

PATHOLOGY AND QUANTITATION OF CUTANEOUS INNERVATION

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Table of Contents

INTRODUCTION	
ANATOMICAL FEATURES OF THE SKIN	
METHODS OF CUTANEOUS NERVE ANALYSIS	
Choice of biopsy location	
Biopsy methods	
Staining skin biopsy specimens	
Skin blister methods	
Epidermal sheet preparations	
PCR techniques in skin	
MICROSCOPY	
Characteristics of cutaneous nerves	
Epidermal nerves	
Variation in nerve fiber density	
Epidermal nerves in skin blisters	
Other cutaneous nerves	
Morphological changes in sensory neuropathy	
Correlation with tests of unmyelinated nerve function	
Correlation between epidermal nerve and sural nerve	
Utility of skin biopsy	
CONTRIBUTIONS OF SKIN BIOPSY TO DIAGNOSIS	
Painful sensory neuropathy	
Diabetic neuropathy	
Diabetic truncal neuropathy	
HIV-associated sensory neuropathies	
Friedreich ataxia	
Restless Legs syndrome	
Familial Dysautonomia (Riley-Day syndrome, HSAN Type III)	
Congenital insensitivity to pain with anhidrosis (CIPA, HSAAN Type IV)	
Psoriasis	
Port wine stains	
Leprosy	
Fabry disease	
Sensory ganglionopathies	
Post-herpetic neuralgia	
CADASIL	
Chronic inflammatory demyelinating polyneuropathy (CIPD)	
Postural tachycardia Syndrome (POTS)	
Other conditions	
Neuroaxonal dystrophies	
RESEARCH USES OF SKIN BIOPSY	
Human models of regeneration after nerve injury	
Skin blister: a model mechanical denervation	
Capsaicin: a model for chemical denervation	
Comparison of nerve function with structure	
Animal models using skin biopsy	
CONCLUDING REMARKS	
REFERENCES	
LEGENDS AND FIGURES	

INTRODUCTION

Evaluation of unmyelinated cutaneous nerves obtained by skin biopsy has emerged as a useful method to diagnose and study peripheral nerve disorders.^{27, 45, 48, 52, 63} Until recently work with peripheral nerves was restricted to studies of myelinated nerve fibers because methods available for assessment of small, unmyelinated fibers were limited. With standard electrodiagnostic studies unmyelinated fibers remain “invisible” because nerve conduction studies assess only large sensory and motor fibers. Even assessment by sural nerve biopsy is problematic; electron microscopy is required to visualize unmyelinated fibers and nerve biopsy only gives a “window” into one location along the nerve at a single time-point. Finally, from a functional standpoint, cutaneous sensation is transduced by identifiable nerve fibers that enter their “targets” in the skin; these cannot be identified by nerve biopsies. These limitations and the observations that individuals with sensory neuropathies have spontaneous acral neuropathic pain with marked allodynia stimulated development of methods to identify and quantify unmyelinated nerves in the skin.

Early studies of cutaneous nerves obtained by punch skin biopsy defined the density and distribution of Meissner’s corpuscles and their myelinated nerves in different age groups and in several hereditary disorders. Unmyelinated nerves were not visualized.^{7, 20} The development of sensitive immunohistochemical techniques offered a new approach to identify and quantify small sensory nerves. In particular, immunostaining of skin biopsies for protein gene product 9.5 (PGP 9.5), a neuronal ubiquitin carboxy terminal hydrolase,¹⁰⁶ has been used by a number of groups to visualize the subpapillary plexus of small myelinated and unmyelinated nerve fibers.

Epidermal nerve fibers have received the greatest scrutiny, mainly because they appear to be early indicators of neuropathy and adequate samples can easily be obtained for quantitation.^{18, 46, 63, 110} These fibers include both A delta and C fibers that convey “slow” nociception. We developed robust normative data,⁶¹ and have demonstrated a usually distally dominant pattern of epidermal nerve fiber loss in diabetic neuropathy,⁴⁷ HIV-associated sensory neuropathies,⁸² and idiopathic small fiber sensory neuropathies.³⁵ In patients with severe neuropathy, decreased epidermal densities are most severe distally with less marked reductions found at progressively more rostral levels and prominent predegenerative axonal swellings identified even at asymptomatic sites. In fact, abnormalities of cutaneous innervation are found in some individuals with normal tendon reflexes at the ankles, normal sural nerve action potential amplitudes, and normal quantitative sensory tests. Epidermal denervation also occurs in individuals with spontaneous allodynic pain.^{34, 78} While the precise structural correlates of allodynia remain uncertain, it can clearly occur even with marked depletion of A delta and C fibers in the skin.

The association of neuropathic pain with epidermal nerve loss is, en face, paradoxical because nerve fiber loss is traditionally related to loss of sensation without pain. Neuropathic pain has been correlated with epidermal nerve loss in small fiber sensory neuropathy, post herpes neuropathy, HIV sensory neuropathy and diabetic neuropathy. Explanations for this apparent paradox include possible changes in both peripheral and central nervous systems. In peripheral neuropathy caused by persistent action of an etiological agent, continuous spontaneous pain can result from ectopic discharges in peripheral nociceptive fibers.⁷⁷ Alternatively, sensitized nociceptors responding with reduced threshold to weak stimuli that normally evoke touch, warm or cool sensations also evoke pain (hyperalgesia). Capsaicin induced mechanical and heat hyperalgesia is one example. Another is the neuronal excitability that results from increased voltage-gated Na⁺ currents after exposure to serotonin, prostaglandin E₂ or adenosine, agents that produce tenderness or hyperalgesia.²⁶ Likewise, products of nerve degeneration might increase nerve excitation by unmasking proteins normally hidden from immune surveillance (e.g. P0, P2). Resultant immune cell activation exposes surviving nerves to inflammatory cytokines and other substances that alter threshold to stimuli.¹¹¹ There is growing evidence that injury to nociceptors leads to increased firing, not only in injured axons, but also in uninjured neighboring axons¹¹⁵ and perhaps in dorsal root ganglia neurons at an adjacent level.⁴ In fact, selective injury of a ventral root causes hypersensitivity of C fiber afferents in an adjacent root.¹¹⁶ Subsequent activation of nociceptors is believed to produce central sensitization and secondary hyperalgesia. One mechanism is by central terminals of injured A δ fibers that develop nerve sprouts from deep dorsal horn lamina into superficial laminae. If these synapse with nociceptive neurons the neurons could become susceptible to activation by otherwise innocuous peripheral stimuli.¹¹⁴ In addition, a shift of descending modulation from inhibition to facilitation can contribute to hypersensitivity.⁸⁴

ANATOMICAL FEATURES OF THE SKIN

Skin separates the external environment from internal tissue while providing sensory input, protection from pathogens and allergens and a water barrier. The layered structure of skin imparts characteristics essential to these functions (Fig. 1). The distinct dermal and epidermal layers of skin comprise the major compartments of cutaneous structure. The epidermis is the topmost living layer of skin and consists of layers of keratinocytes that are replaced in approximately a 28 day cycle in man as they are desquamated to form the cornified surface layer of dead cells that provides the water barrier. Intermixed among the vital keratinocytes are basal melanocytes, dendritic Langerhans cells, and sensory nerve terminals. The epidermis is separated from the dermis by the dermal-epidermal basement membrane. The area of dermis

immediately internal to the epidermis, the papillary dermis, contains a capillary plexus with periodic loops that reside within dermal protrusions into the epidermis called dermal papillae. Within the matrix of the dermis, cutaneous structures include hair follicles with associated arrector pilorum muscles and sebaceous glands, blood vessels, nerve bundles, and sweat glands (Fig. 1).

The nerves to skin arise from sensory and motor neurons residing in dorsal root ganglia and sympathetic ganglia. Bundles of nerves enter the skin deep in the dermis and course toward the skin surface giving off axons to innervate the associated end organs. Unmyelinated nerve fibers comprise the vast majority of cutaneous innervation to the above dermal structures. The few myelinated nerve fibers terminate at hair follicles, Meissner's corpuscles and Merkel complexes. When the vertically oriented nerve bundles enter the superficial papillary dermis they form a horizontal subepidermal neural plexus (Fig. 2). Epidermal nerve fibers branch from this plexus and, while penetrating the dermal-epidermal basement membrane to enter the epidermis, they lose their Schwann cell ensheathment and collagen collar.¹² Within the epidermis they extend between keratinocytes to the surface of the vital epidermis. Autonomic nerves to sweat glands enwrap the coiled sweat tubules in such a dense pattern that they virtually cover much of the surface (Fig. 1 Sg). The autonomic innervation of the arrector pilorum muscles is easily distinguished within the muscle by a characteristic wavy pattern. The complex innervation pattern of hair follicles is comprised of both myelinated and unmyelinated fibers, with specialized nerve endings at the base and along the shaft of the hair.^{22, 28, 85} Fine unmyelinated nerve endings form a mesh-like network covering larger arteries in the deep dermis, but only one or two nerves are on the more superficial smaller arterioles (Fig. 1 Ar).

Epidermal nerve fibers are constantly remodeling because of the turnover of the epidermal layers due to migration and shedding of keratinocytes. In addition, epidermal nerve fibers respond to injury by regenerative regrowth and collateral sprouting. The neurotrophic requirements of epidermal nerve fibers have been studied and 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. The other half are predominantly GDNF-responsive neurons that express the receptor c-ret⁶⁵ as well as a series of other markers. These markers include the ability to bind a specific Griffonia lectin for fucosyl moieties, isolectin B4, that provides a convenient, but not completely specific, marker for the GDNF-dependent population. Finally, most nociceptors bear the receptor for the chili pepper toxin, capsaicin. This receptor, vanilloid receptor type 1 (VR1), is the basis for the burning pain elicited by 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.¹⁰ A related receptor, vanilloid receptor-like receptor type 1, has recently been identified; primarily on somewhat larger sensory neurons probably giving rise to A delta fibers. Vanilloid receptor-like receptor type 1 responds to very high temperatures ($>52^{\circ}\text{C}$).¹¹ In the human, almost all of the epidermal axons are VR1-positive.⁸² Epidermal fibers containing the peptides CGRP and substance P are NGF-dependent axons. In some animals these commonly enter the epidermis but in human skin they usually terminate near capillary loops in the dermal papilla. Attempts to visualize immunostain for other peptides in human nerves, including the isolectin B4, have been unsuccessful.

