Assessing function and pathology in familial dysautonomia: assessment of temperature perception, sweating and cutaneous innervation

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Summary

This study was performed to assess cutaneous nerve fibre loss in conjunction with temperature and sweating dysfunction in familial dysautonomia (FD). In ten FD patients, we determined warm and cold thresholds at the calf and shoulder, and sweating in response to acetylcholine iontophoresis over the calf and forearm. Punch skin biopsies from calf and back were immunostained and imaged to assess nerve fibre density and neuropeptide content. Mean temperature thresholds and baseline sweat rate were elevated in the patients, while total sweat volume and response time did not differ from controls. The average density of epidermal nerve fibres was greatly diminished in the calf and back. There was also severe nerve loss from the subepidermal neural plexus (SNP) and deep dermis. The few sweat glands present within the biopsies had had reduced innervation density. Substance P immunoreactive (-ir) and calcitonin gene related peptide-ir (CGRP-ir) were virtually absent, but vasoactive intestinal peptide-ir (VIP-ir) nerves were present in the SNP. Empty Schwann cell sheaths were observed. Temperature perception was more impaired than sweating. Epidermal nerve fibre density was found to be profoundly reduced in FD. Decreased SP and CGRP-ir nerves suggest that the FD gene mutation causes secondary neurotransmitter depletions. Empty Schwann cell sheaths and VIP-ir nerves suggest active denervation and regeneration.

Keywords: familial dysautonomia; temperature perception; sweating; skin biopsy; epidermal nerves

Abbreviations: CGRP = calcitonin gene related peptide; CT = cold threshold; ENF = epidermal nerve fibre; FD = familial dysautonomia; ir = immunoreactive; PGP = protein gene product; QSART = quantitative sudomotor axonal reflex test; SNP = subepidermal neural plexus; SP = substance P; VIP = vasoactive intestinal peptide; WT = warm threshold


Introduction

Familial dysautonomia (FD; Riley-Day syndrome) is an autosomal recessive disorder characterized primarily by variable autonomic dysfunction with accompanying sensory loss. (Axelrod et al., 1974; Axelrod and Hilz, 2000.) The disease appears to affect development and survival of the unmyelinated and small myelinated neurons resulting in decreased numbers of sensory, sympathetic and some parasympathetic neurons (Axelrod and Hilz, 2000; Anderson et al., 2001). Even in the youngest subjects, extensive consistent pathology is evident, including reduced sural nerve fascicular area with markedly diminished numbers of non-myelinated and small diameter myelinated axons, grossly reduced size of dorsal root ganglia and sympathetic ganglia due to decreased neuronal population, and small intermediolateral grey columns in the spinal cord (Pearson et al., 1974, 1978; Axelrod and Hilz, 2000). With increasing age, there is further depletion of neurons in dorsal root ganglia and an increase in residual nodules of Nageotte (Pearson et al., 1978).

Neurological dysfunction is clinically evident at birth and progresses during ageing (Axelrod et al., 1981; Axelrod and Hilz, 2000). Protean functional abnormalities exist due to the pervasive nature of the autonomic nervous system, including episodic hyperhidrosis with anxiety and during sleep. The main sensory manifestations are diminished pain and temperature...
perception (Axelrod et al., 1981; Hilz et al., 1998; Axelrod and Hilz, 2000).

Three mutations in the \textit{IKBKAP} (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein) gene, located on chromosome 9 (9q31), have been recently shown to cause FD (Anderson et al., 2001; Slaugenhaupt et al., 2001; Leyne et al., 2003). The most common mutation is a single base substitution in intron 20. Over 99% of FD patients are homozygous for this mutation, which affects the splicing of the \textit{IKBKAP} transcript. The splicing alteration may be tissue (i.e. neuron) specific as RNA isolated from FD lymphoblast cell lines is primarily wild-type, whereas RNA isolated from post-mortem FD patient brain samples is primarily mutant (Slaugenhaupt et al., 2001).

The function of \textit{IKBKAP} is not completely understood, but it may function within a larger complex, with additional proteins, to facilitate or promote the expression of other genes or neurotransmitters (Holmberg et al., 2002). Although FD penetrance is complete, there is marked variability in the expression of the disease. It is possible that the variability is due, in part, to relative expression of neurotransmitters such as substance P (SP) affecting sensory perception or vasoactive intestinal peptide (VIP) affecting autonomic function.

