Epidermal innervation: changes with aging, topographic location, and in sensory neuropathy

Giuseppe Lauria\textsuperscript{a,d}, Neil Holland\textsuperscript{a}, Peter Hauer\textsuperscript{a}, David R. Cornblath\textsuperscript{a}, John W. Griffin\textsuperscript{a,b}, Justin C. McArthur\textsuperscript{a,c,*}

\textsuperscript{a}Department of Neurology, Johns Hopkins University, Meyer 6-109, 600 N. Wolfe St., Baltimore, MD 21287-7609, USA
\textsuperscript{b}Department of Neuroscience, Johns Hopkins University, Baltimore, Maryland, USA
\textsuperscript{c}Department of Epidemiology, Johns Hopkins University, Baltimore, Maryland, USA
\textsuperscript{d}Istituto Nazionale Neurologico 'C. Besta', Milan, Italy
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\textsuperscript{b}Department of Neuroscience, Johns Hopkins University, Baltimore, Maryland, USA
\textsuperscript{c}Department of Epidemiology, Johns Hopkins University, Baltimore, Maryland, USA
\textsuperscript{d}Istituto Nazionale Neurologico 'C. Besta', Milan, Italy

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Abstract

In previous work we demonstrated little effect of aging on the density and spatial pattern of epidermal innervation, however, this was restricted to two sites proximal and distal in the leg. To expand on these observations, we used punch skin biopsy in ten healthy controls to examine the variation in intra-epidermal nerve fiber (IENF) density at multiple specific sites in the leg. There was a consistent gradient in IENF from proximal to distal sites in all subjects, but minimal effect of age was noted. In the older age group (≥70 years), the IENF densities ranged from 28.6±1.9 IENF/mm\textsuperscript{2} at the trunk to 15.5±1.5 at the distal leg. In a group of six patients with painful sensory neuropathy, we confirmed a length-dependent reduction in IENF. We observed what may be a predegenerative change, namely increased branching of epidermal nerve fibers at clinically unaffected sites. These data suggest little age-related change in IENF, at least up to age 75 years, in healthy normals. The increased branching complexity noted in unaffected sites in patients with sensory neuropathies implies that this may be a predegenerative change, preceding the actual loss of nerve fibers. Skin biopsy may be a useful tool for assessing the topographic extent and degree of nerve fiber damage in sensory neuropathies and its quantitative interpretation should be little affected by aging changes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Skin biopsy; Aging; Sensory neuropathy; Epidermal fibers

1. Introduction

The presence of nerve fibers within the human epidermis was debated for about 130 years after Paul Langerhans published his first description of intra-epidermal nerve fibers (IENF) using the gold chloride method [1]. Although his findings were subsequently confirmed by other authors [2–5], the limitations imposed by the relative insensitive staining methods led several investigators to deny the existence of free nerve fibers in the epidermis [6–8]. Recently, IENF have been definitively demonstrated by immunohistochemical staining using the panaxonal marker, anti-protein gene product 9.5 (PGP9.5) as well as antibodies to neurofilament (NF), substance P (SP), calcitonin gene-related product (CGRP), and neuron specific enolase (NSE) [9–11].

Skin biopsy has become an attractive technique to evaluate the terminal regions of small nerve fibers. There is extensive innervation of the skin by both sensory and autonomic fibers as demonstrated by staining for the panaxonal marker PGP9.5 [10–12]. The ability of skin biopsy to assess the features of unmyelinated C and small-myelinated A\textsubscript{\textalpha} nerve fibers makes it a useful tool for studying sensory neuropathies which affect small nerve fiber classes [13]. IENF can be reliably quantified [14] and IENF
density correlates with both the presence and the severity of the disease [12,15,16]. We recently published a relatively large normative series based on 98 controls with biopsies from two sites in the leg. Interestingly, little effect of age was seen in this group, except for higher densities in youngest individuals (10–19 years) [17].

