibers from glabrous skin with a variety of methods in healthy control populations[9-11] and in patients with peripheral neuropathies.[12-16] In this review, we will discuss the development of skin biopsy for investigation of normal and diseased dermal myelinated nerves. We highlight recent key publications to our best knowledge and discuss further applications of skin biopsy in light of such work in order to better understand the utility of the procedure and the extent of pathology on these most distal myelinated fibers.

Cutaneous neuroanatomy

Small fibers

The underlying structures of the skin include a variety of both autonomic and sensory organs innervated by axons of varying calibers that can be functionally differentiated (Fig. 1). Free nerve endings, which are found in both the dermal and epidermal layers, are small caliber fibers that convey thermal stimuli in addition to noxious stimuli. Thinly myelinated (Aδ) or unmyelinated (C) fibers branch off from the subepidermal neural plexus and either terminate in the dermis or penetrate the dermal-epidermal basement membrane and course between the keratinocyte cells of the epidermis as unmyelinated fibers.

Large fibers

Mechanical sensory perception, in contrast, is conveyed by large, myelinated Aβ caliber fibers, which innervate cutaneous mechanoreceptors (Table 1). These thickly myelinated afferents in the reticular and papillary layers of the dermis have different principal sensory terminals in hairy skin compared to those in glabrous (non-hairy) skin (Fig. 1). In hairy skin, Aβ fibers innervate vascular structures like arteriovenous anastosomes (AVAs) and hair follicles that act as mechanical sensory receptors by detecting changes in hair position. Myelinated fibers are much more abundant and homogenous in glabrous skin because of the increased numbers of mechanoreceptors found only in glabrous skin (Fig. 2).[9, 11]

Mechanoreceptors

Meissner corpuscles (MCs) and Merkel complexes are 2 mechanoreceptive sensory end terminals of myelinated afferents that are located respectively in the apex and the base of dermal papillae in the subepidermal region immediately below the basement membrane. MCs respond to low-frequency stimuli and convey tactile sensations of vibration and light touch. They are found exclusively in glabrous skin in a decreasing distal-proximal gradient. Thus, higher densities are observed in the fingertip, for instance, compared to more proximal locations on the palm.[17]

Intrapapillary myelinated endings (IME) are the large, thickly myelinated fibers that typically furnish between 1 and 3 MCs per dermal papilla. They can be easily quantified on account of their regular vertical courses to the apex of dermal papillae, where they may innervate several relatively large corpuscles (30 × 80 μm).[9] It is generally accepted that Schwann cells lose the ability to myelinate as axons enter the receptor corpuscle; however, recent studies in human[18] and primate digits[19] report MBP immuno-reactivity within the corpuscle. About 25% of the MCs from human digital skin contained MBP-immunoreactive nerve endings.[10] Even so, they rarely appear to reach the apex of the papillae and purportedly do not actually supply the corpuscle. To the best of our knowledge, subsequent investigation of this discrepancy has not been reported in humans.

Similar large myelinated fibers can be found terminating at the base of dermal papillae in the disk-like endings of Merkel cell-neurite complexes. These slowly-adapting mechanoreceptors convey prolonged sensations of touch and pressure and are found near the bases of hair follicles in hairy skin and the bases of dermal papillae in glabrous skin. The quantification of MC afferents as an indicator of dermal fiber density has proven more suitable than those of Merkel complexes, in part because MCs are more abundant and regularly spaced with long, linear axonal paths compared to the folded, sigmoidal paths of fibers that furnish Merkel cells.[9]

Pacinian corpuscles and Ruffini endings are also low-threshold mechanoreceptors located in glabrous skin, yet they are typically not visualized through common procedures of skin biopsy due to their position in the deep dermis and subcutaneous tissue. Biopsies are typically collected at a relatively shallow depth in order to avoid severing larger subcutaneous blood vessels in the vicinity of these mechanoreceptors. In recent postmortem studies, however, Pacinian corpuscles and their afferents have been characterized in primates[19] and in the amputated digits of humans.[10]

Resident Cells of the Skin

Cell types in the skin that are implicated in conveying sensory information include Merkel cells, terminal Schwann cells, and basal keratinocytes in the epidermis. Merkel cells are scattered in the basal layer of the epidermis as well as the root sheath of hair follicles.[20] The function of the Merkel cell-neurite complex is not completely understood, as it is either a mechanoreceptor itself or else plays a modulatory role in the sensory function of neurons.[21] Interestingly, unlike epidermal Merkel cells, those located in the outer root sheath of hair follicles express the capsaicin receptor TRPV1 (transient receptor potential vanilloid 1) and the neurotransmitter Substance P. They express multiple glutamate receptors and neuropeptide receptors[22] in addition to P/Q type voltage –gated calcium channels but lack synaptic connections and neurotransmitters.[20]

Basal keratinocytes in the epidermis are the most plentiful cell type in the skin and have also been shown to express TRPV1 as well as the vanilloid thermoreceptors TRPV3 and TRPV4.[23] Mechanical stress on keratinocyte cultures increases intracellular calcium levels which can be blocked by a TRP antagonist, suggesting a role of TRP in signal

transduction.[24] Keratinocytes also exhibit a trophic effect in cell culture models of sensory neurons.[25] Keratinocytes may have a role in pain signal transduction as well as nerve regeneration, although the mechanism of communication with peripheral dermal nerves is still unknown; thus, direct evidence is still lacking.

