

Assessment of cutaneous innervation by skin biopsies

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Skin biopsies that are immunostained to identify nerve fibers provide a new tool for assessing the small caliber nociceptors that terminate in the epidermis, as well as other cutaneous nerve fibers. Skin biopsies can be performed in multiple sites and can be repeated over time, so that a spatiotemporal profile of epidermal innervation can be constructed. This approach may help assess the progression of fiber loss in disease and of regeneration and re-innervation with treatment. *Curr Opin Neurol*

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Abbreviations

DRG	dorsal root ganglia
GDNF	glial cell-line derived neurotrophic factor
IENF	intraepidermal nerve fiber
NGF	nerve growth factor
VR1	vanilloid receptor type 1

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Introduction

In the 1960s, electrodiagnostic methods emerged that allowed an assessment of the large sensory and motor fibers. The techniques could be performed in a repeatable fashion, were acceptable to most patients, and became routine diagnostic tools. Tools for assessing small caliber fibers lagged. Electrophysiological techniques were either technically daunting, such as the collision techniques, or required in-vitro recording after excision of the nerve. Therefore, although routine electrodiagnosis changed the practice of neuromuscular medicine profoundly, it left the small nerve fibers 'invisible'. However, the small fibers outnumber the large fibers by approximately 8:1 in the cutaneous sensory nerves, which are responsible for important roles such as protective sensibility, interoception, and autonomic function.

This gap has been partly filled by autonomic nervous system measures, such as quantitative sweat testing to measure the function of postganglionic sympathetics, and the measurement of heart period variability and Valsalva responses to assess cardiovascular regulation. For assessment of small sensory nociceptors, the venerable technique of axon reflex testing by wheal and flare is available, but it is difficult to quantitate and is little used. Several instruments capable of quantitatively testing aspects of sensory psychophysics have been developed. The modalities that can be measured include thermal sensibility (warming and cooling) and heat pain, as well as vibratory sensibility, which is subserved by larger fibers.

With some fascinating exceptions [1], classical histological techniques suggested that epidermal innervation was scanty, and 'missed' most of the small sensory fibers. Beginning approximately 10 years ago, immunostaining for a series of markers, especially the panaxonal marker, protein gene product 9.5, presented a very different picture [2–6]. The epidermis was seen to be extensively innervated. Simple punch biopsies [3–5,7,8] and epidermal biopsies obtained by suction blisters [9] were well accepted by patients, and a rapidly increasing literature has examined the roles of assessment of epidermal innervation in such diverse disorders as diabetes, HIV infection, leprosy [10], Fabry's disease, pharmacological neurotoxicity, postherpetic neuralgia, and idiopathic neuropathies. This review will summarize the current understanding of skin innervation, assess the reproducibility and reliability of current techniques, evaluate recent reports on specific diseases, and look towards future approaches.

Organization of cutaneous innervation

The small sensory fibers that provide protective sensibility include A δ and C fibers. The former are small myelinated fibers, whereas the latter are unmyelinated axons that are arranged in Remak bundles. Remak bundles are defined as a non-myelin-forming Schwann cell and the unmyelinated C-fiber axons that it ensheaths. The number of unmyelinated axons in a single Remak bundle may number from one to more than 10. Axons exchange among Remak bundles as they pass from the dorsal root ganglia (DRG) to their targets, and postganglionic sympathetic fibers may be admixed with sensory fibers in a single Remak bundle. At least three trophic factors affect C-fiber nociceptors: nerve growth factor (NGF), glial cell-line derived neurotrophic factor (GDNF), and insulin-like growth factor type 1. The neurons that depend on NGF, approximately half the small sensory neurons in rat DRG, express the high affinity NGF receptor *trkA* as well as the low affinity receptor p75 [11]. The other half are predominantly GDNF-responsive neurons that express the receptor c-ret [11] as well as a series of other markers. These markers include the ability to bind a specific *Griffonia* lectin, isolectin B4, a ligand for fucosyl moieties that provides a convenient (but not wholly specific) marker for the GDNF-dependent population. Finally, most nociceptors bear the receptor for the pepper toxin, capsaicin. This receptor, vanilloid receptor type 1 (VR1), is the basis for the burning pain elicited by the topical application of capsaicin. It normally responds to heat and to protons; mice genetically engineered to lack VR1 fail to respond to heat and acid [12]. A related receptor, vanilloid receptor-like receptor type 1, has recently been identified, primarily on somewhat larger sensory neurons probably giving rise to A δ fibers. Vanilloid receptor-like receptor type 1 responds to very high temperatures (>52°C) [13].