The effect of aging on epidermal innervation needs to be delineated. Aging produces several modifications to both neuronal and non-neuronal structures: smoothing of the dermal–epidermal junction [18], loss of fibers to sweat glands [19] and Meissner corpuscles [20–22], as well as loss of small myelinated and unmyelinated fibers in the distal sural nerve [23,24] and reduction in the number of dorsal root ganglia neurons [25]. Furthermore, experimental studies reported that older animals show a shrinkage of the sensory fields related to small fiber function and decreased collateral sprouting [26]. Whether these last changes are also present in human beings is still unknown.

Most sensory neuropathies, including those related to diabetes mellitus and HIV infection, are characterized by distally predominant symptoms and axonal degeneration of cutaneous sensory nerves that is most severe distally in the leg [13,16]. Relatively little attention has been paid to morphologic changes in unaffected sites, which might serve as predegenerative ‘markers’ of neuropathies affecting small caliber nerve fibers.

The objectives of this study were: (1) to quantify the degree of variation in IENF density with respect to rostral/caudal orientation and age at multiple sites in healthy controls; and (2) to identify ‘predegenerative’ changes in patients with small fiber sensory neuropathies. We anticipated that this information would improve the ability to use skin biopsy as a valid diagnostic technique to assess small caliber nerve fiber damage in neuropathies.

2. Subjects and methods

2.1. Group 1

Ten subjects (five males and five females, ages 23–75 years) were recruited from a group of healthy volunteers, after a directed neurological examination to exclude systemic and neurological diseases. Subjects were specifically recruited to fall within the younger or older age groups for comparison of age-related changes: ≤45-year-old (five subjects aged 23–45 years) and ≥70-year-old (five subjects aged 70–75 years). None of the participants had signs or symptoms suggestive of neuropathy nor a history of diabetes mellitus, alcoholism, exposure to neurotoxic drugs, vitamin deficiencies, HIV infection, or other possible neuropathic risk factors. Each subject gave their informed consent, using a biopsy protocol approved by our institutional review board.

All normal subjects had skin biopsies at each of five sites: distal leg, proximal calf, distal thigh, proximal thigh and trunk. The distance between each site and the L4 spinous level was measured and recorded. Punch biopsies (3-mm Acupunch [Acuderm, FL]) were performed using a sterile technique, following the local injection of 2% lidocaine with epinephrine anesthesia. The specimen was fixed in 2% paraformaldehyde–lysine–periodate (2% PLP) for 24 h. Fixed specimens were serially cut by freezing microtome to obtain approximately 50-μm sections. Three sections selected randomly from the sectioned punch were immunostained with a free-floating protocol with PGP9.5 (Ultraclone, UK) as previously described [12].

2.2. Group 2

We identified six patients with sensory neuropathy (four males and two females, ages 41–72 years) who had recently undergone skin biopsies from the same multiple sites. All patients complained of distal painful dysesthesia and numbness. Four of them had idiopathic sensory neuropathies (three male and one female, ages 41–72 years) and two diabetic neuropathies (one male and one female, ages 50 and 60 years, respectively). Clinical, electrophysiologic and QST assessments were obtained on all.

For each biopsy, the number of IENF in three sections was counted by a single observer blinded to the site and to the diagnosis, using previously published techniques [12,14,17]. Following the same counting rules that we have previously published, we counted individual epidermal nerve fibers as they traversed the dermis/epidermis junction. Subsequent secondary branching was not incorporated into the density measure. The total linear length of the epidermis in the counted sections was measured using the Bio-Quant V instrument (R&M Biometrics, Nashville, TN) and the linear density of IENF/mm obtained. Some sections were re-counted by the same observer and by a second blinded one, to assess the intra- and inter-observer reliability. In all subjects IENF density was quantified in three sections from each of the five biopsied sites. Nineteen sections were counted twice by the same observer and 13 also by a second observer, both blinded to the site and clinical diagnosis. Spearman's correlation coefficients were 0.90 (P<0.001) and 0.89 (P<0.001), respectively, thus indicating high intra- and inter-observer agreements. We assessed the relationship between anatomical site and IENF density by relating the density to the distance of each biopsy site from the spine, corrected to the mean leg length of all subjects.