Melanocytes, which produce photoprotective melanin, are also in close contact with sensory endings, and electron microscopy studies support a possible synaptic communication.[26, 27] Langerhans cells are antigen-presenting cells in close proximity to Merkel cells. They do not express TRPV or TRPM (transient receptor potential melastatin) channels but are sensitive to thermal stimulation.

Terminal Schwann cells are present in the skin as modified Schwann cells that comprise the capsules of MCs. Both Schwann cells that make up the tightly wrapped compact myelin formed along dermal myelinated nerves, and those that form the noncompact single encirclement of small fibers are S100 immunoreactive. Compact myelin may be distinguished by the staining of compact myelin proteins such as Myelin basic protein (MBP). Schwann cells can also be identified using an anti-p75 nerve growth factor receptor antibody or a neural cell adhesion molecule (NCAM/CD 56), both of which allow for the evaluation of decreases in terminal Schwann cells in patients with neuropathy.[28, 29]

Technical considerations for skin biopsy procedure

Punch Biopsy

In myelinated fiber investigations, punch biopsy is typically applied to glabrous skin, such as the lateral aspect of the finger,[30] fingertip[9] or palm.[17] Although the sole of the foot is also a potential site, fear of infection and prolonged recovery time are logistical concerns, particularly in patients with neuropathy. The fingertip is a highly sensitive place, and biopsies in the wrist and forearm presumably provide a lesser chance to see Meissner receptors. As a compromise, our lab prefers to do the procedure on the lateral aspect of the finger between the first and second interphalangeal joints with a small diameter punch (2 mm) positioned at least 2 to 3 mm from the border of hairy skin.[30] The drawback to this method is that there is the potential for injury to the digital nerve;[31] however, this has not occurred in our experience. Biopsies are collected at a depth of approximately 3-5 mm under sterile conditions after intradermal injection of local anesthetic (i.e. xylocaine or lidocaine), and at this depth, injuries to the digital nerve are unlikely. Wounds heal within 1 week without the need of sutures, and over time, only slightly visible scarring is evident. The procedure is well-tolerated by patients. Although the infection rate in glabrous skin biopsies has not been investigated on a large scale, it is most likely comparable to the relatively low rate observed in skin biopsies from the lower extremity 1.9:1000.[32]

Unique aspects in dealing with skin biopsies

Myelinated nerve bundles located deep in the dermis travel roughly parallel to the surface of the skin, with individual myelinated fibers branching off more superficially in the dermis where they course perpendicular to the skin’s surface to innervate mechanoreceptors and terminate in dermal papillae (Fig. 2).[33] Unlike teased fiber preparations, capturing dermal fibers requires taking into account the undulating course of fibers throughout the biopsy. Combining a z-stack of images taken in small increments throughout thick sections allows three-dimensional visualization of large cutaneous structures. Thus, MCs and fibers spanning multiple internodes may be projected wholly in focus, enabling investigators to quantify the morphometrics of dermal myelinated nerves. Subsequent tissue processing procedures may be selected based on the method of visualization required to address a particular scientific question.

Skin biopsy and gene expression

Real-time polymerase chain reaction (rtPCR) has been used to evaluate expression levels of different genes in skin biopsies at the transcriptional level.[30, 34, 35] Increased cytokine gene expression has also been recently demonstrated in hairy skin biopsies from patients with small fiber polyneuropathy and chronic inflammatory demyelinating polyneuropathy (CIDP).[36, 37] When studying neural specific proteins such as PMP22, it is likely that the mRNA transcripts obtained are neural specific. However, this technique is limited in that mRNA expression may be largely derived from numerous non-neural cells, especially given the small fraction of neuronal tissues present in skin. Several technical caveats may limit the reliability of this approach, including the standardization of mRNA levels, secondary effects from axonal loss, and the aforementioned majority of non-neuronal cell types.

Immuno-electron microscopy (immuno-EM) is an alternative approach to quantifying protein expression in skin biopsy that allows investigators to avoid these difficulties and localize proteins to areas of compact myelin. Although technically challenging, quantification of proteins specific to myelin may be expressed in the number of conjugated particles per mm^2 of myelin analyzed. Multiple proteins may be studied in the same nerve fiber by using species-specific secondary antibodies conjugated with gold particles of distinctly different sizes. This avoids any secondary effect of axonal degeneration that would decrease levels of any gene expression in neural tissues and would be difficult to exclude with real-time PCR. Using this technique, PMP22 expression has been shown to be increased and variable in patients with Charcot-Marie-Tooth disease type 1A (CMT1A) and decreased in patients with hereditary neuropathy with liability to pressure palsies (HNPP).[30] There are significant technical barriers to immuno-EM that include difficulties with trimming away the large portion of non neural tissue in the skin in order to focus on myelinated nerve fascicles. Furthermore, tissue preparation procedures may be deleterious to antigens.[38]