METHODS OF CUTANEOUS NERVE ANALYSIS

Choice of biopsy/blister location

The minimal invasiveness and negligible scarring remove most cosmetic objections to skin biopsy and skin blister procedures. When choosing a biopsy site, the susceptibility to nerve loss with neuropathy and the availability of normal data should be considered. Areas subject to trauma or with scars should be avoided. Site selection also depends upon the purpose for which the biopsy/blister is being considered, for example for diagnosis or for longitudinal study. Diagnosis of small fiber pathology can be made from a distal skin location where the clinical examination shows decreased sensation; particularly decreased sensitivity to mechanical (pin) and thermal (hot) pain stimuli. In patients with neuropathy, biopsies from the dorsum of the foot, distal calf and proximal calf frequently contain a reduced number of epidermal nerves or may be devoid of epidermal nerve fibers altogether. The foot has the advantage of being more distal, but its nerve is subject to trauma. Supplementary biopsies at more proximal levels, e.g. calf, thigh or upper extremity, can yield additional information about the severity and distribution of small fiber loss (Fig. 3a-d). If the plan is to follow the course of neuropathy or the response to therapy it is best to select a biopsy location with a definite reduction of epidermal nerves, but with a sufficient nerve residual in the epidermis and subepidermal neural plexus to either provide a substrate for nerve regeneration or allow documentation of further degeneration. This can usually be accomplished by removing biopsies from the upper sensory level, if present, and from one or two more proximal locations, keeping in mind that the loss of epidermal nerves is generally more severe than would be inferred from the clinical symptoms or findings. Future biopsies for comparison should be performed horizontally adjacent to the original biopsy at least 5-10 mm away from scarring and within the same peripheral nerve distribution. Normal epidermal nerve fiber values have been determined for proximal and distal anterolateral thigh, proximal and distal posterior-lateral calf, dorsum of the foot over the extensor digitorum brevis muscle, over the

first dorsum interosseus muscle, proximal volar forearm and the paraspinal area at T4 - T5. The most frequently biopsied locations selected to assess a suspected length-dependent neuropathy are thigh and calf. The flexibility of the technique allows biopsy or blister to be performed safely almost anywhere on the body. Thus, a localized sensory complaint, e.g. a thoracic radiculopathy, can be examined by biopsy of the symptomatic site and a mirror-image control, with postherpetic neuralgia being a possible exception because there may be contralateral changes in the unaffected side.⁷⁶

Biopsy methods

Skin biopsy is commonly performed with a 3 mm diameter punch instrument (Fig. 4) (Acupunch, Acuderm Fl.) with local anesthetic. The wound rarely requires cautery or suture, and the infection rate in the lower extremity is <1:200. A shallow biopsy is adequate if interest is limited to epidermal nerves. A deeper biopsy (3-4 mm) is necessary to acquire sweat glands, the full hair follicle, subcutaneous fat, and larger arterioles. The biopsy is immediately placed into 4° C fixative and stored overnight then transferred to 20% sucrose phosphate-buffered saline for cryoprotection and storage. The biopsy can be held in sucrose phosphate-buffered saline at 4° C for up to a month or frozen for more prolonged storage. Formaldehyde based fixatives provide the best tissue and antigen preservation. Glutaraldehyde destroys the antigens and should not be used. Zamboni (2% paraformaldehyde, picric acid), Lana (4% formaldehyde, picric acid) and PLP (paraformaldehyde, lysine, periodate) fixatives all provide optimal preservation. If these fixatives are not available, 10% formalin preservation for 12-18 hours is an alternative, but produces a more fragmented appearance of the epidermal nerves and is sub-optimal. For multicenter studies or trials, where the biopsy will be performed at a location geographically removed from the processing laboratory, the specimen should be placed into fresh cold fixative for 12 to 24 hours, then transferred into cryoprotectant for overnight shipping on wet ice.

Staining skin biopsy specimens:

Nerve evaluation from skin biopsy relies on immunohistochemical localization of neural antigens within skin tissue sections. This is accomplished by applying a primary antibody directed against the antigen of interest followed by a labeled secondary antibody directed to the primary antibody. The major primary antibody used for the localization of nerves in skin biopsy recognizes the pan neuronal marker PGP 9.5 (Ultraclone, Wellow, UK; Chemicon, Temecula, CA) that is present in all cutaneous nerves. Localization of additional antigens can aid in diagnosis, but may not be necessary in all situations. Several antigens can be localized within a single tissue section by using secondary antibodies that recognize and react with the

immunoglobulin (IgG) of the primary antibodies. Typically rabbit, mouse and goat primary antibodies are localized with donkey antiserum specifically prepared to recognize IgG from only a single species (rabbit, mouse, or goat). Complementary markers such as fluorophores or enzymes label each of the species-specific secondary antibodies. Each fluorophore's emission wavelength peak must be distinct so that it can be viewed individually with appropriate light filters (Fig. 5). Alternatively, enzymes such as peroxidase or alkaline phosphatase are reacted with a variety of available substrates to provide reaction products of different colors. All secondary antibodies should be derived from the same species (eg. donkey) and must be from a species different than hosts of the primary antibodies. Secondary antibodies used for multiple staining must be pre adsorbed with IgG from all species other than the primary antibody being localized.

Frozen 50-100 μm thick sections are cut perpendicular to the epidermal surface. The thicker sections provide greater sampling and visualization of anisotropic nerve fibers as they weave through the skin. Sections are floated in staining reagents containing triton X-100 to promote penetration of antibodies. Samples are washed and antibodies are diluted in a solution of phosphate-buffered saline, 1% normal donkey serum, and 0.3% triton X-100. Sections are incubated in 5% normal donkey serum so that nonspecific binding of IgG will be "blocked". Sections are incubated in primary antibodies (e.g. rabbit anti PGP 9.5 and mouse anti type IV collagen (Chemicon, Temecula, California). with gentle rotation for 5 hours at room temperature and overnight at 4 °C then washed in three changes of wash buffer, 1 hour each with gentle rotation. This is followed with overnight incubation in labeled secondary antibody to the rabbit and mouse primary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania) then washed three times. The secondary antibodies are labeled with either peroxidase or a stable fluorescent marker.

Peroxidase markers are reacted enzymatically to produce a precipitate at the site of the antigen. The peroxidase reaction product is visible by bright field microscopy and by electron microscopy. With the peroxidase reaction the basement membrane is localized as being just below the basal membrane of basal keratinocytes. Fluorescent markers require no further reaction before being studied by fluorescence or confocal microscopy. A new generation of fluorescent markers, Cy dyes 2, 3 and 5 (Jackson ImmunoResearch) are very bright and remain stable for several years in permanent mountants. The markers are useful for labeling two or three antigens within the same section. Labeling nerve with Cy 3 and basement membrane with Cy 2 and endothelial cells with *Ulex europaeus* agglutinin type I with Cy 5 provides a vivid image of skin structure, innervation and vasculature (Fig. 6).

Non-immune serum specificity controls are essential and should be run with each biopsy. Accurate counts of epidermal nerve fibers, interpretation and quantitation are

difficult when sections fold or curl during drying of the epidermis. For optimal viewing and quantification the epidermis should lie flat on the slide. Premounting sections in a small drop of Nobel agar prevents drying, adheres the sections, and holds the surface of the stratum corneum perpendicular to the slide. The premounted section is dehydrated in alcohol, cleared with methyl salicylate and mounted on a cover slip in DPX (Fluka). This provides a permanent preparation and is useful for slides labeled with Cy fluorophores and some enzyme reactions. Glycerol can be used to coverslip tissue without dehydrating the specimens and is useful for short-term storage.

Skin blister method

Skin blister is a painless, bloodless method to acquire, quantify and plot distribution of epidermal nerve fibers.⁴³ The blister roof, which consists of pure epidermis and epidermal nerve fibers, separates from the dermis on a plane between the basal membrane of basal keratinocytes and the dermal-epidermal basement membrane. Blisters are made by applying suction to a blister capsule (WR Medical Electronics, Stillwater, MN) that is adhered to shaved, cleansed skin with double sided tape (Fig. 7). The surface of the capsule resting on skin contains one or more round openings to accommodate the desired number and size of blisters. Tegaderm tape (3M, Maplewood, MN) disks, cut slightly smaller than the size of the desired blister (e.g. 3 mm diameter), are placed on the skin surface to conform to the openings in the blister capsule. These prevent over stretch of the blister roof. The capsule is secured to skin with an elastic bandage, leaving the transparent top of the capsule uncovered to permit the examiner and subject to observe blister formation and the capsule is evacuated to a negative pressure of 300 mm Hg. The small volume of the capsule makes it imperative to have tight seals to prevent leakage. A reservoir placed in series with the tubing between the pump and capsule enhances blister formation by equilibrating small leaks. We use a commercially available mechanical pump to evacuate the capsule but an inexpensive hand held pump is adequate when a single capsule is used. Initially small blisters form, then coalesce to form a full blister after 20 to 40 minutes. The time is less with increasing subject age and skin temperature but also depends upon skin location, and successful maintenance of negative pressure. Warming the area with a heating pad hastens blistering. After the capsule is removed the blister roof with tape disk intact is excised with micro scissors and placed in cold Zamboni fixative overnight at 4°C. Fixed blisters are cryoprotected with 20% sucrose in 0.1M phosphate buffered saline until processed. Prior to processing, blister roofs are frozen on dry ice. Specimens are processed as whole mounts without sectioning, otherwise the procedure is identical to that for thick sections.

Epidermal sheet preparations

This is a modification of the standard punch technique that allows for separation of the epidermis from the dermis before fixation.⁵ The technique is particularly useful for examining the epidermis in the horizontal plane without dermal vessels or nerves. In addition, the epidermal sheet can be used for protein or mRNA assays, as described below, rather than immunocytochemical staining. Briefly, after obtaining the punch biopsy, the specimen is bathed in EDTA for 2 hours, then the epidermis can be easily removed from the dermis, fixed, and immunostained as above. This preparation permits the identification of the nerve fiber terminals within the epidermis without blood vessels or the dermal structures. The sheet can be stained in exactly the same way to identify PGP immunoreactive nerve fibers.