The ratio of epidermal fibers to branch points (‘branching ratio’) and number of branched fibers at each site were compared between healthy controls and neuropathy cases. In four control subjects (ages 23, 32, 72, 75 years) and in all six sensory neuropathy patients the number of single points of ramification of each fiber crossing the dermal–epidermal junction (branch points) and the total number of separate branched IENF (i.e. excluding the unbranched
fibers) were counted in three sections from the distal leg, distal thigh and proximal thigh.

Nonparametric statistical analysis was performed utilizing the SPSS package (Statistical Program for Social Sciences). Intra- and inter-observer agreements were obtained by analysis of Spearman's correlation coefficient. Individual IENF densities, number of branch points, number of branched fibers and branching ratio were treated as continuous variables and compared between groups by analysis of variance (ANOVA). P values lower than 0.05 were considered significant.

3. Results

3.1. Topographical variation in intra-epidermal nerve fiber density

There was a rostral-caudal gradient of decreasing IENF density in the leg. The overall mean density in Group 1 healthy subjects was 34.88/mm (95% confidence intervals [CI] 30.99–38.77) at the trunk, 23.09/mm (95% CI 20.89–25.28) at the proximal thigh, 14.60/mm (95% CI 13.43–15.78) at the distal thigh, 10.96/mm (95% CI 9.89–12.03) at the proximal calf and 15.34/mm (95% CI 13.56–17.13) at the distal leg. IENF density at the distal leg, calf and distal thigh was significantly lower than that at the proximal thigh and trunk (P<0.001), and IENF density at proximal thigh was significantly lower than at trunk (P<0.001). IENF density at the calf was not significantly lower than at distal thigh and distal leg (Table 1).

3.2. Effects of aging on normal cutaneous innervation

When the two age-groups were analyzed separately, in both we found the same gradient of length-dependent epidermal innervation in both age groups (Fig. 1): IENF density was significantly lower at the distal leg, calf and distal thigh than at the proximal thigh and trunk (P<0.001). The presence of a rostral-caudal gradient of epidermal innervation was confirmed by the linear relation-

![Fig. 1. IENF density in normal controls. Epidermal innervation shows a rostral-caudal gradient in both the groups with significant differences noted for trunk vs. proximal thigh (*), trunk vs. distal leg (**), and proximal thigh vs. distal leg. (***). Comparison between the age groups showed a significant difference only at the trunk (##) (P<0.001, ANOVA).](image)

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![Fig. 2. IENF density in normal controls. Comparison with distance from spine corrected for leg length. Overall, the regression line slopes for the two age groups were not significantly different.](image)

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<table>
<thead>
<tr>
<th></th>
<th>Control subjects (IENF density±S.E.M.)</th>
<th>Sensory neuropathy subjects</th>
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<tbody>
<tr>
<td></td>
<td>≤45 year (n = 5)</td>
<td>≥70 year (n = 5)</td>
</tr>
<tr>
<td>Trunk</td>
<td>41.12 (±2.37)</td>
<td>28.64 (±1.94)*</td>
</tr>
<tr>
<td>Proximal thigh</td>
<td>25.10 (±1.31)</td>
<td>21.21 (±1.55)</td>
</tr>
<tr>
<td>Distal thigh</td>
<td>15.28 (±0.87)</td>
<td>13.92 (±0.73)</td>
</tr>
<tr>
<td>Calf</td>
<td>11.29 (±0.43)</td>
<td>10.63 (±0.96)</td>
</tr>
<tr>
<td>Distal leg</td>
<td>15.17 (±0.89)</td>
<td>15.52 (±1.53)</td>
</tr>
</tbody>
</table>

* Statistical comparisons were performed by comparing individual IENF densities by analysis of variance (ANOVA) between the two age-groups.
* P<0.001 comparing densities at trunk.
** P<0.001 compared to controls.