Meissner corpuscle observations and suggestions as a neuropathy biomarker

Aβ fibers lose their myelin sheaths as they enter MCs and course between modified Schwann cells, outlining the lamellar structure of the corpuscle in addition to dense small fiber innervation;[39] thus, MCs have been described as polyinnervated structures with nociceptive peptidergic and nonpeptidergic C fiber innervation in addition to the large Aβ afferents responsible for mechanosensation.[40] A number of investigators[12, 14, 16, 19, 41] have made observations of reduced densities of MCs with persisting receptors exhibiting disease-related neuropathy and marked atrophy (and less frequently, hypertrophy). Such abnormalities are described as a range of anomalies in size, orientation, and lamellar structure. MC density has been suggested to be a potential biomarker of peripheral neuropathy[42] on account of the susceptible extreme distal location of corpuscles, yet typically abundant and homogenous distribution in the glabrous skin of healthy controls.[9] Care must be taken in sampling, because marked reductions and inconsistencies in size and distribution have also been noted as a result of aging.[43] Significant discrepancies were reported, for example, in a comparison of a 76 year-old woman (3.1 MCs/mm^2), a 43 year-old man (7.3 MCs/mm^2), and a 4 year-old (49.3 MCs/mm^2).[42] Nolano et al.[9] reported the density of MCs in fingertip biopsies of healthy volunteers between the ages of 22 and 53 years (mean, 33.7 ± 9.2 years) to be 33.02 ± 13.2 MCs / mm^2, with a mean density of their myelinated afferents, designated intrapapillary myelinated endings (IME), to be 59.0 ± 29.3 fibers/ mm^2. Reports of gender differences in MC density may be attributed to a differences in finger surface area between men and women rather than an innate gender difference in tactile sensitivity.[44] It should be noted, however, that some investigators have not seen a

significant correlation between either gender and MC[45] or fiber density.[46] This is an important issue that warrants further investigation.

Abnormalities in myelinated Aβ fibers in more proximal sensory nerves (i.e. sural, radial, ulnar) may be detected by nerve conduction studies.[47] Electrophysiologic correlations are observed in MCs by eliciting tactile responses on the fingertip with a square-wave generating vibrator and recording near nerve sensory responses.[41] Correlations were found between evoked tactile responses and electrical potentials of areas with normal MC density, and conversely, no correlations were found between abnormal corpuscles and tactile responses.[41] These findings support the role of MCs in detecting tactile sensations like vibration. In addition, morphological abnormalities of MCs correspond to mechanoreceptor dysfunction evidenced by reduced tactile responses recorded in the same area before biopsy in patients with Freidrich ataxia and diabetes.[13] Interestingly, MC density has been reported to parallel epidermal fiber reductions in patients with a sensory neuropathy presumed to be restricted to small fibers only.[48] Skin biopsies from patients with systemic sclerosis revealed a reduction of MCs and marked denervation of both sensory and autonomic fibers, indicating the previously unknown involvement of both large and small fibers.[16] In spinobulbar muscular atrophy patients, there was a similar reduction in MCs and a widespread loss of small myelinated and unmyelinated autonomic and sensory fibers, even in seemingly unaffected skin.[13] Sensory deficits were also evidenced in the glabrous skin biopsies of patients with Parkinson disease.[49] Together, these findings illustrate the importance of reevaluating biopsies in regards to large fibers rather than quantifying small sensory fibers alone. Due to the extreme distal location of dermal myelinated fibers, more subclinical findings may surface in a number of neurological disorders.

Quantification of MC density has also been achieved *in vivo* by noninvasive reflectance confocal microscopy (RCM). This has been done at the distal phalanx of digit V and the thenar eminence in small samples of healthy controls, patients with HIV,[50] and inherited neuropathy patients with CMT1A.[51] A strong correlation was found between MC density and touch-pressure thresholds at the thenar eminence ($r= -0.77$; $r^2 = -0.59$) as well as a trend toward correlations with vibration threshold.[51] Densities calculated through this method appear to be significantly lower than those calculated by histology in the same location,[9, 52] suggesting further work is needed to compare findings between *in vivo* confocal microscopy and biopsy samples to validate this method. However, its noninvasive nature makes RCM appealing as a possible sensory measure for neuropathy patients.

A large array of receptors, including those associated with nociception and growth factors, are present in myelinated and non-myelinated nerve fibers and mechanoreceptors.[40, 53] Neurotrophin 3 (NT3) and brain-derived neurotrophic factor (BDNF) are trophic factors that support the health of Merkel complexes and MCs, respectively.[54] Animals with genetic mutations which prevent BDNF expression have been shown to have severe developmental deficits in MC formation in digital glabrous skin.[53] The lamellar cells of human MCs have been shown to express BDNF colocalized with S100 as well as the transmembrane tyrosine kinase receptor TrkB, a high-affinity receptor for BDNF.[55, 56] A three-fold decrease in TrkB expression was shown to coincide with the well known reduction in corpuscle density as a result of aging.[55] Furthermore, reductions of BDNF and other trophic factors have been reported in a patients with neuropathy,[57] leading to an interesting question of whether the dysregulation of growth factors required for the normal function of large diameter fibers and mechanoreceptors could be correlated with cutaneous pathology from glabrous skin biopsies in patients with peripheral neuropathy.