At the dermal–epidermal junction the Remak bundles lose their Schwann cell investment, and the unmyelinated axons ascend through the epidermis between adjacent keratinocytes as true ‘free nerve endings’ [6]. Most epidermal axons extend to the stratum corneum, where they terminate. Some epidermal fibers contain the peptides CGRP and substance P; these are NGF-dependent axons. Many lack peptides, including the isolectin B4-positive GDNF-dependent axons. In the human, almost all of the epidermal axons are VR1-positive (Polydefkis, unpublished observation). Sufficient amounts of capsaicin topically applied to the skin results in the destruction of these fibers, with a consequent loss of heat pain sensibility [14,15]. These fibers can gradually regenerate [1,15]. In rat hairy skin there is evidence for a small population of epidermal fibers that are postganglionic sympathetics [16–18].

Measurement of epidermal innervation in the human

Two methods of sampling the epidermis are available. The least invasive is removal of the epidermis by placing a suction capsule over the skin; the epidermis separates cleanly at the dermal–epidermal junction [9]. This approach is painless and occurs without bleeding because all of the blood vessels terminate beneath the epidermis in the dermal papillae. For these reasons it may be particularly safe on, for example, the feet of diabetic patients. The resulting preparations can be viewed in sections or in horizontal orientation of a whole mount, giving a bird’s eye perspective. There are two disadvantages to this technique: it is time-consuming, taking 30–90 min to develop the blister, and by definition no data can be obtained about dermal innervation.

The second approach is simple punch biopsy of the skin. This is also well tolerated. If the biopsy diameter is restricted to 3 mm or less no suture is needed. The biopsy site heals by granulation and leaves a small circular scar that gradually resolves. The epidermal innervation can readily be quantitated [19], using either confocal microscopy [20,21] or the direct counting of chromogen-stained specimens [19,22]. Counting criteria have included epidermal fiber length [20,23], the number of fibers crossing the dermal–epidermal junction [19,22], and stereological methods [23]. With training, technicians can achieve high inter-rater reliability ($R=0.941$, $P<0.001$) and intra-rater reliability ($R=0.993$, $P<0.001$) [22].

In the leg, the density of epidermal fibers is greatest most proximally and declines in normal individuals with distance down the leg [24]. At any specific site the density declines only modestly with age [22,24]. This contrasts with such large fibers as those innervating Meissner corpuscles. There is wide biological variability in density among individuals, but excellent inter-interval reliability at a given site for a normal individual (M. Polydefkis, C. Yiannoutsos, S. Shriver, *et al.*, unpublished observation). Using the value of the lowest 5% as the cut-off value to identify normal, the percentage correctly classified (diagnostic efficiency) was 88%, the specificity 97%, the positive predictive value 92%, and the negative predictive value 90% [22]. The high positive predictive value and sensitivity increase the value in identifying the presence of a disease process in the absence of other diagnostic criteria.

The data from skin biopsies has been compared with the assessment of small fibers in sural nerve biopsies, as measured by morphometry of plastic sections and electron micrographs [25]. There is excellent correlation between epidermal nerve fiber density and small

myelinated fiber densities in sural nerve biopsies. The correlation is somewhat less good with unmyelinated fibers, partly reflecting the difficulties with electron micrograph morphometry in nerve biopsies, and partly the fact that sympathetic fibers are included in the nerve biopsy counts. It is noteworthy that some patients with clinical manifestations of neuropathies involving small sensory fibers had normal unmyelinated axon densities in the sural nerve, and extremely low epidermal nerve fiber densities at the level of the ankle [24,25]. This pattern probably reflects fiber loss that is restricted to the terminal and preterminal regions of the small sensory axons. For trials of therapeutic agents that are intended to promote regeneration, such patients might represent an especially attractive population, because detectable reinnervation might occur relatively early. A recent editorial [26] commented that, 'there is growing evidence that simple unmyelinated fiber counts in nerve biopsies fail to reveal the degree of distal, unmyelinated nerve degeneration in the skin'.