PCR techniques in skin

It is feasible to quantify neurotransmitter or neurotrophin mRNA levels in either the epidermis (using the sheet preparation), or the dermis (using the standard punch biopsy). In HIV/AIDS there is interest in the concept that specific antiretrovirals may affect mitochondrial DNA levels by inhibition of gamma DNA polymerase.¹³ Subcutaneous fat is a rich source of mitochondria. We use the subcutaneous fat, which is usually trimmed and discarded from punch skin biopsies, to measure levels of mitochondrial DNA with real-time PCR²³ in HIV-seropositive patients receiving antiretrovirals. Specific antiretrovirals, the dideoxynucleosides, reduce mitochondrial DNA levels by inhibition of gamma DNA polymerase,⁶⁰ and thus this technique may provide a convenient measure of the severity of dideoxynucleoside toxicity and the metabolic complications common in individuals treated with these drugs long-term.¹⁴

MICROSCOPY

Thick sections are advantageous for viewing the full meandering course of nerves from the nerve trunk in the dermis throughout their ramifications to the endings in sweat glands, arrector pili, hair follicles, arterioles or epidermis. Sections are first scanned under a 10x objective in the microscope to evaluate the orientation and flatness of the section and staining of nerves, blood vessels and cutaneous organs. The course and ending of single unmyelinated nerve fibers are better visualized with a 20x (or higher) objective, as for example following epidermal nerve fibers through basement membrane and the pathway between keratinocytes to their endings under stratum corneum. Images taken from thick sections are often marred by out-of-focus blur because the focal plane of a 20x objective is about 2 μ m. Objects in 50 to 100 μ m sections that are above and below this focal plane are out of focus. Some laboratories use thinner sections to obtain sharp images by conventional microscopy. This is counterbalanced by the inability to follow nerves for more than a short

distance in a section and reduced sampling.

Confocal microscopy of thick sections is one solution to this problem, although the added complexity may not always be necessary for simple clinical questions. The confocal microscope takes in-focus images while retaining the advantages of working with thick sections. This is accomplished by “optical sectioning” the fluorescent labeled thick sections into a series of smaller increments that correspond to the focal length of the lens being used. A confocal z-series comprised of multiple images taken at incremental focal planes through out the tissue eliminates out of focus blur and provides an in focus three dimensional view of the tissue (Fig. 8). The operator can select the top and bottom of the section to be imaged, the size of the z-focus increments, and the fluorophores to image before collecting the z-series. A laser scanning confocal microscope requires about 30 minutes to scan double stained sections (two antibodies) at 2 μm increments through 50 μm of tissue. The non-laser spinning disk confocal system (eg. CARV) can collect equivalent images in about 3 minutes. The z-series is projected into a single in-focus image for viewing. The series can also be “paged through” to follow nerves throughout the tissue, or the series can be rendered into a three dimensional object.

QUANTITATION OF CUTANEOUS NERVES

Epidermal nerves

Standard punch biopsies removed from anatomical locations that are often sampled to diagnose neuropathy contain an adequate sample of unmyelinated epidermal nerves and subepidermal neural plexus for detecting neuropathic changes. Epidermal innervation can be readily quantified using either confocal microscopy^{45, 78} or direct counting of chromogen-stained specimens.^{16, 32} The diagnosis of neuropathy is dependent upon finding a reduced density of epidermal nerves. Abnormalities of nerve morphology provide valuable secondary help. Epidermal nerve fibers can be quantified accurately because after they leave the nerve bundles of the subepidermal nerve plexus and before they enter the epidermis, they separate as single nerve fibers. In thick sections the entire intraepidermal segment of most epidermal nerve fibers can be followed rather than isolated nerve segments as is necessary in thinner sections. A set of almost identical counting rules evolved simultaneously in both laboratories (Fig. 9): A) Count each nerve as it crosses the basement membrane of the epidermis. Because epidermal nerve fibers often branch below the basement membrane as well as in the epidermis it is necessary to establish a standard counting site. Most investigators have agreed in practice to count as one unit each nerve that penetrates the basement membrane, even if there is branching within the epidermis. In peroxidase-stained sections the membrane and cytoplasm of basal keratinocytes stain darker than surrounding tissue. As the basal membrane is only a few μm above the dermal-epidermal basement membrane it can be used as the

site for counting penetrating epidermal nerve fibers. In sections that are double stained for PGP 9.5 in nerve and type IV or type VII collagen for basement membrane and labeled with compounds that fluoresce at different wavelengths it is possible to directly observe the epidermal nerve fibers as they penetrate basement membrane. If confocal images of thick sections are available the optical section in which the epidermal nerve fiber penetrates the basement membrane is located by paging through the z series of optical sections. B) Nerves that branch after crossing the basement membrane are counted as one. Epidermal nerve endings in normal subjects are usually quite simple – often free of branches or with minimal branching as they course through the epidermis. Nerves of patients with neuropathy appear more complicated with increased branching. To address both nerve loss and collateralization of surviving axons, the number of fibers crossing the basement membrane is used to provide counts (how many nerves) while analysis of tracing data provides information about the complexity (length and branching pattern) of the nerve (how much nerve). C) Nerves that split below the basement membrane are counted as two units. Single epidermal nerve fibers arise as branches from nerve bundles of the subepidermal neural plexus. Each branch is counted as it penetrates the basement membrane. D) Nerves that appear to branch at the basement membrane are counted as two units. E) Nerve fragments that cross the basement membrane are counted. Epidermal nerve fibers that appear short are counted. They may be short fibers or the roots of fragments that terminate in adjacent sections. F) Nerve fibers that approach but do not cross the basement membrane are not counted. Nerve fibers that terminate immediately proximal to the basement membrane are common in neuropathy. They frequently follow the basement membrane a short distance before ending with a terminal swelling. G) Epidermal nerve fragments that do not cross the basement membrane are not counted. This rule has been followed in all publications by the Minnesota group. In previous publications by the Johns Hopkins group, such epidermal nerve fragment were included in the total numbers, but will not be included in future counts.

Tracings made of epidermal nerve fibers with a software program (NeuroLucida, MicroBrightField, Vermont) provide the number of nerve fibers, branch points, nerve fiber length and coordinates of basement membrane penetration (Fig.10). This information is useful because in some patients nerve branching and length increase in apparent compensation for a decrease in nerve number. The data are also useful for analysis of nerve distribution.

Results of epidermal nerve fiber density are commonly expressed as number of epidermal nerve fibers/mm of epidermis, providing a “linear density”. Comparison of such values from different reporting laboratories requires that the thickness of the section be known as thicker sections contain more nerve fibers. Results can also be

reported as epidermal nerve fibers per sq. mm area of skin surface for easier comparison.^{32, 45} Measurement accuracy is potentially adversely influenced by compression of the section between the slide and cover slip and tissue shrinkage during preparation. For accurate comparison, these factors must be taken into consideration. Our laboratories report epidermal nerve fiber density based on nerve counts made in the equivalent of 50 μm thickness of sectioned tissue, making adjustments for changes resulting from dehydration and coverslip compression.

Manual counting should be performed on at least 3-4 non-adjacent sections taken from different places within the biopsy. After manual counting the number of individual epidermal nerve fibers, the length of the epidermis is measured using Bioquant (Bioquant V, R&M biometrics, Nashville, TN). Counting rules should be established a priori for each laboratory, and preferably only one observer used for research studies involving serial specimens. We have compared manual counting with light microscopy to stereological techniques and demonstrated that the manual counting produced comparable densities, but was much quicker.¹⁰¹ Reliability is highest when at least four sections at each anatomical site are counted. A technician can be trained to perform the manual quantitation, with excellent inter- and intra-observer reliability (>0.95).⁶¹ We have also demonstrated that different laboratories can achieve reasonable inter-laboratory reliability.⁹⁸ For example, mean (\pm SEM) inter-observer reliability was 12.2% \pm 1.1 for each biopsy site and mean intra-observer variability was 10.2% \pm 1.5 for individual sections. The variability between laboratories was 25.5% and the correlation coefficient for both intra-observer and inter-observer reliability was 0.98. The correlation coefficient between sites was 0.94. There was no relationship between absolute intra epidermal nerve fiber counts and reliability.

Normal epidermal nerve fiber density

The density of epidermal nerves varies depending upon the biopsy site.⁴⁰ In general a consistent gradient in intra-epidermal nerve fiber density exists from proximal to distal sites in the lower extremities with minimal effects of race or sex. Significantly higher intra-epidermal nerve fiber densities were noted in a younger group aged 10 to 19 years, but over the age of 20 yrs there is little change up to 80 years of age (Fig. 11).^{15, 78} Stereological quantitation has been compared with linear density estimation and is significantly associated with a correlation coefficient of 0.79 ($p=0.001$). With formalin fixation (which tends to underestimate by about 20% compared to PLP fixation) the normative range for intra-epidermal nerve fiber density (including intra-epidermal fragments) in healthy normal controls is 21.1 ± 10.4 per mm (mean and standard deviation) in the thigh, with a 5th percentile of 5.2 fibers/mm. At the distal leg the normative range is 13.8 ± 6.7 , with a 5th percentile reading of 3.8 fibers/mm. Using the cutoff values for the 5th percentile for

intraepidermal nerve fiber density at the distal part of the leg, the diagnostic efficiency (percentage correctly classified) was 88%, with a specificity (percentage true negative) of 97% and a sensitivity (percentage true positive) of 45%. Positive predictive value is 92% and negative predictive value is 90%. Although there is a relatively wide biological variation in the intraepidermal nerve fiber density, the distribution of densities at the distal part of the leg permits the delineation of lower limit of normal based on the 5th percentile value for healthy controls. The high specificity of the measure increases the positive predictive value of the test, and is therefore ideal for its anticipated use, namely to verify the presence of a disease for which there may be little clinical or electrophysiological evidence, or when the clinician must be virtually certain of a diagnosis, for example, before initiating some form of therapy. The relatively low sensitivity of the measure implies that a normal intra-epidermal nerve fiber density does not rule out the presence of a sensory neuropathy. Normative values epidermal nerve fiber density for six body locations have been determined at the Kennedy laboratory (Table 1).