Compartments of dermal myelinated nerve fibers and their pathogenic aberrations

Like all myelinated nerve fibers, dermal myelinated fibers are highly compartmentalized into discreet regions with unique protein compositions. Exploiting this fact with immunohistochemical staining allows for differentiation and morphological analysis of such regions as the node, paranode, juxtaparanode and internode, which appear to be similar in structure as well as composition in both the skin and in more proximal nerve bundles.[30, 58] The following is a review of the efforts that have been made to characterize these compartments of dermal myelinated fibers in order to better understand normative parameters and the presence of disruptions frequently seen in a number of peripheral nerve diseases and animal models of demyelinating neuropathy.

Internodal length

During development, myelinating Schwann cells migrate along the axons, make contact with the axon, and elongate longitudinally to form individual internodes. Large diameter axons are typically wrapped by thicker myelin and form longer internodes; thus, while axons become smaller in their diameters toward their terminals, internodes become shorter.[59] Data from human skin biopsy studies reflect this expected anatomical gradient.[60] For instance, internodal measurements of distal fibers in the glabrous fingertip (79.1±13.8 μm)[9] are shorter than those in the proximal phalanx (94.5±28.6 μm),[14] also see Table 2.

Provitera et al.[11] compared morphometrics of glabrous skin and hairy skin, and found similarities in internodal length, yet slight anomalies were found in regards to axon diameter and nodes (e.g. slightly thicker axons and shorter nodes in glabrous skin). This is likely due to anatomical differences necessitating differing nerve courses rather than evidence of structural differences between the fibers of different skin types.[11] Even so, hair follicles are the primary mode of mechanosensation in hairy skin at the depth of skin biopsies, such that sampling of myelinated fibers from hairy skin biopsies is difficult due to the relatively distant spacing of follicles, particularly in comparison to the ease of sampling such fibers innervating the much more dense population of mechanoreceptors in glabrous skin (Fig. 2).

Skin biopsy offers an exceptional opportunity to study internodal architecture in humans, as it is a minimally invasive outpatient procedure. Skin biopsy is typically sectioned perpendicular to the skin surface to reveal internodes from a similar vantage point as previously seen only with teased nerve fiber preparations of the sural nerve. Due to the shorter length of dermal internodes, even the small area of a skin biopsy section can capture multiple sequential internodes.[11] For this reason, segmental demyelination can be identified using this approach as well.[14]

It is possible that using the finger as a biopsy site may have less pathological findings in patients who lack significant electrophysiological findings in the upper extremities. However, our experience in patients with CMT and diabetic neuropathy suggests that this site is distal enough to demonstrate abnormalities even when there are minimal exam findings in the hands.[14, 61] Furthermore, it avoids the difficulty in sampling that may be encountered in cases of severe neuropathy where no fibers are present in the legs or feet. Future studies that apply this technique to demyelinating neuropathies such as CIDP or Guillain-Barré syndrome (GBS) would be informative.

A number of pathogenic mechanisms may cause shortened internodal length, including remyelination resulting from primary axonal degeneration or primary demyelination[62] and developmental defects of myelinating Schwann cells in internodal lengthening.[63] Differentiating the mechanism is feasible in acquired conditions, since it is known that a

developmental problem is not present. Thus, shortened internodes are only related to the event of remyelination. However, this issue becomes complicated in inherited neuropathies (e.g. CMT), in which both a developmental defect and remyelination may play a role. Moreover, the chronic nature of CMT with insidious demyelination could make detection of segmental demyelination more difficult.

Our lab has investigated internodal length in patients with CMT1A using skin biopsies. We have found dermal internodes to be shortened compared to healthy controls as well as compared to patients with the primarily axonal type of CMT (type II) (Fig. 3).[14] Interestingly, segmental demyelination was absent in the dermal nerves of CMT1A patients, while it was detectable in patients with CIDP by the same technique. This finding has led us to hypothesize that shortened internodes in dermal myelinated nerve fibers are caused by a developmental defect of internodal lengthening of Schwann cells in the patients with CMT1A. Because segmental demyelination is a known feature in sural nerve biopsies from patients with CMT1A, this finding in skin biopsies suggests that terminal Schwann cells of sensory nerve fibers may possess biological properties distinctive from those in proximal nerve fibers. If this is the case, this distinctive feature has likely rendered these terminal Schwann cells insensitive to segmental demyelination in CMT1A. Alternatively, chronic insidious demyelination may accumulate over time and contribute to the shortened internodes in CMT1A.[14]

Paranodal length

Each node of Ranvier is flanked by 2 hemi-paranodes, which are typically symmetric under light microscopy. After segmental demyelination, remyelination occurs to re-establish new internodes as well as new hemi-paranodes.[64] The newly established internode and paranode differ in internodal length from the adjacent internode that remained intact without experiencing de-/remyelination.[65] In this situation, the hemi-paranode on the remyelinated side would appear asymmetric from that on the intact side.