Examination of cutaneous nerve morphology provides objective quantitative measurements of unmyelinated epidermal sensory endings and autonomic nerves. The pan neuronal marker anti-protein gene product 9.5 (PGP 9.5) (Thompson et al., 1983) shares homology with ubiquitin carboxy terminal hydrolase (Wilkinson et al., 1989), and provides a powerful tool to localize virtually all nerve fibres including the finest nerve endings. Double and triple immunofluorescent staining with PGP 9.5, the basement membrane marker anti type IV collagen and antibodies to various neuropeptides reveals the elaborate and delicate innervation of cutaneous organs.

Thus, using the same subjects, we performed quantitative assessments of sensory and autonomic functions (temperature perception and sweating) and examined cutaneous innervation to characterize the extent of nerve depletion and to determine if there was any relation between clinical and histological features.

**Methods**

**Patients**

Ten patients with FD were examined. There were seven females and three males aged 18 to 47 years (mean age: 34.4 ± 10.44 years (SEM)). The diagnosis was confirmed at the New York University Dysautonomia Treatment and Evaluation Center using established clinical diagnostic criteria that included Ashkenazi Jewish ancestry, decreased or absent deep tendon reflexes, absence of overflow tears, absence of fungiform papillae of the tongue and absent axon flare response following intradermal histamine injection. Four patients had required fundoplication and gastroscopy. Scoliosis was present in all patients and three patients had undergone spine fusion surgery. All patients had a history of supine hypertension and orthostatic hypotension. Seven of the participating patients were known to suffer from dysautonomic crises; three of the seven patients had crises more than once a month. Genetic analysis showed that all patients were homozygous for the common intron mutation.

The procedures were approved by Internal Review Boards at New York University School of Medicine and the University of Minnesota. Informed consent was obtained, with a parent signing for patients younger than 21 years.

**Temperature perception**

Thermal perception thresholds were determined in all patients by means of a Thermotest\textsuperscript{TM} (Somedic, Stockholm, Sweden) according to the ‘method of limits’ (Fruhstorfer et al., 1976; Hilz et al., 1999b). Starting from a baseline temperature of 32°C, five consecutive warm or cold stimuli were applied in ramp fashion at 1°C/s with a thermode which had a stimulating surface of 1.5 cm \( \times \) 2.5 cm. Limits of stimulation were preset at 5°C and 50°C. As soon as the tested person indicated stimulus perception by pushing a button, the temperature reversed to the 32°C baseline. A computer calculated the difference between the baseline and the signalled peak temperature. Warm and cold perception thresholds (WT and CT) were determined at the distal medial calf and at the mid-shoulder by averaging from the responses to warm or cold stimuli. Thermal perception thresholds were compared with an age-related normative database of 491 volunteers (Hilz et al., 1999b).

**Sweating assessment**

The quantitative sudomotor axonal reflex test (QSART) was used to assess postganglionic sudomotor function at baseline and in response to acetylcholine iontophoresis (Low et al., 1983) on the left calf and arm. The measured increase of sweat production due to acetylcholine iontophoresis over time, expressed as \( \mu \text{moles/min} \), was plotted and compared with known normal and abnormal values.

A baseline sweat rate was assessed after the patient was supine for 20 min. Then, 10% acetylcholine was iontophoresed through the skin for five min by means of a 2 mA constant current. The response was monitored during current flow and for an additional 5 min to observe the return to baseline. As the return to baseline may be delayed or prolonged in FD patients as a result of denervation supersensitivity, the recording was continued for up to 15–20 min. Values obtained in FD patients were compared with values of age-matched controls (Bickel et al., 2004).

**Skin biopsies**

After performing the temperature perception and sweating assessments, 3 mm punch biopsies were obtained from the calf and mid scapular level over paraspinal muscles. Age- and sex-matched control values were provided by the Kennedy Laboratory.