Epidermal nerves in skin blisters

Whole-mounted blister and epidermal sheet preparations have sampling advantages over sectioned skin biopsies. The “bird’s eye” perspective of epidermal nerve fibers in these 3 mm diameter (area = 7.1 mm²) preparations presents for visualization and analysis the same number of nerves contained in the combined sections of a 3 mm biopsy (Fig. 12). Using a 20x objective lens we count the epidermal nerve fibers in 5 representative segments of the blister roof, each measuring 0.4 x 0.3 mm. The 0.6 mm² skin surface area is approximately 12% of the whole blister roof. The results, expressed as counts of epidermal nerve fibers per surface area of skin, vary according to body location in the same manner as that observed with biopsies. Epidermal nerve fiber density determinations from biopsies and blisters show a high degree of correlation ($r = 0,71$; $p < 0.005$).⁴⁹ The blister roof also provides an advantageous oversight of the horizontal territory of individual epidermal nerve fibers and the distribution of all epidermal nerve fibers within the specimen. Changes in epidermal nerve distribution caused by reorientation of nerves in their course through the epidermis by elongation, branching or loss of branching probably occurs normally during the continual, regeneration of the epidermis, perhaps similar to normal reshaping of the terminals of motor axons.⁵⁷ We suspect that exaggerated redistribution of epidermal nerves may be the earliest reaction of epidermal nerves to neuropathy (Waller, L personal observations).

Other cutaneous nerves

In general, dermal nerves are more difficult to quantify than epidermal nerve fibers. The sub epidermal neural plexus that occupies the papillary dermis, is often less

dense in subjects with neuropathy. These bundled nerve fibers cannot be accurately counted or traced. The volume of nerve in the subepidermal neural plexus has been estimated based on the total fluorescence⁵⁵ but accurate quantification of nerves in the subepidermal neural plexus by thresholding from background is complicated by the positive immunoreactivity of dermal fibroblasts for PGP 9.5. A semi-quantitative rating system can be used to assess the density of the plexus (Fig. 13): 0 = normal, -1 = mild (50%) loss, -2 = severe (75%) loss, -3 = a trace, -4 = no nerve, +1 = hyperinnervation. A similar semi-quantitative rating system can be used to quantify sudomotor nerves. These rating systems require that the viewer have an excellent interpretation of “normal”. Other techniques utilizing image analysis to quantify cutaneous nerves have recently appeared¹⁰⁷ but have not yet been widely accepted.

The density of sudomotor nerves around secretory tubules of sweat glands can be quantified by calculating nerve volume per sweat gland volume in confocal images.⁴⁵ Sudomotor nerve density is below normal in patients with severe neuropathy (Fig. 14) whereas sweat glands often appear well innervated in mild or moderate neuropathy, even though the secretion of sweat is decreased. This suggests that quantitation of sudomotor nerves may not be helpful for early diagnosis of diabetic neuropathy. A possible explanation for this apparent discrepancy is that sweat glands in non hairy human and murine skin receive multiple sudomotor nerves. Multiple innervation facilitates collateral reinnervation after a partial nerve lesion⁴⁴ but sweat volume may be compromised. The occasional finding of increased sweat gland innervation⁴⁵ and of increased sweat secretion⁵⁸ in proximal skin locations of diabetic subjects may represent localized nerve growth in response to a general stimulus for collateral reinnervation during the early denervation stage of neuropathy.

We have not attempted to quantify the innervation of arrector pilorum muscles, hair follicles or arteries. Quantitation of the nerves to arrector pilorum muscles is possible with the quantitative methods described for sweat glands. This may be useful in the clinical diagnosis of peripheral autonomic nerve disease if adequate sampling of these muscles is achieved. Hair follicles have an abundant supply of associated myelinated and unmyelinated nerve fibers but the relatively few follicles visualized in biopsy of non-hairy skin create a sampling problem. Evaluation for clinical purposes may be possible in the scalp, depending upon the number of follicles available for analysis. The wide distribution of cutaneous arteries and arterioles and the variable number of nerves per vessel complicate attempts to quantify vasomotor nerves.

Morphological changes in sensory neuropathy

In healthy subjects, epidermal fibers arise from subpapillary nerve fiber bundles and run towards the stratum corneum in either branched or unbranched patterns.

The fibers are usually thin, sometimes varicose, and often end with a single club-like enlargement. In cases of sensory neuropathy intraepidermal nerve fibers show distinct morphological changes. Fibers frequently show more tortuous courses and more complex ramification.^{16, 53} In addition to the increased branching complexity, there often is increased varicosity with stubby, rounded projections, intra-axonal swellings, and claw-like terminals. In general, these morphological features, particularly the intra-axonal swellings, are more evident at the thigh than in regions below the knee (Fig. 3c). The composition of intra-axonal swellings remains uncertain. Neurofilament stains are negative and EM studies to date have been relatively inconclusive, suggesting that the swellings represent collections of organelles. Because of their proximal location to sites with epidermal denervation, we believe that swellings represent pre-degenerative changes. In models of epidermal regeneration, clusters of terminal nerve fiber swellings appear more commonly in neuropathic individuals suggesting that such clusters of swellings may represent aberrant efforts at nerve sprouting, or microneuromas.

The interaction of nerve with basement membrane may be altered in neuropathy as evidenced by morphological irregularities found in patient biopsies. Some nerve fibers follow a course along the dermal side of the basement membrane but do not penetrate – frequently ending in a bulb-like swelling (Fig. 3d). In patient biopsies with low nerve fiber density, nerves entering the epidermis often branch at the point of penetration through the basement membrane and form a tuft of nerve endings that extend into epidermis. Similarly, clustering of epidermal nerve fibers is frequently seen in disease states, wherein multiple nerve fiber penetrations are interspersed with relatively long segments of nerve-free epidermis.

Correlation with tests of unmyelinated nerve function

Relatively few studies have examined the relationship of epidermal nerve fiber densities to other tests of nerve function.

Heat pain has been examined in humans using a chemical (topical capsaicin) denervation model. While no correlation was found with heat pain sensitivity and epidermal nerve fiber density using a large (900 mm²) diameter probe, statistically significant correlations between fiber density and heat pain sensitivity were found when a small (7.1 mm²) probe delivered the stimulus.⁵⁰ This apparent incongruity is believed to result from the spatial summation of surviving epidermal nerves plus stimulation of the proximal stumps of degenerated epidermal nerves in the dermis (some of which may be sensitized by the degeneration of the nerve ending) that occurs when the larger probe heats a deeper area. The smaller probe heats a more isolated shallow area, with fewer nerve endings activated.

Comparisons of intraepidermal nerve fiber densities with just noticeable difference (JND) thresholds for cooling and vibration levels were made in HIV

sensory neuropathy using the CASE IV instrument (WR Company, Stillwater, MN) and were inversely correlated. Since high JND indicates decreased sensitivity to sensory stimuli, it is not unexpected that the correlation estimates were negative. A correlation analysis based upon the percentile distribution rather than the JND is another statistical approach, but given that the sample size was relatively small and that many of the subjects had mild neuropathy, this analysis was based on the categorization of subjects as normal/abnormal based on their JNDs. Surprisingly, intraepidermal nerve fiber density inversely correlated more closely with vibratory threshold than with cooling threshold. Vibration is subserved by A-beta fibers that are not present in the epidermis while cool threshold detection is mediated by A-delta fibers, which may be included in measurements of intraepidermal nerve fiber densities. An association with C-fibers, which subserve noxious heat and mechanical pain and represent the bulk of epidermal nerve fibers was not included in the QST battery. The inverse correlation of intraepidermal nerve fiber density and vibratory threshold may be partially explained by concurrent involvement of large and small fiber nerves. Furthermore, the fact that only a minority of subjects (43%) had abnormal epidermal nerve fiber densities at the distal leg at baseline suggests that the severity of the neuropathy in these subjects was relatively mild (and perhaps that the epidermal denervation in these subjects had not progressed to the ankle).⁸² In another study of 32 patients with painful, burning feet there was no concordance between the results from QSART, QST (vibration and cooling by CASE IV) performed on the upper and lower extremities and skin biopsy from the distal calf in which 28 (87.5%) had a reduction of intraepidermal nerve fibers.⁷⁸

Intra-epidermal nerve fiber densities have also been assessed in small fiber and autonomic neuropathies, and compared with autonomic screening tests. In a study that was designed to quantify the severity of autonomic impairment in patients with painful neuropathy excellent correlation was observed between the quantitative sudomotor axon-reflex test (QSART) and cooling abnormalities and loss of intraepidermal nerve fiber density.⁷³ The QSART quantitatively evaluates the post ganglionic sympathetic sudomotor axon.⁵⁸ Also, see under Postural tachycardia syndrome.

Correlation between epidermal nerve and sural nerve

Twenty six patients with neuropathic complaints had sural nerve morphometry and determination of epidermal nerve fiber density at the distal part of the leg. Non-parametric correlations were used because of the non-linearity of the values. Intraepidermal nerve fiber density correlated with the densities of total myelinated fibers within the sural nerve ($r=0.57$, $p=0.0011$), small myelinated ($r=0.53$, $p=0.029$), and large myelinated fibers ($r=0.49$, $p=0.0054$). There was a trend towards an association between intraepidermal nerve fiber density and sural nerve unmyelinated

nerve fiber densities ($r=0.32$, $p=0.054$). Sensory nerve action potential amplitudes and large myelinated nerve fiber densities were highly correlated ($r=0.87$, $p<0.0001$). Intraepidermal nerve fiber density and sural nerve small fiber measures were concordant in 73% of patients. In 23% of cases, reduced intraepidermal nerve fiber density was the only indicator of small fiber depletion. It was usually normal in acquired demyelinating neuropathies and where the clinical suspicion for neuropathy was low. The inference is that determination of distal leg intra-epidermal nerve fiber density may be more sensitive than sural nerve biopsy in identifying small fiber sensory neuropathies and that intra-epidermal nerve fiber density and assessments of large nerve fibers by nerve biopsy and electrophysiology are all useful for characterizing sensory and sensory-motor neuropathies.³¹

Utility of Skin Biopsy

It is important in clinical practice to have a good sense of the utility (and limitations) of skin biopsy for evaluation of the peripheral nervous system. The biopsy can only provide information about the most distal terminals of sensory nerves and autonomic where they meet their target organ. Interpretation can be affected by local trauma, other diseases or biopsy artifacts. Nonetheless, biopsy of proximal and distal sites can provide inbuilt intra-individual controls. We have found the punch skin biopsy technique to be clinically useful for defining presence and severity of focal neuropathy, of distally dominant sensory neuropathy and differentiation of neuropathy from radiculopathy. It remains to be conclusively demonstrated that skin biopsy can track changes in neuropathies over time, or with regenerative treatments, although the technique has this potential.

CONTRIBUTIONS OF SKIN BIOPSY TO DIAGNOSIS

Quantitation of epidermal nerve fiber density and morphology has been helpful for evaluating several clinical disorders.