By immunohistochemical analysis using antibodies against the paranode-specific protein, Caspr, we have quantified the length of paranodes as well their degree of asymmetry. We found that the index of asymmetry of the hemi-paranodes was increased in the skin biopsies of patients with both CMT1A and CIDP.[14] Thus, chronic, insidious segmental demyelination may have taken place in these nerve fibers. Taken together, investigating cellular compartments on dermal myelinated fibers has the potential to assist us in understanding the pathogenic processes of demyelinating neuropathies and the subsequent remodeling of myelin.

Nodal Gap

The nodal gap is a small but critical structure in the genesis and propagation of action potentials. The size of the gap directly affects the capacitance of the nodal membrane, thereby influencing the safety factor of action potential production.[66, 67] Moreover, septate junctions along with paranodal myelin membrane physically insulate the node from the juxtaparanode, where voltage-gated potassium channels reside. Disruption of the paranodal membrane allows outward current of potassium channels to counteract the depolarizing inward current at the node and may result in failure of action potential propagation.[68] Because axonal diameters vary minimally in dermal myelinated nerve fibers, the length of the nodal gap would reflect the area of the nodal axolemma.[69] By using immunohistochemistry with antibodies against MBP, normative values have been reported.[9, 11] Nodal lengths may be overestimated when they are measured with MBP staining alone, as it has been shown to poorly stain the paranodal regions flanking the node.[9] Without staining the paranodes, nodal gap measurements are slightly exaggerated from true

values; however, current normative data (Table 2) demonstrate that consistency between labs does not appear to be an issue, even in relatively small sample sizes. Future studies utilizing antibodies against Caspr would, however, improve accuracy, since it is specifically localized to the paranodal region.[70] Future studies with larger sample sizes and assessments of reliability would enhance these data as well.

It has been well documented that the molecular architecture of nodes of Ranvier are altered in certain peripheral nerve disorders.[2, 4] For instance, after segmental demyelination, voltage-gated sodium channels may spread out of the nodal region to paranodes or even juxtaparanodes.[71] Subsequent remyelination will restore the sodium channel localization to the nodal region. Interestingly, during this process, heminodes may be formed, and subtypes of sodium channels may be altered, including up-regulation of $Na_v1.8$.[6, 72] Additionally, blocking this subtype has been shown to inhibit neuropathic pain in some animal models.[73, 74] It is reasonable to believe that such molecular changes could also be investigated in human skin biopsies.

Axon caliber and myelin thickness

The caliber of individual axons and the thickness of their myelin sheath can be quantified using immunofluorescent staining at high power magnification (100×)[9] or with the higher resolution afforded by EM or semi-thin sectioning.[14] In the case of immunofluorescent staining, software was used to trace an outline of longitudinally orientated axons and myelin to calculate a G-ratio of axon diameter/fiber diameter (0.73 ± 0.04) in fingertip skin biopsies.[9] In a related measure, axonal density of myelinated fibers in nerve fascicles was calculated with EM analysis of cutaneous fibers captured at a perpendicular orientation ($7.73 \times 10^{-3} \pm 1.9$ axons/μm^2).[14] Myelinated fibers become highly branched and tapered as they progress distally toward the skin, so the average diameter of dermal fibers (3.4±0.6 μm) is much smaller than what would be expected in the more proximal location of the sural nerve (≈ 5-10 μm).[62]

Diagnostic alternative to nerve biopsy

Nerve biopsy is invasive and often clinically unjustified without an alternative for research purposes. Skin biopsy is minimally invasive and can be repeated in different areas of the body with little discomfort to the patient, thus it is suitable for longitudinal investigations. Similar morphological parameters may be assessed as with teased fiber preparations, including detection of a number of pathogenic disease markers (summarized in Table 3).

Skin tissue can be processed similar to nerve biopsy cross sections to reveal dermal fascicles containing myelinated and unmyelinated axons. Semi-thin cross sections dyed with methylene blue and examined by light microscopy allow quantification of axonal density. Similar EM preparations as with sural nerve biopsy can be done to show myelin compaction with the ability to quantify the periodicity as well as the G-ratio of myelin thickness. In a comparative study of the 2 procedures, it was found that reductions in either large or small fibers in the sural nerve were nearly always reflected in the epidermal fiber content of the skin, although a reduction in epidermal fibers may exist while sural nerve morphometry is normal.[75] This reflects the particularly susceptible terminal location of epidermal fibers. Furthermore, a recent study from Dacci et al demonstrates that skin biopsies can reveal morphological changes that are not yet detectable in sciatic nerves, thus appearing to be a more sensitive approach for detecting pathological changes.[58]

In other studies, Immunoglobulin M (IgM) deposits were found on dermal myelinated fibers immunoreactive for various myelin proteins (e.g. PMP22, MBP, and MAG) in patients with anti-myelin-associated glycoprotein (anti-MAG) neuropathy, suggesting the development of

skin biopsy as a potential procedure for diagnosis.[15, 76] Even so, the diagnostic utility of skin biopsies in myelin-related neuropathies is still in its infancy and warrants further investigation.

Expansion of skin biopsy applications

With the advance of molecular medicine, skin biopsies have been applied rapidly to more areas in neurological research. Some advances will be briefly discussed below.