Cutaneous innervation and neuropathic pain

The most prominent clinical manifestation of most painful neuropathies is burning in the feet. Present spontaneously and continuously, it is usually most troublesome at night, when it interferes with sleeping. It may be associated with restless legs [27]. 'Lightning pains', sudden shock-like or 'ice-pick-like' pains in small and varying spots in the leg, are often described. Hyperalgesia and allodynia are prominent in some painful neuropathies. The pain may increase while walking. The same patients that have spontaneous pain often have elevated thresholds to elicited pain, and consequently a predilection for painless injuries such as foot ulcers and Charcot joints. Painless injuries are seen most often in diabetic, leprosy, and amyloid neuropathies.

Among the neuropathies in which neuropathic pain can be a prominent clinical feature are diabetic polyneuropathy [28–30], the sensory neuropathy of AIDS [5] (M. Polydefkis, C. Yiannoutsos, S. Shriver, *et al.*, unpublished observation) amyloid neuropathy, and the idiopathic painful polyneuropathies, a very frequent problem that especially affects elderly individuals [21,24]. Other less common neuropathies include Fabry's disease [31], and some paraproteinemic neuropathies. Neuropathic pain can also be prominent in traumatic and physical injuries to the peripheral nervous system, angiopathic disorders such as vasculitis, and herpes sensory ganglionitis.

The mechanisms contributing to the development of neuropathic pain are incompletely understood and undoubtedly manifold, almost certainly differing in important details among the various inciting disorders [32,33,34–39]. Peripheral sensitization, with increased

excitability of the nociceptor itself, can occur. In addition, central sensitization caused by changes in the dorsal horn is well documented. Central sensitization can be driven by increased electrical activity of the nociceptors. Important insights have come from experimental models of partial nerve injury. In partial nerve injury spontaneous activity can develop within the proximal stump of interrupted nerve fibers [32,40,41]. In addition, there are changes in activity in uninjured intact nociceptors that neighbor degenerating nerve fibers [33,34].

Skin biopsy in neuropathies

Several general comments apply to many nerve disorders. First, although neuropathic pain and the loss of pin and thermal sensibility may be restricted to the feet, there can be loss of epidermal innervation at more proximal sites. In patients with burning pain that is restricted to the feet, skin biopsy abnormalities are usually present at the level of the ankle above the lateral malleolus, and changes are often seen in the thigh [22,24]. Second, in regions in which the epidermal densities remain normal there may still be pathological changes, consisting of predegenerative changes such as axonal swellings within the epidermis and dermis [24], clumping of epidermal fibers with non-innervated patches between, and epidermal axons that extend horizontally beneath the stratum corneum or at the dermal–epidermal junction [42]. None of these changes are easily amenable to quantitation, but they provide supporting evidence for neuropathy.

Diabetic neuropathy

Diabetic neuropathy has been most extensively studied. Small fibers have long been recognized to be involved in diabetic polyneuropathy, and skin biopsies have confirmed that epidermal denervation can occur relatively early. When multiple sites are biopsied, in general the most distal site is most severely affected [43]. This rule does not apply to diabetic truncal neuropathy; the affected areas have been shown to have reduced epidermal nerve fiber densities, indicating that this disorder affects postganglionic fibers, rather than being a radiculopathy [42].