Painful sensory neuropathy

In 20 patients with painful sensory neuropathies, intra-epidermal nerve fiber density was significantly reduced compared with age-matched controls, even in regions proximal to the areas of clinically identifiable sensory abnormalities. For example, patients with grade 1 neuropathy in whom sensory abnormalities were restricted to the toes and feet had significantly reduced intraepidermal nerve fiber densities at the calf. Cooling thresholds on quantitative sensory threshold testing (CASE IV device) did not correlate with intraepidermal nerve fiber density or the clinical grade of sensory neuropathy (Fig. 15). Intraepidermal nerve fiber density

from calf and thigh correlated against clinical estimates of neuropathy severity.³⁵

The clinical features, natural history, and neuropathology were described for 32 patients presenting with “burning feet,” thought to represent idiopathic small fiber sensory neuropathies. Two clinical patterns were apparent based on natural history and anatomical location of cutaneous denervation. Most patients (28 of 32) presented with neuropathic pain that was initially restricted to the feet and toes, but extended more proximally to involve the legs and hands. Intra-epidermal nerve fiber density was most severely reduced distally, with more normal intra-epidermal nerve fiber densities at proximal sites. The minority (4 of 32) presented with abrupt onset of generalized cutaneous burning pain and hyperesthesia. In these patients intra-epidermal nerve fiber densities were reduced at both proximal and distal sites. For the entire group, intraepidermal nerve fiber densities were reduced at the calf site below the 5th percentile in 81% of all patients.³⁴ Fig. 16 compares nerve densities in biopsies of the skin and sural nerve from one patient from each group.

Skin biopsy was obtained in a separate study of 57 patients with painful, burning feet, minimal signs of neuropathy and normal nerve conduction studies. Of these 44 (77%) had a reduced number of intraepidermal nerves at the distal calf. Some nerves ended abruptly just below the basement membrane, others had enlarged, swollen terminal nerve endings. Reduced ankle reflexes were found in 11%, pinprick loss on toes and feet in 45% and loss of vibration sense in 43%. Only three patients had conditions known to be associated with peripheral neuropathy.⁷⁸

Diabetic neuropathy

Earlier work in painful diabetic neuropathy suggested that there was a predominance of involvement of unmyelinated and small myelinated nerve fibers in the sural nerve.⁸ Small fibers have long been recognized to be involved in diabetic polyneuropathy, and skin biopsies have confirmed that epidermal denervation can occur relatively early, and generally in a length-dependent manner. The total length of epidermal nerve per volume of epidermis correlates with the overall clinical severity (Fig. 17). Some of the surviving epidermal nerves have morphological abnormalities, particularly axon swellings.^{47, 80} The subepidermal neural plexus is often thinner than in control skin, and fibers within the sub-epidermal plexus may have a thickened, dystrophic appearance. In some neuropathies single nerve fibers are seen ending in a terminal enlargement just under the basement membrane.⁴⁵ These fibers appear to be unsuccessfully attempting to penetrate basement membrane en route to regeneration into the epidermis, although it is also possible that they are in the process of dying back. Sweat gland innervation is sometimes abnormal or completely lost in diabetic patients (Fig. 17), but it appears not to be useful as an early indication of neuropathy.⁴⁵

Epidermal nerve fibers also appear to be prominently affected in patients with impaired glucose tolerance (IGT). Several groups have reported an increased prevalence of IGT among patients with painful sensory neuropathy^{74,97} and randomly selected patients with sensory neuropathy and IGT were found to have reduced epidermal nerve fiber densities.⁹⁹ Of 97 patients with clinically diagnosed predominantly sensory neuropathy of undetermined etiology evaluated with oral glucose tolerance tests, 36% were classed as IGT and 20% as diabetes. Both the IGT and diabetes group had reduced distal leg IENF densities. The neuropathy associated with IGT was less severe than neuropathy associated with diabetes. Patients with predominantly small fiber neuropathy had a shorter duration of neuropathy symptoms and most had IGT. The results suggest that small caliber nerve fiber loss may be the earliest detectable sign of neuropathy in glucose dysmetabolism.¹⁰³

Diabetic truncal neuropathy

In diabetic truncal neuropathy skin biopsies from symptomatic regions show a loss of intraepidermal nerve fibers. After clinical recovery there may be nerve fiber regeneration.⁵³

HIV associated sensory neuropathies

Sensory neuropathies of HIV/AIDS affect at least 30% of individuals with AIDS.⁹⁰ The distal sensory polyneuropathy associated with HIV overlaps clinically with the toxic neuropathy provoked by specific antiretrovirals. The sensory neuropathies produce paresthesias or burning pain in the feet, often associated with hyperalgesia and lightning pains. Unlike HIV-associated dementia, the incidence rates have not declined with the advent of highly active antiretroviral therapy, probably because the dideoxy nucleoside analogues produce peripheral neurotoxicity.⁶⁶ Punch skin biopsies were originally developed at the JHU site to study HIV-associated sensory neuropathies that had previously been shown by sural nerve biopsy to be associated with prominent involvement of unmyelinated nerve fibers.¹⁷ The most systematic survey of skin biopsies in HIV-sensory neuropathy comes from a trial of recombinant human nerve growth factor. Skin biopsies were included in this trial as an outcome measure, the first reported application of skin biopsy to measure treatment effect. 62 of 270 patients with sensory neuropathies who participated in the trial of recombinant human nerve growth factor were included in a substudy examining the density of intra-epidermal nerve fibers.⁶² Fiber density was inversely correlated with neuropathic pain as measured both by patient and physician global pain assessments, but not using the Gracely Pain Scale. The reproducibility of the technique, as assessed from intra-subject correlation between baseline and the week 18 densities, was 81% in the distal part of the leg, and 77% in the upper thigh.

Decreased intraepidermal nerve fiber density at the distal leg was associated with lower CD4 counts and higher plasma HIV RNA levels. There was no treatment effect over the relatively short period of 18 weeks, and a few biopsies studied out to 70 weeks remained stable over time. Although no significant treatment effect was observed over the 18 weeks, the reproducibility of the technique appears to be good, suggesting that this technique could be incorporated as an outcome measure in future trials of regenerative agents for sensory neuropathies.

Friedreich ataxia

Friedreich ataxia is an autosomal recessive inherited ataxia. Hyperexpression of a GAA trinucleotide on the causative gene (X25) localized to chromosome 9 results in reduction of the protein frataxin.⁹ Pathology of large diameter myelinated nerve fibers in peripheral nerves is consistent with the loss of vibratory sense, deep tendon reflexes and severely reduced sensory action potentials. Small fiber involvement was postulated but unproven¹⁹ until severe loss of unmyelinated sensory nerves and of the autonomic innervation to sweat glands, arrector pili and arterioles was uncovered in thick sections of skin biopsies.⁷⁰ The small fiber loss correlated with reduced mechanical and thermal nociception.⁷¹

Restless Legs syndrome

The restless legs syndrome (RLS) is characterized by a compelling urge to move the extremities. It is often associated with paresthesias or dysesthesias, motor restlessness, worsening of symptoms with rest, relief by activity, and worsening of symptoms in the evening or night. Two forms are described: *crurum dolorosum* characterized by pain and *crurum paresthetica* characterized by paresthesias.²¹ Numerous risk factors are reported for RLS, and there is a suggestion that early-onset (age 45 or younger) and late-onset RLS differ etiologically.^{1,2} Nerve biopsies performed in an unselected group of primary patients suggest that the incidence of peripheral neuropathy may be underestimated.³⁷ Twenty-two consecutive patients with RLS were evaluated for evidence of large-fiber neuropathy (LFN) and small sensory fiber loss (SSFL). Neuropathy was identified in eight (36%). Three patients had pure LFN; two had mixed LFN and SSFL; and three had isolated SSFL. Patients with SSFL had a later onset, reported pain in their feet more frequently, and tended not to have a family history of RLS. Patients without SSFL did not associate onset with age, family history, or presence of pain. The report suggested that two forms of RLS exist: one is triggered by painful dysesthesias associated with SSFL, has a later onset, and a negative family history; the other does not have SSF involvement, has an earlier onset, a positive family history, and is without pain.⁷⁹

Familial Dysautonomia (Riley-Day syndrome, HSAN Type III)

Familial Dysautonomia^{3, 86} is an autosomal dominant disorder of sensory and autonomic nerves found in Ashkanazi Jews that is caused by mutation of the IKBKAP gene on chromosome 9q31.⁶ Clinical features include increased sweating, absent overflow lacrimation, vomiting crises, decreased sensitivity to pain, depressed Achilles reflexes, postural hypotension, absence of tongue fungiform papillae and of the axon flare response to intradermal histamine. Biopsy of non glabrous skin shows severe loss of unmyelinated sensory and autonomic nerves at the calf and moderate loss in the paraspinal region of the upper back.³³

Congenital insensitivity to pain with anhidrosis (CIPA, HSAN Type IV)

CIPA is a rare condition caused by mutation of the TRKA (NTRK1) gene on the 1q 21-22 chromosome³⁸ characterized by mental retardation, congenital analgesia that leads to self mutilation, multiple scars and fractures and anhidrosis with repeated bouts of fever.⁸⁷ Nerve biopsy shows loss of unmyelinated and small myelinated fibers.²⁵ Recently a 10 year old girl was reported with a low IQ, pain and thermal insensitivity. The remainder of the neurological examination, sensory nerve conduction velocities, cardiovascular reflexes and visual, brainstem and somatosensory evoked potentials were normal. Sural nerve biopsy was devoid of small myelinated nerves and contained only rare unmyelinated nerves. Epidermal nerve fibers were essentially absent in skin from the thigh, back and calf. The few sweat glands present were hypotrophic and without innervation.⁶⁹

Psoriasis

Psoriasis is a common skin disorder characterized histologically by epidermal hyperproliferation, dilated tortuous capillaries and a lymphocytic infiltrate. Skin biopsy shows that the epidermal nerves are greatly elongated through a thickened epidermis to the stratum corneum.³⁹ There are several treatment modalities available; therapy that targets epidermal proliferation, immunomodulators, or physical treatment, such as the use of lasers. The pulsed dye laser at 585 nm wavelength targets blood vessels and selectively destroys dilated vessels in the dermal papillae. Laser therapy is followed by remodeling of the epidermis and subsequent regeneration of capillaries and epidermal nerves with return to a more normal appearance.¹¹⁸

Port wine stains

It is proposed that the pathogenesis of port wine stains might be related to a lack of innervation around the ectatic blood vessels.⁸⁹ Biopsy of the involved skin shows a deficiency of innervation that is inversely proportional to the size of the vascular spaces. Moreover, nerve density may be predictive of the response of port wine stain lesions to laser treatment.¹¹⁷

Leprosy

Severe loss of immunoreactivity to PGP 9.5 and neurofilament antibodies occurs in leprosy. Loss is greatest in the tuberculoid variety of leprosy, less in lepromatous and least in the indeterminate variety.⁴¹ Curiously, there is almost complete loss of reactivity for neuropeptides CGRP, SP and NPY. This raises the possibility that neuropathy may be detected early by loss of immunoreactivity to some antigens while reactivity to other antigens remains, at least temporarily.