Intracellular organelle trafficking

Intracellular organelles are constantly in motion, typically using microtubules as a "railway," and many neurological diseases are related to abnormal intracellular organelle trafficking.[77, 78] To examine this aspect of cell biology, one would have to use live cells from human subjects, which can be achieved by culturing cells from skin biopsies. We have recently described a new subtype of CMT, called CMT type 4J, which is caused by recessive mutations of the *FIG4* gene. By taking a skin biopsy, it was possible to culture the fibroblasts of affected individuals to create an *in-vitro,* patient-specific model to further investigate the biology of FIG4 deficient human cells.[79] As a result, it was discovered that there was excessive accumulation of vacuoles in the CMT4J fibroblasts that physically obstructed organelle trafficking.[79] This finding reveals a new pathogenic mechanism of the disease and suggests the possibility of further applications of skin biopsy to studies of cellular function and pathology.

Pharmacological advancements

Glabrous skin biopsy may also prove to be a valuable indicator of drug therapy effectiveness. The dermal innervation of mouse footpads was recently used as a measure of neurotrophin-induced remyelination and consequent attenuation of behavioral and pathological signs of diabetic neuropathy in streptozocin (STZ) induced hyperglycemic mice.[80] If a therapy that promoted remyelination in hyperglycemia-damaged fibers were to advance to clinical trials, glabrous skin biopsy could provide a relatively painless and affordable method of tracking responses and effectiveness on a structural level.

Several pain syndromes, including inherited erythromelalgia, idiopathic small fiber neuropathy, and paroxysmal pain syndrome have been linked to mutations in sodium channel subtype 1.7, leading to hyperexcitability.[81, 82] The sodium channel dysregulation observed in neuropathy patients, in combination with theextensive role of sodium channels in other forms of pain, suggests a promising avenue for exploring new treatments for painful neuropathy. Activation of potassium channel currents by drugs such as retigabine may also provide new avenues in analgesia.[83] TRPV-1 expression has also been implicated in diabetic neuropathic pain[84]. Most studies have been performed in rodent models, but skin biopsy could be utilized in diagnosis of ion channel subtype mutations as well as the efficacy of treatments in patients with symptoms of painful neuropathy.

Limitations of Glabrous Skin Biopsy

Although this review has focused on the expanding field of skin biopsy, there are also significant disadvantages to using glabrous skin biopsy to study histology. It is possible some patients may have such severe neuropathy that no identifiable fibers are demonstrated. This has not been found to be a deterrent in the literature or in the authors' experience, as even patients with severe neuropathy have some dermal myelinated fiber endings which can be measured, but it significantly affects the interpretation of results. While glabrous skin has a significantly greater number of myelinated fibers than hairy skin, glabrous skin biopsies do not contain nerves large enough to study fascicular anatomy. Furthermore, there are fewer

nerve fibers than a typical sural nerve, and they do not typically contain motor nerves to assist in the study of motor neuron-specific disorders. In addition, the typical amount of tissue obtained is less than that needed for protein quantification using typical scientific methods such as Western blot, which is not an issue with nerve biopsy. More work on normative data is also needed, with larger normal subject studies before these evaluations can be used for clinical diagnostic testing.

Conclusion

While a few diseases affecting the peripheral nervous system continue to be diagnosed primarily by whole nerve biopsies, many can be reliably diagnosed by electrophysiologic tests and/or genetic screening without the need to ever evaluate the molecular pathology of the nerves themselves. Evaluation of dermal myelinated fibers could provide both an inexpensive and relatively painless method of gaining a better understanding of the underlying molecular pathology of a variety of disorders causing cutaneous demyelination, mechanoreceptor atrophy, and axonal degeneration. This further application of skin biopsy can be added to ongoing evaluations of epidermal fibers, thereby expanding the assessment of neurological damage by providing a means to evaluate morphology, protein expression, and disease pathogenesis in unmyelinated and myelinated fibers during the onset and progression of peripheral neuropathies.

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Abbreviations

PNS	peripheral nervous system
CASPR	contactin-associated protein
Kv	Potassium channel
MPZ	myelin protein zero
PMP22	peripheral myelin protein 22
MBP	myelin basic protein
IME	intrapapillary myelinated ending
MC	Meissner corpuscle
PCR	polymerase chain reaction
CIDP	chronic inflammatory demyelinating polyneuropathy
EM	electron microscopy
CMT	Charcot-Marie-Tooth disease
HNPP	hereditary neuropathy with liability to pressure palsies
RCM	reflective confocal microscopy
NT	neurotrophin
BDNF	brain-derived neurotrophic factor
Trk	tropomyosin-receptor-kinase
GBS	Guillain-Barré syndrome

MAG	Myelin-associated glycoprotein
IHC	immunohistochemistry
NaV	Sodium channel
IgM	immunoglobulin M
SKP	skin-derived precursor
iPS	induced pluripotent stem cells