Sensory neuropathy of AIDS and antiretroviral toxic neuropathy

The sensory neuropathy of AIDS affects at least 30% of individuals with AIDS [44,45]. It usually produces burning pain in the feet, often associated with hyperalgesia and lightning pains. Unlike HIV dementia, its numbers have not declined with the advent of highly active antiretroviral therapy, probably because a similar neuropathy is produced by some antiretroviral agents, especially dideoxy compounds. Pathological studies of the peripheral nerves have shown a roughly equal loss of

large and small nerve fibers, and early skin biopsy reports identify the distal loss of epidermal innervation. Polydefkis and colleagues (unpublished observation) reviewed 47 individuals who received two biopsies over 18 weeks in an NGF treatment trial. The intraepidermal nerve fiber (IENF) densities were remarkably reproducible over this interval. There was an association between severe pain and lower IENF densities. There were strong associations of higher CD4 cell counts and lower viral loads with higher IENF densities. How well patients performed on tests of just noticeable differences in cooling and vibration sensibility were inversely correlated with IENF density.

A recent trial of NGF in the sensory neuropathy of AIDS included measures of epidermal innervation. Although there was some improvement in pain as assessed by a visual analog pain scale, there was no evidence of cutaneous reinnervation (M. Polydefkis, C. Yiannoutsos, S. Shriver, *et al.*, unpublished observation) [46].

Idiopathic small fiber sensory neuropathy

Recent reports have established the value of skin biopsies in evaluating painful sensory neuropathies of unknown etiology. Such patients may have normal tendon reflexes, large fiber sensibility, and electrophysiology, but marked epidermal denervation [24,47,48]. Undoubtedly, multiple disorders are lumped within this category. Most of the patients are older, and there is interest in the extent to which glucose intolerance may be a factor in some of these patients (C. Sumner and D. Cornblath, personal communication).

Leprosy

The first large-scale studies of skin biopsies to measure cutaneous innervation were carried out in leprosy [10]. In all forms of leprosy affected areas have profound denervation, usually affecting both the dermis and epidermis [10,49,50]. The approach is being evaluated to assess dermal nerve damage during multidrug chemotherapy for leprosy [50].

Fabry disease

Fabry disease is an X-linked recessive disorder caused by α -glucosidase A deficiency, with consequent accumulation of ceramide trihexoside. In addition to vascular complications of renal insufficiency and occasional myocardial and cerebrovascular damage, Fabry disease produces a painful neuropathy. Scott *et al.* [31] studied 20 patients with intact renal function. There was a marked loss of epidermal innervation to a level approximately 27% of the normal mean. Sural nerve biopsies in three patients showed that only small fibers, both myelinated and non-myelinated, were lost. By quantitative sensory testing this disease was associated with a disproportionate loss of cold perception [31].

Congenital insensitivity to pain

Nolano *et al.* [51] demonstrated profound denervation of cutaneous structures, including sweat glands, arrector pili, and epidermis, in a patient with this disorder.

Postherpetic neuralgia

Shingles represent reactivation of latent varicella-zoster virus with a sensory ganglion, causing the loss of a variable proportion of the sensory neurons. In some patients, neuropathic pain persists for months or years (postherpetic neuralgia). Hyperalgesia and allodynia to light touch are often prominent. Two studies have examined the demonstration that the epidermal fiber density in areas of allodynia are reduced, although they came to differing conclusions. Rowbotham and Fields [52] felt that higher fiber densities correlated with pain, an observation that might suggest that intact hyperactive nociceptors were responsible. Oaklander [53] found that pain correlated with lower IENF densities, and interpreted the results as more compatible with central sensitization. Some recent experimental data from the Chung model of partial nerve injury also found that the more thoroughly denervated regions of the rat hind paw have more hyperalgesia [34].

Conclusion

The validity of skin biopsy for assessing the extent of epidermal innervation and especially nociceptors appears to be established. Skin biopsy provides a means of assessing the numbers of C-fiber nociceptors. Easily applicable and robust quantitation of the dermal innervation, sweat glands, hair shafts, and other potentially instructive nerve fiber populations remain to be developed. An area of great interest is the potential value of skin biopsy in clinical trials of agents that are intended to promote the regeneration of nerve fibers.

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