Fabry disease.

Fabry disease is an X-linked recessive disorder caused by deficiency of alpha-galactosidase A activity. Hemizygotes develop deposits of neutral glycosphingolipids, principally ceramide trihexoside, throughout the nervous system but predominantly in vascular endothelial cells. A painful small fiber neuropathy develops that is difficult to detect and quantitate by conventional methods. The neuropathy can develop in children as young as 5 years of age, with characteristic episodes of acral burning pain. Twenty Fabry patients (hemizygotes, ages 19 to 56 years) with preserved renal function were found to have normal nerve conduction studies and large fiber quantitation by sural nerve biopsy. By contrast, involvement of small cutaneous fibers in these patients was easily demonstrated and quantified by punch skin biopsy. All patients showed severe loss of intraepidermal innervation at the distal part of the leg with a density of 0-2.4/mm, versus a density for control subjects at this site of 4.7-6.5 fibers/mm. Fiber loss at the distal thigh was proportionately less severe. Fabry patients underwent biopsy at 6-month intervals for periods ranging from 1 to 3 years with no significant longitudinal changes in density except in two patients who demonstrated a rapid decrement in innervation density following an increase in spontaneous pain.⁹²

Sensory ganglionopathies

Ganglionopathy patients tend not to show the normal gradient in intraepidermal nerve fiber density between the proximal thigh and the distal part of the leg i.e., they show a non-length dependent process. One cohort presented with predominant gait and limb ataxia and proprioceptive sensory loss; 8 of 16 patients had positive sensory symptoms. Causes of the ganglionopathy were paraneoplastic in six, idiopathic in seven, and one had a hereditary sensory autonomic neuropathy. In ganglionopathies, the mean intraepidermal nerve fiber density did not differ between the proximal thigh (10.37/mm (10.11)) and the distal leg (10.41/mm (10.46)). Compared to other sensory neuropathies and controls, the intraepidermal nerve fiber density was significantly lower at the proximal thigh site in patients with ganglionopathies.⁵⁴

Post-herpetic neuralgia

Shingles represent reactivation of latent varicella-zoster virus in a sensory ganglion causing 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 demonstrated that the density of epidermal nerves in areas of allodynia are reduced, although they came to differing conclusions. One study found that higher fiber densities correlated with pain, an observation that might suggest that intact but hyperactive nociceptors were responsible. The reduction in intraepidermal nerve fiber density correlated with the magnitude of thermal sensory deficits.⁸⁸ By contrast, a second study⁷⁵ found that pain correlated with lower intra epidermal nerve fiber densities, and interpreted the results as more compatible with central sensitization. Interestingly, there was also a reduction of intraepidermal nerve fiber density in the contralateral, unaffected epidermis.¹¹⁵

CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) is one example of how skin biopsy can be used for analysis of non neural substances in investigation of neurological disease. CADASIL is an autosomal dominant disorder that presents as migrainous headaches, cerebral ischemia, and multi-infarct dementia. An abnormality on chromosome 19q12 produces a mis-sense point mutation in the notch-3 gene. Punch skin biopsy has been used to identify characteristic granular electron-dense osmiophilic material attached to vascular smooth muscle cells.¹⁰⁹ However, a commercial laboratory test for the genetic abnormality is now available from Athena Labs (Worcester, MA) which should provide improved specificity and sensitivity.³⁰

Chronic inflammatory demyelinating polyneuropathy (CIDP)

Patients with CIDP were recently shown to have about 50% reduction in intra-epidermal nerve fiber density compared to age and gender matched controls, suggesting that small diameter sensory nerves as well as large caliber nerve fibers are affected in CIDP.¹⁵

Postural tachycardia syndrome (POTS)

A collaborative study by Johns Hopkins University and the Mayo clinic examined the relationship between the QSART and epidermal nerve fiber densities in patients with postural tachycardia syndrome (POTS).⁹⁶ Inclusion criteria for POTS in the study included: orthostatic heart rate increment (30 beats/minute to an absolute heart rate of 120 beats/minute within five minutes of head up tilt); symptoms of orthostatic intolerance; distal abnormal QSART and abnormalities or

loss of vasoconstrictor response. In POTS, distal autonomic fibers are affected in isolation sparing somatic fibers with consistently normal nerve conduction studies.⁹¹ Intraepidermal nerve fiber density correlated with peripheral post-ganglionic sudomotor status assessed by the Composite Autonomic Severity Score (CASS)¹⁰² both in the category of the sudomotor sub scores and the overall total score, which is a composite index of combined sudomotor, adrenergic, and cardiovagal function. In POTS the mean intraepidermal nerve fiber density was normal, while the CASS was abnormal. Although three of the eight patients showed some minor morphological abnormalities, the normalcy of the epidermal nerve fiber densities suggests that distal involvement of autonomic fibers occurs in isolation in POTS.

Skin biopsies have been less widely-studied in pediatric neurological disorders, but there are some conditions where examination of the cutaneous innervation has characteristic changes. Two examples are provided.

Giant Axonal neuropathy

Giant axonal neuropathy (GAN) is an autosomal recessive neurologic disorder characterized by the development of a severe polyneuropathy, CNS abnormalities and characteristic tightly curled hair. Mutations in the gigaxonin gene have been identified as the underlying genetic defect.⁵¹ Ultrastructurally, accumulations of neurofilaments and osmiophilic aggregates are found in giant axons. Intermediate filaments accumulate in Schwann cells, perineural cells, fibroblasts, endothelial, and epithelial cells in both nerve and skin biopsies.¹⁰⁴ We have observed giant swellings within dermal nerve fibers, although the density of epidermal nerves appears to be normal.

Other conditions

Depletion of epidermal nerve fibers has been demonstrated in a variety of conditions with dermatological manifestations. Image analysis detected a significant decrease in PGP 9.5 immunoreactive nerves in the epidermis and subepidermal layers and in CGRP-ir nerves around the capillaries within dermal papillae of patients with Raynaud's Phenomenon, although the changes were not always apparent by visual screening.¹⁰⁵ Patients with systemic sclerosis were found to have decreased CGRP and PGP 9.5 immunoreactive nerves in all skin areas and of VIP-ir nerves in the sweat glands.¹⁰⁵ An increased number of PGP 9.5-ir fibers has been reported in the dermis and dermal-epidermal junction in atopic dermatitis, a condition in which the sensation of itching is prominent.¹⁰⁸ It has been suggested that increased dermal innervation could explained the pruritus, burning pain and hyperalgesia in notalgia paresthetica¹⁰⁰ and that an increase of sensory neuropeptides SP and CGRP might be involved in the pathogenesis of nodular

prurigo.⁶⁴ No changes of innervation were found in patients with lichenified eczema.
64

Neuroaxonal dystrophies

Neuronal intranuclear inclusion disease has no defined metabolic abnormalities, but it affects the peripheral nervous system, which could therefore be used to identify morphological abnormalities.²⁴ In infantile neuroaxonal dystrophy, there is also an accumulation of intermediate neurofilaments, as well as mitochondria and organelles with vesiculotubular profiles.⁵⁹ There is limited experience with the use of skin biopsies in this condition, however, some children have shown dystrophic changes in the subepidermal neural plexus.

RESEARCH USES OF SKIN BIOPSY

The existence of unmyelinated sensory nerves within a few microns of the skin's surface has provided opportunities to study the reactions of peripheral nerves to mechanical, thermal and chemical stimuli under safe, minimally invasive circumstances. Early experiments, using methylene blue staining, localized the itching sensation caused by cowhage spicules and injected proteases to the subepidermal neural plexus.⁹³ Use of immunohistochemical staining has allowed expansion of research into the correlates of sensory testing, effects of mechanical and chemical trauma to cutaneous nerves and regeneration of sensory nerves.

Human models of nerve regeneration

Skin biopsy variations:

We developed two models to study reinnervation of the epidermis.⁸³ One model uses a circular incision that transects the subepidermal plexus, resulting in Wallerian degeneration of the nerve fibers that enter the incised cylinder, leaving a defined zone of denervated dermis and epidermis. The earliest reinnervation of epidermis occurred by collateral sprouting from the terminals of epidermal axons from just outside the incision line. These terminals extended horizontally across the incision line and through the superficial layers of the epidermis, beneath the stratum corneum. By 13 days, numerous regenerating axons appeared in the deeper dermis derived from transected axons. The latter regenerating axons grew towards and ultimately into the epidermis, so that epidermal axonal density had normalized by 30-75 days. The invasion of these axons was associated with regression of the horizontally growing collateral sprouts. The second model utilizes an identical incision followed by removal of the incised cylinder of skin, leaving a denervated area in which Schwann cells are absent. New fibers arose by terminal elongation of the epidermal axons outside the incision line, as in the incision model, and especially by collateral branching of epidermal fibers at the incision margins. These collaterals

reached the epidermal surface of the basal lamina at the dermal-epidermal junction, and then grew slowly toward the center of the denervated circle. In contrast to the first model complete reinnervation was not achieved even after 23 months. These, and other models might be used to study reinnervation of denervated skin in man in different injury models and have relevance for exploring the stimuli for axonal growth and remodeling.