Biopsied tissue was immediately fixed in chilled Zamboni’s fixative, refrigerated overnight, transferred to a sucrose-PBS (phosphate-buffered saline) solution and shipped overnight on ice to the University of Minnesota where samples were processed for double and triple immunofluorescent localization of antibodies according to previously described protocols (Kennedy et al., 2000). Thick sections (60 \( \mu \text{m} \)) were cut with a freezing sliding microtome. Antibodies to PGP 9.5 (Biogenesis, Poole, UK), calcitonin gene-related protein (CGRP) (Amersham, Arlington Heights, IL, USA), SP and VIP (DiaSorin, Stillwater, MN, USA), myelin basic protein (Ultralene Limited, Wellow, Isle of Wight, UK) and type IV collagen (Chemicon, Temecula, CA, USA) were localized by double or triple staining using Cy2, Cy3, and Cy5 labelled secondary antibodies.
Control values obtained from Hilz et al. Non-specific antibody adhesion was blocked by incubation with 5% normal donkey serum in diluent/wash solution (PBS, 0.3% Triton X-100, 1% normal donkey serum). Sections were floated in wash and antibody solutions in spot plates. Gentle rotation enhanced antibody penetration. Specificity controls were run with non-immune serum for each fluorophore. Sections were incubated simultaneously in each primary antibody dilution for 5 h at room temperature and overnight at 4 °C. Samples were washed at room temperature in three changes of the wash solution over a period of at least 3 h. Secondary antibodies, labelled with fluorophores, were applied simultaneously and incubated at room temperature for 5 h and overnight at 4 °C. Samples were washed three times over 3 h, then adhered to cover slips with agar, dehydrated with alcohol, cleared with methyl salicylate and mounted in the synthetic resin DPX (a mixture of distyrene, tricresyl phosphate and xylene).

Confocal images were acquired to determine epidermal nerve fibre (ENF) density. Epidermal nerves were imaged with a CARV non-laser confocal microscope (Atto Biosciences, Rockville, MD, USA) equipped with a multi wavelength automatic filter changer using a 20 x plan apochromat objective (NA 0.75). The z series of 16 optical sections was acquired at 2 μm increments. Sampling for quantification of nerve fibre density was carried out by collecting four images from each sample. If distribution abnormalities were found, the sampling was extended to include a montage of images across an entire section. In samples with no nerves seen on preliminary inspection, one image of each sample was taken.

Nerve fibre density was determined with Neurolucida Software (MicroBrightField, Colchester, VT, USA) by tracing from the intersection of the nerve fibre (PGP-9.5-immunoreactive; PGP-9.5-ir) with the dermal–epidermal basement membrane (type IV collagen-ir) to the ending within the epidermis. A counting unit was identified when a nerve crossed the basement membrane (Kennedy et al., 1996).

Tissues sections were observed with a Nikon epifluorescence microscope for visual evaluation of nerve and neuropeptide content of the SNP in the papillary dermis, sweat glands (if present) and epidermal sweat duct pores (acrosyringia). A rating scale was used to classify innervation: 0 = normal; –1 = mild loss of up to one half of normal nerve density; –2 = moderate loss of more than half of normal nerve density; –3 = sparse innervation; and –4 = no nerve present. CGRP, VIP and SP were evaluated as either present or absent. The number of sweat glands and acrosyringia were mapped from serial sections through each biopsy.

Statistics
A Mann–Whitney U-test was used to analyse qualitative differences between the temperature perception and sweating results of controls and patients. The χ²-test was used for quantitative differences, e.g. symptom prevalence. The level of significance was set at P < 0.05. A commercially available statistics program was used for data analysis (SYSTAT, Evanston, IL, USA).

Results
Temperature perception
Temperature thresholds for both warm and cold were elevated in the seven tested FD patients at both calf and shoulder, and were significantly higher than in the control persons (Table 1). Two of the 10 FD patients were insensitive for cold stimulation at the distal medial calf, while one was also insensitive for cold stimulation at the back. Three of the 10 FD patients were insensitive for warm stimulation at the distal medial calf and two of the 10 for warm stimulation at the shoulder. Mean CT in FD patients was 10.5 ± 4.8 °C (normal volunteers = 2.9 ± 1.7 °C) at the calf and 12.6 ± 4.5 °C (normal volunteers = 1.3 ± 0.6 °C) at the shoulder. Mean WT in FD patients was 8.3 ± 3.5 °C (normal volunteers = 3.5 ± 2.0 °C) at the calf and 8.6 ± 2.7 °C (normal volunteers = 1.8 ± 0.9 °C) at the shoulder.

Sweating
There was no significant difference in total sweat volume or response time between the group of patients with familial dysautonomia and healthy controls (Table 2) (Mann–Whitney: P > 0.05). However, marked intra-group variability among the FD patients was noted in both response time and total sweat volume. Although FD sweat response time tended to be shorter in the forearm than the calf, the total sweat volume was greater in the calf.