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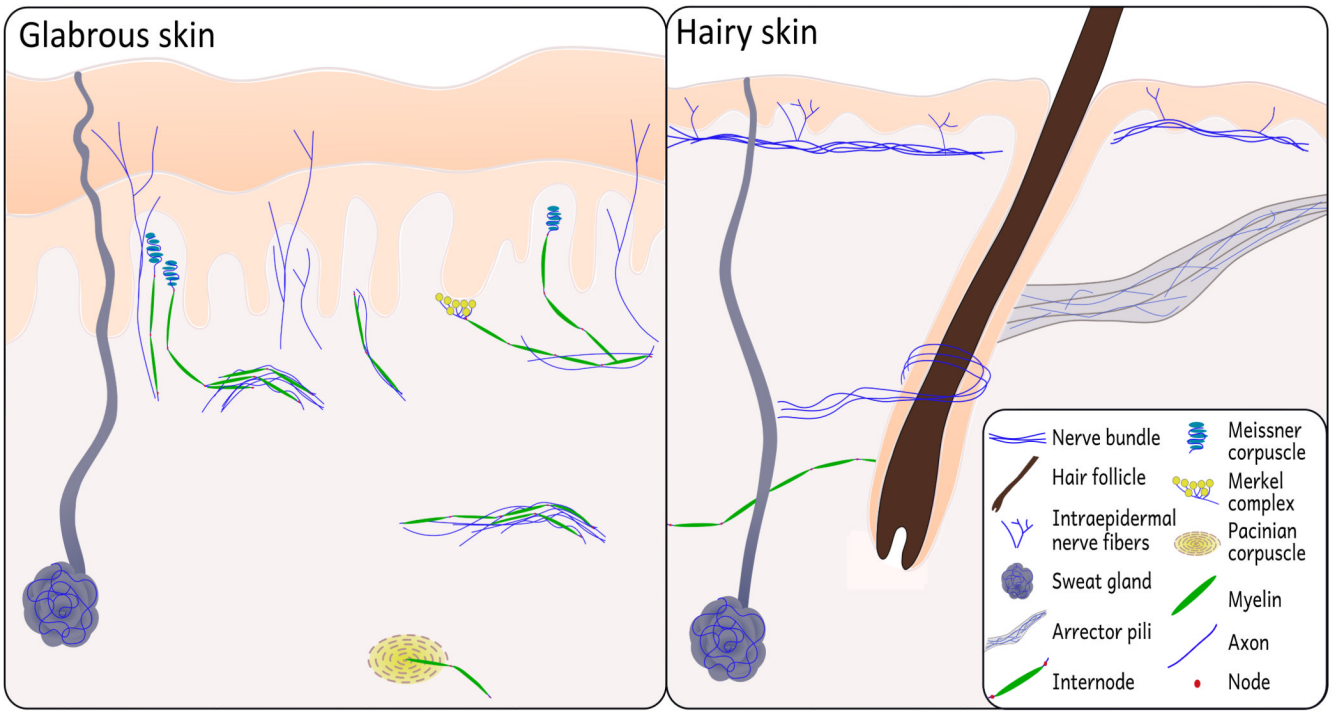

Figure 1.

Schematic of neural structures present in glabrous skin and hairy skin. Small sensory fibers branch off of dermal bundles to innervate the epidermis in both hairy and glabrous skin. In addition, autonomic nerve fiber innervation can be seen in sweat glands and arrector pili muscles. The presence of large myelinated fibers in hairy skin is largely restricted to mechanoreceptive hair follicles; glabrous skin has a comparatively high density of mechanoreceptive organs and afferent Aβ caliber myelinated fibers.

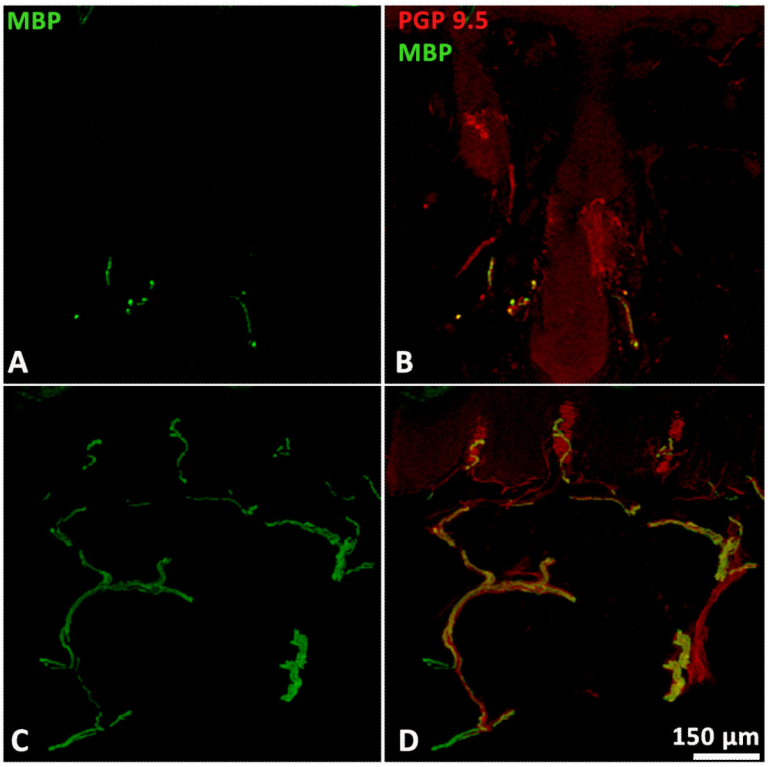

Figure 2.
Confocal images of MBP-immunoreactive myelinated fibers in hairy (A) and glabrous skin (C). In glabrous skin, fibers are prevalent and homogenously spaced as they furnish Meissner corpuscles in the dermal papillae (D). In hairy skin, MBP-ir fibers are typically much less prevalent and frequently cluster around hair follicles (B).