Skin blister: a model of mechanical denervation

Mechanical denervation of the terminal 40 to 60 μm of epidermal sensory nerve fibers can be produced to study wound healing and sensory reinnervation by removing the epidermis with a skin blister.⁴⁹ Regeneration of new epidermis occurs within three days after a 3 mm blister wound. The new epidermis is initially innervated by collateral sprouts arising from epidermal nerve fibers in the normal skin surrounding the blistered area. Slightly later, the proximal stumps of epidermal nerves at the base of the blister begin to enter the new epidermis, first near the edge of the lesion, later at the center. Eventually, regenerated nerves from the proximal stumps in the base reinnervate the epidermis in near normal numbers and the collateral branches disappear.¹¹² Reinnervation is accompanied by return of sensation within 30 days. Similar reinnervation occurs in patients with diabetic neuropathy, even in areas of reduced sensation, but the return of epidermal nerve fibers is only to the level of the pre lesion innervation.⁴²

Capsaicin: a model for chemical denervation

When capsaicin, a pungent ingredient in hot chili peppers, is applied topically it causes warm, painful sensations. These are replaced after several hours by a period of hyposensitivity. Sensation returns toward normal in two to three weeks.⁹⁵ This sequence was initially believed to result from desensitization of nociceptive receptors but was later shown to be induced by the influx of calcium ions with osmotic changes as well as activation of calcium sensitive proteases resulting in axonal degeneration.¹¹³ More recently skin biopsy showed that capsaicin administered intradermally is soon followed by depletion of epidermal nerves and superficial dermal nerves within 24 hours (Fig. 18). Reinnervation of the epidermis reached approximately 50% of normal and heat pain sensation reached 60% of normal after four weeks.⁹⁴ Capsaicin administered topically also causes loss of epidermal and superficial dermal nerves and decreased sensitivity to noxious heat and mechanical (pin) stimulation. Serial biopsies reveal that nerve regeneration after topical capsaicin is faster than after intradermal delivery. The return of epidermal nerves and sensation is nearly complete in 30 days (Fig. 19).⁷² These findings strengthen the hypothesis that epidermal nerves are polymodal nociceptors. The detection of a loss and subsequent recovery of sensitivity to noxious heat was

possible when stimuli were delivered by a small probe (2 to 3 mm diameter) but not by the larger 30 x 30 mm probe of the type common on commercial instruments.⁵⁰ Both of our groups have developed reproducible systems for applying capsaicin and examining the patterns and rate of regrowth of epidermal nerve fibers. In normal volunteers treated with topical capsaicin epidermal nerve density returns at an average rate of 0.185 fibers/day (range: 0.023 – 0.362 fibers/day). The rate of nerve fiber regeneration is significantly decreased in diabetics (0.069 fibers/day, range: 0.002 – 0.258 fibers/day, $p < 0.001$), and there was a trend for a lower rate of regeneration with increasing severity of neuropathy (Fig. 20).⁸¹ These models have potential as outcome measures in trials of regenerative agents and for improving understanding of trophic factor regulation after nerve injury.

Comparison of nerve function with structure

Comparisons have been made between the morphology of cutaneous nerve receptors and the results of electrophysiological stimulation. Highly significant correlations were found between the density of normal Meissner corpuscles and proximally recorded sensory nerve action potential areas whether generated by electrical stimulation of the digital nerve or by tactile stimulation of the finger pad. The density of abnormal Meissner corpuscles correlated significantly with the sensory action potential area but not with tactile potential area, whereas the density of papillary myelinated nerve fibers correlated with tactile but not with electrical stimuli. The natural stimuli appeared to be transmitted centrally more effectively by normal receptors than by atrophic receptors.⁷¹

Animal models using skin biopsy

Several groups have now used skin biopsy techniques in animal models to examine denervation and regeneration after various surgical models of nerve injury.^{67, 68, 36, 56, 115} Early experiments showed that rat sciatic nerve transection resulted in denervation, thinning of the epidermis and increased expression of PGP 9.5 by Langerhans cells.³⁶

A mouse model has been devised to correlate the reinnervation time of muscles, sweat glands and skin with the time required for return of function to these organs (Fig. 21). After sciatic nerve crush sequential biopsies of foot muscles and footpads from the hind paw of the same mouse can be examined to detect the time of reappearance of alpha motor, sudomotor and epidermal nociceptor nerves. Reappearance of end organ function can be tested at the same time intervals by sciatic nerve stimulation recording muscle, pilocarpine (ip) stimulation to detect reappearance of sweat droplets and pin prick to skin to detect return of nociception.⁶⁸ The model has potential for testing the efficacy of therapeutic agents to facilitate nerve regeneration.

Reinnervation of epidermal nerves in pigskin was tested after removal of a 3 mm skin biopsy and immediate replacement (autotransplantation). The first nerves that entered the newly regenerated epidermis over the wound were collateral branches of epidermal nerves in the normal uninjured epidermis surrounding the wound. These nerves grew on the epidermal surface of the basement membrane toward the center of the wound, then turned at an acute angle toward the stratum corneum. Nerve regeneration from the severed subepidermal plexus nerve trunks along the sides of the lesion began much later (weeks) and extended slowly toward the center of the lesion. Reinnervation never approached normal.²⁹ The same experiment on one human subject gave identical results (personal observation).

An important experimental study demonstrated that intraepidermal nerve fiber density is significantly reduced after dorsal root ganglionectomy or sciatic nerve transection in rats, but not after dorsal and ventral radiculotomy, sympathectomy, or spinal motor neuron lesion.⁵⁶

CONCLUDING REMARKS

The validity of skin biopsy/blister for assessing the extent of cutaneous innervation, and especially epidermal nociceptors, has now been established. These techniques provide a reliable and reproducible 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.

Skin biopsy/blister methods have opened new opportunities to learn about the response of unmyelinated nerve fibers to a number of disease entities and experimental conditions. The minimally invasive nature of the biopsy, blister and epidermal sheet techniques makes it possible to study reactions of unmyelinated nerves to experimental conditions directly in human subjects. The methods are useful for diagnosis and may find a place in the longitudinal evaluation of disease progression. An area of particular interest is the potential value of serial skin biopsies in clinical trials of agents that are intended to promote regeneration of nerve fibers.

Figure legends

1. Human skin innervation and vasculature: Confocal image of 150 μm thick section of human skin immunostained to demonstrate nerves (PGP 9.5—green and yellow) and basement membrane (type IV collagen—red). Image is a projection of 31.5 μm optical sections acquired with a 10x objective lens. Basement membrane outlines cutaneous structure including the basement membrane separating the dermis from the epidermis (Ep), capillary (Cap) loops of the papillary dermis and vasculature throughout the dermis, the central hair follicle (HF) and associated sebaceous gland, arrector pili muscles (AP), and sweat glands (SG). Nerve bundles (NB) arise from the deep dermis to innervate cutaneous structures. Characteristic nerve networks associated with arteries (Ar), sweat glands, arrector pili muscles, the hair follicle and the sub epidermal neural plexus provide sufficient morphologic criteria for identification of these structures based on their innervation patterns.
2. Normal human epidermal and papillary dermis innervation. Nerves are localized with antibody to PGP 9.5 and basement membrane is demarcated with antibody to type IV collagen. Vasculature is labeled with Ulex europaeus agglutinin type I. Epidermal nerve fibers appear aqua and lie within the blue epidermis (E). The subepidermal neural plexus (SNP) appears green or yellow. The dermal epidermal junction appears red. Capillaries (C) appear magenta. Nerve fibers (green and aqua) course in bundles through the dermis and branch in the papillary dermis to form the sub-epidermal neural plexus. Fibers arise from this plexus and penetrate the epidermal-dermal basement membrane to enter the epidermis. Please note some non-neuronal fibroblasts appear green. Epidermal nerve fibers are abundant and uniformly distributed in normal human skin.
3. Cutaneous Innervation of Normal and Diabetic Subjects. Confocal microscope images of sectioned superficial skin of a normal (A, B) and a diabetic (C, D) subject. Epidermal nerve fibers arise from a subepidermal neural plexus which extends horizontally immediately below the dermal-epidermal basement membrane. Numerous nerve fibers cross the basement membrane to innervate the epidermis of a control subject, (A – thigh, B – calf). Diabetic subjects display a range of epidermal nerve fiber density depending on the body location and the degree of neuropathy. Skin biopsies from a diabetic subject have normal density of innervation in the thigh (C), however several abnormalities are observed. Swellings in the nerve fibers are common in the SNP and some nerve fibers are longer and more branched. The bulbous nerve endings are not seen at other depths of the dermis. The calf biopsy of the same subject contains no epidermal nerve fibers (D). A single nerve fiber in the SNP branches at the dermal epidermal junction, but does not cross into the epidermis. (Kennedy, Wendelschafer-Crabb, & Walk, 2000)
4. Punch Biopsy Tool: A 3 mm punch biopsy is made on clean, shaved, skin that has been swabbed with Betadine and numbed with local anesthetic. The biopsy tool is inserted with a twisting motion to approximately half the depth of the metal collar. The depth of the biopsy depends on the thickness of the skin and the sample content desired. Sweat glands lie deep in the dermis and require a full thickness biopsy while shallow biopsies are sufficient for analysis of epidermal nerve density.
5. Fluorophore Emission Wavelength Profile: Fluorophores with distinct wavelengths that can be isolated visually by use of optical filters are used to label antibodies for multi-antigen immunofluorescent staining applications. Nerves are usually labeled with Cy 3,

fluorescence peaking at 565, while basement membrane is labeled with Cy 2, fluorescing at 520. By applying a blocking filter between 540 and 550 the two antigens can be viewed independently; while applying a long band filter the two antigens can be viewed simultaneously and distinguished microscopically by their colors. (Jackson ImmunoResearch, West Grove, PA)