Table 1

Normal distribution of IENF density in healthy control subjects and subjects with sensory neuropathy*
and proximal thigh and grouped them into four patterns: (1) unbranched fibers (Fig. 3A, B); (2) fibers branching just above the dermal–epidermal junction with secondary rami running toward the surface (Fig. 3C, D); (3) fibers branching farther from the dermal–epidermal junction with secondary rami running toward the surface (Fig. 3E, F); (4) fibers branching closer to the epidermis–stratum corneum junction with secondary rami parallel to the skin surface (Fig. 3G, H).

In the neuropathy cases, IENF showed distinct morphological changes. Branching patterns similar to those found in normal controls could be recognized, although the fibers frequently showed more tortuous courses and more complex ramification (Fig. 3A–F). In addition to increased branching, increased varicosity with stubby rounded projections, intraxial swellings, and claw-like terminals were also frequently noted. These features, particularly the intraxial swellings, were more evident at the thigh than in the regions below the knee, where the remaining fibers often appeared excessively beaded.

The number of both branch points and branched fibers showed a linear relationship with the number of IENF in both normal controls and neuropathy patients. We assessed

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**Fig. 3.** (a) IENF in normal controls. Morphological classification by the major branching pattern. Drawing made by Camera Lucida (Olympus 1.25×), magnification ×60. Derm, dermis; Epi, epidermis; Sc, stratum corneum. (A, B) Unbranched fibers, (C, D) fiber branching just above the dermal–epidermal junction with secondary rami running toward the skin surface. In C, note the unbranched fiber arising from the same dermal nerve bundle. (E, F) Fiber branching within the stratum spinosum with secondary rami running toward the skin surface. (G, H) Fiber branching closer to the epidermal–stratum corneum junction with secondary rami running parallel to the skin surface. In C, G and H note minor lateral ramification of the fibers. (b) Morphological features of IENF in sensory neuropathy patients. Drawing made by Camera Lucida (Olympus 1.25×), magnification ×60. Derm, dermis; Epi, epidermis; Sc, stratum corneum. Fibers clearly showed tortuous course and more complex ramification. The major branching patterns found in healthy subjects were recognizable as (A) possible previous unbranched fibers; (B–D) fiber branching just above the dermal–epidermal junction; (E) fiber branching within the stratum spinosum; (F) fiber branching closer to the epidermal–stratum corneum junction.
the degree of branching between the two groups of subjects using the branching ratio (total number of branch points/total number of IENF). Neuropathy patients had a significantly higher ratio than normal controls (P=0.03) at the proximal thigh despite a normal IENF density, indicating an increased degree of branching relative to IENF density, even though this was a clinically unaffected site (Table 2).

4. Discussion

We found that in healthy subjects the density of IENF showed a rostral-caudal gradient, with a linear relationship with the distance from the spine (Fig. 2). The level of the mid-thigh appeared to separate the rostral regions with higher density from the more caudal areas with lower values. These findings confirm our previous observations [12] and the qualitative observation of Wang et al. [10], who compared the epidermal innervation at the back and distal leg. Arthur and Shelley [5] used the methylene-blue staining and quantified IENF per area at different regions of the leg. These studies also found a significant rostral-caudal gradient.

In the present study, the density at the distal leg and proximal thigh closely correlated with that found in previous works [12,16], even though these earlier studies used formalin fixation. Fixation with PLP tended to give the epidermal fibers a less fragmented appearance, thus facilitating the accurate quantification of single fibers. The method used for the estimation of the total IENF length [14] assured a reliable measure of IENF density, as shown also by the low mean standard errors and the high intra- and inter-observer agreements.

The technique is relatively simple, and its only significant limitation is in the accurate recognition of an epidermal fiber. It appears to be reproducible, with low mean standard errors and high inter- and intra-observer agreement, and valid, correlating well with stereological assessment [14].

At the calf the density was lower than that reported by Kennedy et al. [15] using a different technique which is not directly comparable. These authors used confocal microscopy and a different fixative, as well as different counting rules.