Table 1 Temperature thresholds in ten FD patients compared with control* data

<table>
<thead>
<tr>
<th>FD Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>CT: calf</th>
<th>CT: shoulder</th>
<th>WT: calf</th>
<th>WT: shoulder</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD1</td>
<td>47</td>
<td>F</td>
<td>8.5</td>
<td>5.3</td>
<td>4.6</td>
<td>ND</td>
</tr>
<tr>
<td>FD2</td>
<td>43</td>
<td>F</td>
<td>18.3</td>
<td>15.8</td>
<td>11.9</td>
<td>ND</td>
</tr>
<tr>
<td>FD3</td>
<td>19</td>
<td>M</td>
<td>12.7</td>
<td>9.5</td>
<td>10.4</td>
<td>ND</td>
</tr>
<tr>
<td>FD4</td>
<td>26</td>
<td>F</td>
<td>Insensitive</td>
<td>Insensitive</td>
<td>Insensitive</td>
<td>Insensitive</td>
</tr>
<tr>
<td>FD5</td>
<td>29</td>
<td>F</td>
<td>10.50</td>
<td>9.70</td>
<td>12.70</td>
<td>9.70</td>
</tr>
<tr>
<td>FD6</td>
<td>37</td>
<td>M</td>
<td>17.50</td>
<td>11.30</td>
<td>Insensitive</td>
<td>11.70</td>
</tr>
<tr>
<td>FD7</td>
<td>41</td>
<td>F</td>
<td>Insensitive</td>
<td>19.70</td>
<td>Insensitive</td>
<td>Insensitive</td>
</tr>
<tr>
<td>FD8</td>
<td>38</td>
<td>F</td>
<td>9.90</td>
<td>16.40</td>
<td>8.50</td>
<td>10.20</td>
</tr>
<tr>
<td>FD9</td>
<td>19</td>
<td>M</td>
<td>10.80</td>
<td>8.00</td>
<td>4.20</td>
<td>5.10</td>
</tr>
<tr>
<td>FD10</td>
<td>45</td>
<td>F</td>
<td>3.90</td>
<td>10.60</td>
<td>7.60</td>
<td>6.50</td>
</tr>
</tbody>
</table>

FD mean | 11.5 | 11.8 | 8.6 | 8.7 |
FD SD   | 4.7  | 4.6  | 3.3 | 4.0 |
Control mean | 2.9 | 1.3  | 3.5 | 1.8 |
Control SD | 1.7 | 0.6  | 2.0 | 0.9 |
P value | <0.05 | <0.05 | <0.05 | <0.05 |

*Control values obtained from Hilz et al. (1999b).
The baseline sweat rate was similar on the forearm and the calf. Both were significantly higher in FD patients compared with healthy controls (arm: 6.0 ± 1.6 versus 4.4 ± 0.5 μmols/min, calf: 4.3 ± 0.6 versus 2.4 ± 0.9 μmols/min, Mann–Whitney: *P* < 0.05).

### Skin pathology

#### Nerve density

Biopsies from the FD patients revealed a profound loss of nerve fibres in the epidermis as well as in the subepidermal neural plexus (Figure 1). Eight out of the 10 FD subjects had no remaining epidermal nerve fibres in their calf whereas the other two had few remaining fibres. ENF density on the back was very much reduced in all subjects. Figure 2 shows that ENF density values for FD subjects were well below the normal ranges for both calf and back. The average values for calf of 25 ± 0.53 ENFs/min (SD) and for back 9.31 ± 7.81 ENFs/min (SD) were significantly below the control values for both sites, i.e. 16.41 ± 6.75 ENFs/min (SD) calf and 70.09 ± 22.45 ENFs/min (SD) back; *P* < 0.05 for both sites.

The subepidermal neural plexus (SNP) and deep dermis of all FD samples also displayed severe nerve loss. The calf SNP score on the rating scale was –3.28 ± 0.74 (SD) and the back score was –2.03 ± 0.94 (SD). Normal SNP is rated as 0. There were no nerves below the dermal-epidermal basement membrane, where the SNP is normally located, in six calf and one back sample (–4 rating)—a rare finding in other types of sensory neuropathies.