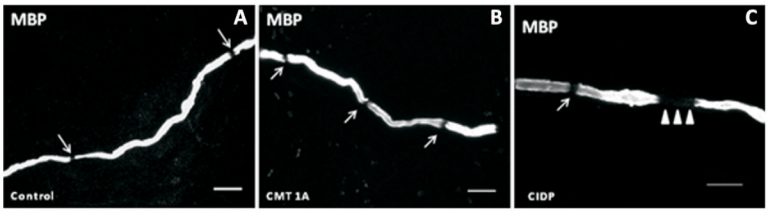

Figure 3.
Immunohistochemical analysis of glabrous skin biopsies has been shown to reveal shortened internodal lengths in patients with CMT1A (B) and CIDP (C) compared to controls (A). Additionally, CIDP patients had signs of segmental demyelination in the paranodal regions, designated by the absence of Myelin Basic Protein staining (C, arrowheads).

Table 1

Innervation, location and perceived sensation of cutaneous small fiber nociceptors and large fiber mechanoreceptors

Cutaneous sensory receptors	Innervating axons	Location	Perceived Sensation
Free Nerve Endings	A6-thinly myelinated C - unmyelinated	Epidermal and dermal layers of all skin	Thermal, mechanical, and noxious stimuli
Meissner corpuscles		Dermal papillae of glabrous skin	Light touch and low frequency vibration
Merkel complexes		Base of hair follicles and dermis of glabrous skin	Prolonged touch and pressure
Ruffini corpuscles	Aβ–thickly myelinated	Deep in the dermal layer of glabrous skin	Stretching of skin and prolonged pressure
Pacinian receptors		Deep in the dermal layer of glabrous skin	Deep pressure and high frequency vibration
Hair follicles		Dermis of hairy skin	Hair displacement

Table 2

Normative data for morphometric characteristics of dermal myelinated fibers and sural nerve fibers. Average age of patients: Provitera et al. 36.3 ± 10.3 years, Nolano et al. 33.7 ± 9.2, Saporta et al. 32.2 ± 12 years.

Reference	Biopsy location	Section [urn]	IME density [fibers/mm^2]	MC density [MCs/mm^2]	Internodal length [um]	Nodal length [um]	Diameter [um]
Provitera et al	Tip of digit III (N = 30)	80		–	83.0 ± 22.5	3.3 ± 0.6	3.4 ± 0.6
	Hairy skin (N = 30)				88.6 ± 23.1	4.6 ± 1.1†	3.0 ± 0.4*
Nolano et al	Tip of digit III (N = 14)	80	59.0 ± 29.3	33.02 ± 13.2	79.1 ± 13.8	3.5 ± 0.8	3.30 ± 0.50
	Tip of digit III, V (N = 1)			33.6, 45.0			
Saporta et al	Lateral aspect of digit II (N = 12)	60		14.6 ± 3.4	94.5 ± 28.6	–	–
Oh	Sural nerve	Teased fibers	N/A	N/A	200 - 1500	–	2-17 μn‡

* $p < 0.01$

† $p < 0.001$

‡ range for myelinated fibers exhibiting a bimodal distribution with peaks at 5 μm and 13 μm

Table 3

Structural aberrations evident in sural nerve biopsies and skin biopsies

Morphological abnormality	Structural location	Common method of detection	Associated disorder(s)	Sural nerve biopsy	Skin biopsy
Tomacula	Paranodes	EM, IHC; teased nerve fiber, longitudinal sections	HNPP	+	+
Onion bulbs	Schwann cells	Toludine-blue staining, modified trichrome, EM; semi-thin cross sections	CMT3	+	-
	basal lamina		CMT1A	+	+
Segmental demyelination	Internodes	IHC; longitudinal sections, teased fibers	CIDP	+	+
Shortened internodal length	Internodes	IHC; longitudinal sections, teased fibers	CMT1A	+	+
Increased myelin periodicity	Schwann cells	EM; thin section	MAG/IgM	+	+
Inflammation and necrosis of the blood vessels	Arteries	hematoxylin and eosin stain; cross section	Vasculitis	+	
Amyloid deposits	Peripheral nerves	Alkaline congo-red stain; paraffin embedded nerve	Amyloidosis	+	
IgM deposits	Myelin sheath	IHC; cross sections or longitudinal sections	Anti-MAG neuropathy	+	+
Small sensory fiber loss	Originate in DRG, epidermal termination	IHC; longitudinal sections	Diabetes, HIV, idiopathic		+

+ = reliable detection, - = not reliably detected, IHC = immunohistochemistry, EM = electron microscopy, HNPP = hereditary neuropathy with liability to pressure palsies, CMT = Charcot-Marie-tooth disease, CIDP = chronic inflammatory demyelinating polyneuropathy, DRG = dorsal root ganglia, MAG = myelin associated glycoprotein, IgM = Immunoglobulin M