The comparison between the two healthy control groups on the basis of age did not show any significant age-related effect on IENF density change, except at the trunk, and the rostral-caudal gradients, as judged by the slope of the regression lines were very similar (Fig. 2). Aging produces several modifications to skin neuronal structures and sensory transducers both in humans [19–22,24] and in rats [26]. However, age-related changes of IENF density had never been reliably assessed. Ridley [27], in 1969, investigated the effects of age on the epidermal innervation of human digital skin using a silver staining, and did not find any particular variation with aging. A recent abstract by Erdem et al. [28] also suggested no age-related effect, at least up to age 60, with a possible decrease after this age.

IENF are sensory axons, as demonstrated by the fact that they disappear from the skin after experimental dorsal root ganglionectomy, but not after dorsal rhizotomy, ventral rhizotomy, or sympathectomy [29]. Our findings of the highest densities of IENF in regions with lower spatial sensory discrimination is an apparent paradox, which has an apparent analogy in the dense innervation of coronal dental tubules of molar teeth, cornea, some glands, sphincters, ducts, chemosensory epithelia and smooth muscles [30]. One possible explanation is that the epidermal nerves do not represent only sensory fibers. Some could have possible efferent roles, including trophic functions [30,31].

4.1. Morphology of epidermal nerve fibers

The morphology of IENF has been examined by most of the recent studies using PGP9.5, but none identified the normal spectrum of profiles and branching patterns. Each of the four major patterns of IENF branching we described could be identified in each healthy subject, without frequency difference at each anatomic site. We did not note a change in branching patterns with age. Early in this century, Bozetzat [3] classified the IENF into seven types, according to the pattern of branching and to the shape of the axons, fibrillar or varicose. At least three of the patterns we presented could be recognized among them:

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>IENF density</th>
<th>No. of branch points</th>
<th>No. of branched fibers</th>
<th>Ratio of branch points to density</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal thigh</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>23.09 (±1.43)</td>
<td>130 (±16.0)</td>
<td>85 (±7.5)</td>
<td>0.54 (±0.03)**</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>19.15 (±2.71)</td>
<td>187 (±24.2)</td>
<td>107 (±11.3)</td>
<td>0.70 (±0.5)**</td>
</tr>
<tr>
<td><strong>Distal leg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>15.34 (±1.03)</td>
<td>94 (±11.2)</td>
<td>60 (±4.5)</td>
<td>0.66 (±0.04)</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>2.70 (±1.18)</td>
<td>82 (±21.3)</td>
<td>45 (±11.7)</td>
<td>0.63 (±0.1)</td>
</tr>
</tbody>
</table>

*Values in parentheses are ±S.E.M.

* P<0.001 controls vs. cases.

** P<0.03 controls vs. cases.
single unbranched, branching close to the dermal–epidermal junction, and branching farther from it. No morphological classification has been published since Bozeta’s work. Later, Kadanoff [32] and Cauna [6] recognized only one type of IENF and, in agreement with Bozeta, distinguished them as smooth or varicose. Similar findings were reported also by Novotny et al. [33], who utilized silver stains, and by Wang et al. [10], Dalsgaard et al. [9], and Kennedy et al. [15] with PGP9.5.

The patients with sensory neuropathy had a significantly lower IENF density than normal controls at the distal leg. However, morphological changes were often evident in regions with an innervation density still within the normal range, in particular at the distal and proximal thigh. At these sites, fibers were more frequently branched, with claw-like endings. Also, large intraaxonal swellings and excessive varicosities were usually much more evident in the regions above the knee. The higher branching ratio found in our patients at the proximal thigh, despite normal epidermal fiber densities, suggests that increased branching may occur early in the course of neuropathy. We have labeled this change ‘predegenerative’ since the density of single epidermal fibers is not reduced at these sites. It may reflect a compensatory mechanism for the ongoing nerve fiber degeneration distally.

In conclusion, we report that IENF density shows a distinct rostral-caudal gradient which is independent of an age-related effect, at least into the 70s. For patients with sensory neuropathy, we also classified the branching patterns of IENF and found that increased branching and axonal swellings appear to represent pre-degenerative changes which may occur in clinically unaffected areas.

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References