#### Neuropeptide content

SP-ir and CGRP-ir nerves normally reside within the SNP such that 10–15 CGRP-ir fibres and one or two SP-ir fibres are visible in the papillary dermis area of normal skin sections. Both neuropeptides were virtually absent in the FD biopsies. VIP is normally confined to sympathetic nerves in sweat glands, arrector pili muscles, near hair follicles and accompanying large vessels. It is almost never present in the SNP. For the FD patients, VIP-ir was consistently present in the SNP of almost all biopsies that still contained a SNP (Figure 3).

#### Schwann cells

Nerves within the dermis, including those in the SNP, are normally ensheathed by a fine calibre Schwann cell sheath. S-100 protein immunoreactivity is useful as a marker for Schwann cells within the dermis. S-100 protein staining normally appears identical to PGP 9.5 neuronal staining for nerves, albeit with a wider diameter since it surrounds the nerves. In double stained sections, several FD samples had S-100 positive sheaths that did not contain PGP 9.5-ir nerve.

#### Sweat glands

Sweat glands were present in the tissue in normal numbers as indicated by the number of acrosyringia (epidermal sweat duct pores) per mm³: back 5.4 ± 2.8 (SD) in FD and 5.9 ± 0.6 (SD) in normal volunteers, calf 5.3 ± 3.3 (SD) in FD and 6.1 ± 0.7 in normal volunteers. However, despite the normal number of acrosyringia and plentiful sweat ducts, few sweat glands were encountered in the dermis of these full thickness biopsies (calf 2.2 and back 1.5 in FD. Ratios of sweat glands to acrosyringia were 0.42 and 0.28 in calf and back, respectively). We assume that most of the sweat glands in these patients resided in deep subcutaneous fatty tissue. The sweat glands present were frequently atrophic and often appeared to be replaced by fat cells. Innervation to sweat glands was sparse and disorganized (Figure 4). Nerve rating for FD sweat glands in calf averaged −2.7 and in back averaged −2.8. Normals for both sites were rated 0.

### Table 2 Sweat function as assessed with QSART in ten FD patients compared with control* data

<table>
<thead>
<tr>
<th>FD patient</th>
<th>QSART forearm</th>
<th>Response time (s)</th>
<th>Baseline sweat rate (μmols/min)</th>
<th>Total sweat volume (μmols)</th>
<th>QSART calf</th>
<th>Response time (s)</th>
<th>Baseline sweat rate (μmols)</th>
<th>Total sweat volume (μmols)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Response time (s)</td>
<td>Baseline sweat rate (μmols/min)</td>
<td>Total sweat volume (μmols)</td>
<td></td>
<td>Response time (s)</td>
<td>Baseline sweat rate (μmols)</td>
<td>Total sweat volume (μmols)</td>
</tr>
<tr>
<td>FD1</td>
<td>30.3</td>
<td>4.6</td>
<td>191.6</td>
<td>60.00</td>
<td>4.10</td>
<td>103.90</td>
<td>27.30</td>
<td>3.80</td>
</tr>
<tr>
<td>FD2</td>
<td>ND28.8</td>
<td>ND5.1</td>
<td>291.5</td>
<td>57.00</td>
<td>4.30</td>
<td>75.60</td>
<td>56.00</td>
<td>4.80</td>
</tr>
<tr>
<td>FD3</td>
<td>ND18.0</td>
<td>ND6.0</td>
<td>287.6</td>
<td>52.30</td>
<td>3.80</td>
<td>84.70</td>
<td>57.80</td>
<td>3.50</td>
</tr>
<tr>
<td>FD4</td>
<td>33.00</td>
<td>3.90</td>
<td>217.60</td>
<td>30.80</td>
<td>4.90</td>
<td>54.60</td>
<td>50.30</td>
<td>4.20</td>
</tr>
<tr>
<td>FD5</td>
<td>6.30</td>
<td>8.00</td>
<td>127.90</td>
<td>25.00</td>
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<td>66.90</td>
<td>25.00</td>
<td>5.50</td>
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<td>FD6</td>
<td>29.30</td>
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<td>345.30</td>
<td>37.30</td>
<td>4.50</td>
<td>52.50</td>
<td>45.40</td>
<td>4.30</td>
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<td>FD7</td>
<td>1.80</td>
<td>9.30</td>
<td>311.80</td>
<td>45.40</td>
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<td>84.80</td>
<td>45.40</td>
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<td>FD10</td>
<td>44.80</td>
<td>5.80</td>
<td>228.40</td>
<td>42</td>
<td>0.9</td>
<td>32.30</td>
<td>42</td>
<td>0.9</td>
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<tr>
<td>FD mean</td>
<td>23.4</td>
<td>6.0</td>
<td>222.3</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>FD SD</td>
<td>12.9</td>
<td>1.6</td>
<td>93.1</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Control mean*</td>
<td>84.5</td>
<td>4.4</td>
<td>167.9</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
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</tr>
<tr>
<td>Control SD*</td>
<td>33.8</td>
<td>0.5</td>
<td>80.6</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*Control values obtained from Bickel et al. (2004).
Other features
Hair follicles were innervated and, on some, there were lanceolate endings. However, there were too few hair follicles in the sections for a definitive evaluation. Myelin basic protein was present in dermal nerves in quantities appropriate to the degree of innervation observed. Capillaries in most samples appeared normal, but in several samples, the capillaries were tortuous.

Discussion
Familial dysautonomia is the most extensively described of a rare group of genetic disorders known as hereditary sensory and autonomic neuropathies, a group of disorders that appear to affect migration, maturation, survival and function of peripheral and central sensory and autonomic tracts (Axelrod et al., 1974; Pearson et al., 1974; Axelrod and Pearson, 1984; Axelrod, 1996). For each disorder, there are specific dermatological manifestations that reflect the neurological deficit (Axelrod and Pearson, 1984). All the disorders lack an axon flare with intradermal histamine. This is attributed to decreased dermal fibres as well as decreased SP and CGRP (Axelrod and Pearson, 1984; Schmelz et al., 1997). However, there is variability between the different disorders in degrees of temperature and pain perception as well as sweating characteristics (Hilz et al., 1999a; Hilz, 2002). Cutaneous nerves convey sensory modalities of touch, warm and cool discrimination, hot and cold pain, vibration and mechanical pain, as well as such sensations as prickle, itch or tickle. Furthermore, cutaneous efferent nerves are responsible for autonomic

Fig. 1 Confocal images of calf biopsies immunostained to reveal nerves (green) and basement membrane (red). Top: Biopsy of calf skin from FD patient. Note the paucity of ENFs and the nerves of the SNP. Bottom: Dermal nerve fibres in normal subject rise to the papillary dermis where they form a horizontal plexus, the SNP. ENFs ascend from this plexus, crossing the dermal–epidermal basement membrane to innervate the epidermis.
responses such as sweating and piloerection. In FD, decreased temperature and pain appreciation results in unrecognized burns and injuries. Autonomic dysfunction results in erythematos blanching of the skin during eating or emotional excitement, predominantly on the face, neck, chest and upper arms. Emotional excitement can cause profuse sweating over the head and trunk, but spares the hands and feet and many patients exhibit excessive sweating during the initial phases of sleep.

Familial dysautonomia is caused by mutation in the \textit{IKBKAP} gene (Anderson \textit{et al.}, 2001; Slaugenhaupt \textit{et al.}, 2001; Fig. 3 VIP immunoreactivity was found in the subepidermal neural plexus of familial dysautonomia biopsies.

\textbf{Fig. 2} Graph of ENF quantification for back and calf for each sample. Average normal values for back and calf are shown; 95% cut-off values are 24.7 ENFs/mm for back and 6.72 ENFs/mm for calf.

\textbf{Fig. 3} VIP immunoreactivity was found in the subepidermal neural plexus of familial dysautonomia biopsies.
Leyne et al., 2003), with >99% of FD patients being homozygous for a single base substitution in intron 20 resulting in a genetically homogeneous population. However, there is marked variability in phenotypic expression, which may be due to IKBKAP’s effect on the expression of other genes or neurotransmitters (Holmberg et al., 2002). Our results confirm the variability of expression not only between patients, but also in different locations on the body in the same individual.

Clinical testing, while useful for diagnosing neuropathy, lacks the sensitivity required for precise staging of neuropathy or as a means of correlating genotype and phenotype. Thus, we combined quantitative assessments of sensory and autonomic functions (i.e. temperature perception and sweating) with immunohistopathological examinations of cutaneous nerves to determine if there was any relation between clinical and histological features.

Temperature perception thresholds for warm and cold sensations were markedly elevated in all tested FD patients and some patients were completely insensitive. This is consistent with the observed marked decrease in unmyelinated peripheral innervation throughout epidermal and subepidermal layers; human neurography experiments have indicated

![Sweat gland innervation. Normal (above): Sweat glands tubules and surrounding capillaries (red), are enwrapped by multiple nerve fibres (green) forming a dense network of autonomic sudomotor innervation. FD (below): Sweat gland is sparsely innervated.](image-url)
that the terminal segments of cold conveying nerves are probably unmyelinated (Campero et al., 2001). The decreased unmyelinated nerve content with virtual absence by immunostaining of CGRP and SP is also compatible with the patients’ decreased nociception because Aδ nociceptors appear to branch into daughter unmyelinated branches proximal to the receptive field (Peng et al., 1999). The findings of Campero et al. (2001) and Peng et al. (1999) are in agreement with the absence of myelinated nerves in biopsies of human hairy skin, except for nerves to Merkel complexes or hair follicles (Kennedy and Wendelschafer-Crabb, 1996). Our thermal sensory and sweat test findings are consistent with previously reported peripheral neuropathology (Axelrod et al., 1974). By using immunostaining methods and confocal imaging for three-dimensional visualizations, we have enhanced understanding by providing objective and quantitative measurements of cutaneous neuropathy.

Sweat gland function was essentially normal in FD patients (except for the excessive baseline volume) and the number of sweat ducts and acrosyringia (and presumably sweat glands) were normal. In contrast, the few sweat glands observed were atrophic and some appeared to have been replaced by fat. These contradictory findings cannot be explained by sweat gland denervation hypersensitivity, because contrary to Canon’s law, QSART testing and the sweat impression techniques rely upon a reduced production of sweat after iontophoresis of acetylcholine or agonist for diagnosis. The acrosyringium to sweat gland ratios of 0.28 (back) and 0.42 (calf) suggest that the majority of sweat glands in the FD patients were deep to the plane of the biopsy, because the acrosyringia counts were not significantly different from the normal number of sweat glands in the same locations as measured from Silastic moulds of pilo-carpine-activated sweating (Willis et al., 1973). The presence of deep hypersecretion suggesting sweat glands could explain the recorded excessive baseline sweat volume. Hypersecretion sweat glands (Low et al., 1983), sweat glands with large sweat droplets (Kihara et al., 1993) and hyperinnervated sweat glands (personal observations) are present in some patients with diabetic neuropathy where they are presumed to be the result of excessive collateral re-innervation (Kennedy et al., 1994).

The presence of VIP in the subepidermal plexus of FD subjects is a new and intriguing finding. VIP is rarely observed in the subepidermal plexus of normal subjects, but has been noted in a few diabetic subjects (G. Wendelschafer-Crabb, unpublished observations; Navarro et al., 1997). This pattern is also reminiscent of results from a prior study on mouse footpad re-innervation following sciatic nerve crush (Navarro et al., 1997; Thompson et al., 1983). VIP-laden fibres normally segregate to sweat glands, with a few following blood vessels or sweat ducts. During re-innervation, the fibres bypass the sweat glands and extend into the SNP and occasionally invade the epidermis. This is rectified within several days as VIP-ir fibres disappear from the superficial dermis, but emerge in the sweat glands. These renegade VIP fibres may be an early indication of attempted re-innervation.

Schwann cells ensheathe all cutaneous nerves, except for the nerve fibre terminations in the epidermis. The Schwann cell marker S-100 protein reveals the typical pattern of dermal nerves and non-neuronal epidermal dendritic (Langerhans) cells. In several FD subjects, we observed what appeared to be empty Schwann cell sheaths probably indicating recent denervation. The presence of empty Schwann cell sheaths and VIP-ir nerves suggests an active denervation process with some regenerative activity and is consistent with the clinical impression that FD is a progressive neurological disorder.

In conclusion, by combining quantitative assessments of sensory and autonomic functions with immunohistopathological examinations of the skin, we confirmed the marked variability of expression of FD. Our findings suggest that some of the variability may result from a process of ongoing denervation and re-innervation superimposed on a lack of initial neuronal development. The absence of immunoreactive CGRP and SP nerves suggests that the FD gene mutation may cause secondary depletions in multiple neurotransmitters whereas the presence of VIP suggests the potential for regeneration.

Acknowledgement

This study was supported by a grant from the Dysautonomia Foundation, New York.

References


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