6. Staining nerve and basement membrane: A. Nerves are immunostained with rabbit anti PGP 9.5 and Cy 3; B. Basement membrane is immunostained with mouse anti type IV collagen and Cy 2; C. Epidermis and vessels are stained with the lectin Ulex europaeus agglutinin type I and Cy 5; D. All images are combined in this colorized representation of the localization of nerves, vessels, dermal-epidermal basement membrane and epidermis. Confocal images are acquired as gray scale image stacks for each antigen-fluorophore combination. These are projected, pseudo colored and combined to create a color image of the entire z series.
7. A blister formed by application of negative pressure in the blister capsule. The blister conforms to the 3 mm opening in the base of the capsule. (Kennedy et al., 1999.)
8. Confocal vs Non-confocal: The confocal microscope uses focused light beams (laser or mercury vapor) to optically section thick tissue sections much like a CAT scanner. A series of many images are acquired at focal increments specific to the objective lens's depth of field. Out of focus blur is eliminated and clear, in-focus images of sections up to 200 μm thick can be collected and used for three dimensional analysis of nerve morphology and quantification.
9. Nerve Counting Rules: A set of concise rules was developed to normalize counting among investigators.
10. Nerve Quantification by Tracing: NeuroLucida software (MicroBrightfield, Williston, VT) uses the confocal image series to identify and trace each nerve fiber from the point where it crosses the basement membrane to its termination within the epidermis. Nerve number, length, complexity, density, and epidermal length and volume can be determined from the acquired data.
11. Effects of age on intraepidermal nerve fiber density at the distal part of the leg by age decile for healthy controls. (McArthur et al. 1998)
12. Blister analysis. A. Blister profile: Confocal image of an immunostained sectioned blister. Nerves (PGP 9.5) are green or yellow, basement membrane (type IV collagen) is red. Epidermis separated from dermis just above the dermal-epidermal basement membrane. Dermal capillaries and the subepidermal neural plexus remained intact (left box). Epidermal nerves, severed from their proximal segment, remained in the blister roof (right box). Scale bar equals 100 μm . (B). Survey confocal image of a 3 mm blister roof immunostained for nerve with antibody to PGP 9.5. Rectangles indicate the areas imaged at 20X for nerve counts. Scale bar equals 500 μm . (C) Higher magnification image is used for quantification of epidermal nerve fibers (from right rectangle in B). Scale bar equals 100 μm . (Kennedy et al., 1999)
13. Sub Epidermal Neural Plexus (SNP) Rating: A semi-quantitative rating system can be used to assess the density of the plexus: **SNP = 0** indicates a normal innervation pattern in the papillary dermis with nerve bundles running subjacent and parallel to the epidermis. **SNP = +1** denotes hyperinnervation, a condition that is encountered in early stages of neuropathy when active regeneration is occurring. It is often associated with

morphological abnormalities such as swellings, clustering of nerves, and truncation of nerve fibers at the dermal-epidermal basement membrane. **SNP = -1** signifies a moderate (50%) loss of SNP innervation, and is usually accompanied by a reduction in epidermal nerve fiber density. **SNP = -2** indicates a severe (75%) loss. **SNP = -3** applies when only a trace of SNP remains. **SNP = -4** indicates that no nerve remains in the papillary dermis.

14. Sweat Gland Nerve Density: These images depict the innervation of sweat glands from normal (top) and diabetic (bottom) human skin. Samples used for quantification are immunostained with anti-PGP 9.5 and imaged confocally. Nerve volume is computer-rendered.
15. Intraepidermal nerve fiber (IENF) density from calf (black circles) and thigh (white squares) skin correlated against clinical estimates of neuropathy severity in patients with painful sensory neuropathy. The data shown are group means with standard error bars. (Holland 97)
16. IENF densities at various sites for chronic progressive idiopathic SFSN and sural nerve morphometry for chronic progressive idiopathic SFSN Patient 1 (top) and 2 (bottom), Left: IENF density (mm^{-1}) at various sites for patients compared with normal controls (mean \pm standard error shown by shaded rectangle and 5th and 95th percentile range shown by bars). Right: Unmyelinated fiber density (axons / mm^2) for patients compared to the 5th and 95th percentile range for normal controls (bars). Patient 1 (top) had normal densities of myelinated and unmyelinated fibers in the sural nerve biopsy specimen (taken from the calf) despite marked cutaneous denervation in skin biopsies. Patient 2 (bottom), with more severe progressive idiopathic SFSN, had reduced densities of small-diameter myelinated and unmyelinated axons in the sural nerve biopsy specimen (taken from the ankle) in addition to marked length-dependent cutaneous denervation. (Holland et al. 1998)
17. Neuropathy Score Correlated with Epidermal Nerve Fiber Density and Sweat Gland Innervation Density in Diabetic Subjects. Correlation of cutaneous nerve quantitation with clinical assessment. The amount of nerve in calf biopsies correlated with the clinical evaluation of most diabetic subjects. The data suggest that the epidermal nerve assessment is useful in evaluating mild to moderate neuropathy, and sweat gland nerve analysis is helpful in later stages of neuropathy. Reduction of epidermal nerve is seen in mild cases of neuropathy, whereas no epidermal nerve is seen in severe cases. Sweat gland innervation remains at normal levels until neuropathy is quite severe. The peripheral and autonomic systems of the diabetic subjects were evaluated from graded results of history and examination, nerve conduction, cardiorespiratory reflexes, warm and cold sensitivity, and sweat testing. (Kennedy & Wendelschafer-Crabb, 1996)
18. Capsaicin-Induced Denervation and Reinnervation: The mean (\pm SEM) change in sensation and number of nerve fibers expressed as percent deviation from normal during and following the course of topical capsaicin application. Note scale for latencies (top) is different than that for other sensitivities and nerve fiber numbers (bottom). Touch was measured using Semmes Weinstein monofilaments. Mechanical pain [pain (%) and pain (V.A.S.)] was evaluated with a spring mounted pin. Heat pain (V.A.S.), cold pain (V.A.S) were tested with a 2 mm probe. Intensity of pain was measured using a visual analog scale (V.A.S.) for mechanical pain, heat pain and cold pain. Epidermal nerve

fiber (ENF) number per area was calculated from analysis of nerves in blister roofs. (Nolano et al., 1999)

19. Capsaicin-treated human epidermis. Nerves degenerate following topical capsaicin treatment as can be seen by comparing control tissue at time 0 (top) with a sample taken after one week of capsaicin application. Epidermal nerve fibers have degenerated, while fibers in the sub-epidermal neural plexus are still plentiful. This provides a good model for the peripheral neuropathy seen in diabetic subjects, where epidermal nerve fibers loss precedes more proximal loss. We will use this model to study basement membrane during nerve regeneration. This model provides denervation without remodeling basement membrane. Scale bar equals 100 μm .
20. ENFD regeneration expressed as a percent of baseline values in diabetic and healthy, non-diabetic control subjects. Healthy volunteers recovered their ENF density at a rate of 0.185 fibers/mm/day (95% CI: 0.15 – 0.21 fibers/mm/day). The rate of nerve fiber regeneration was significantly decreased among those with diabetes (0.069 fibers/mm/day, 95% CI: 0.044 – 0.11 fibers/day, $p < 0.001$) and further reduced among diabetic subjects with neuropathy as compared to those without neuropathy ($p = 0.003$). (Polydefkis et al 2003)
21. Confocal micrographs of PGP 9.5 immunofluorescence in control (A) and reinnervating (B-C) mouse footpads following sciatic nerve crush. A. Nerves in control footpad form a dense network. Sweat glands are very heavily innervated. Large trunks extend through the central sweat gland area to form the subepidermal nerve plexus and Meissner's corpuscles in the tufts of papillary dermis. The epidermis has many fine nerve endings. B. At day 14 post crush, PGP 9.5 immunoreactive fibers returned in large nerve trunks from the base of the pad to the papillary dermis. C. By day 46 post-crush, the pattern of innervation resembles the control, but regenerated nerve fibers to the nerve trunks, subepidermal neural plexus and sweat glands are less well compartmentalized, and the epidermis has shorter nerve fibers. (Navarro et al, 1997)

References

1. Allen, R., LaBuda, M., Becker, P. and Earley, C.: Family history study of RLS patients from two clinical populations. *Sleep Res* 26:309,1997.
2. Allen, R.P. and Earley, C.J.: Defining the phenotype of the restless legs syndrome (RLS) using age-of-symptom-onset. *Sleep Med* 1:11,2000.
3. Axelrod, F.B. and Hilz, M.J.: Familial dysautonomia. In Appenzeller, O. (eds.): *Handbook of clinical neurology; the autonomic nervous system part II, dysfunctions*. Elsevier Science B.V., pp. 144-116,2000.
4. Bajrovic, F., Kovacic, U., Pavcnik, M. and Sketelj, J.: Interneuronal signaling is involved in induction of collateral sprouting of nociceptive axons. *Neurosci* 111:587,2002.
5. Baker, K.W. and Habrowsky, J.E.: EDTA separation and ATPase Langerhans staining in the mouse epidermis. *J Invest Dermatol* 80:625,1983.
6. Blumenfeld, A., Slaughaupt, S.A., Liebert, C.B., Temper, V., Maayan, C., Gill, S., Lucente, D.E., Idelson, M., MacCormack, K., Monahan, M.A., Mull, J., Leyne, M., Mendillo, M., Schiripo, T., Mishori, E., Breakefield, X., Axelrod, F.B. and Gusella, J.F.: Precise genetic

- mapping and haplotype analysis of the familial dysautonomia gene on human chromosome 9q31. *Am J Hum Genet* 64:1110,1999.
7. Bolton, C.F., Winkelmann, R.K. and Dyck, P.J.: A quantitative study of Meissner's corpuscles in man. *Neurology* 16:1,1966.
 8. Brown, M.J., Martin, J.R. and Asbury, A.K.: Painful diabetic neuropathy. A morphometric study. *Arch Neurol* 33:164,1976.
 9. Campuzano, V., Montermini, L., Molto, M.D., Pianese, L., Cossee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A. and et al.: Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science* 271:1423,1996.
 10. Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I. and Julius, D.: Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288:306,2000.
 11. Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J. and Julius, D.: A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398:436,1999.
 12. Cauna, N.: The free penicillate nerve endings of the human hairy skin. *J Anat* 115:277,1973.

13. Chen, C.H., Vazquez-Padua, M. and Cheng, Y.C.: Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. *Mol Pharmacol* 39:625,1991.
14. Cherry, C.L., Gahan, M.E., McArthur, J.C., Lewin, S.R., Hoy, J.F. and Wesselingh, S.L.: Exposure to dideoxynucleosides is reflected in lowered mitochondrial DNA in subcutaneous fat. *J Acquir Immune Defic Syndr* 30:271,2002.
15. Chiang, M.C., Lin, Y.H., Pan, C.L., Tseng, T.J., Lin, W.M. and Hsieh, S.T.: Cutaneous innervation in chronic inflammatory demyelinating polyneuropathy. *Neurology* 59:1094,2002.
16. Chien, H.F., Tseng, T.J., Lin, W.M., Yang, C.C., Chang, Y.C., Chen, R.C. and Hsieh, S.T.: Quantitative pathology of cutaneous nerve terminal degeneration in the human skin. *Acta Neuropathol (Berl)* 102:455,2001.
17. Cornblath, D.R. and McArthur, J.C.: Predominantly sensory neuropathy in patients with AIDS and AIDS-related complex. *Neurology* 38:794,1988.
18. Dalsgaard, C.J., Rydh, M. and Haegerstrand, A.: Cutaneous innervation in man visualized with protein gene product 9.5 (PGP 9.5) antibodies. *Histochemistry* 92:385,1989.

19. Dyck, P.J.: Neuronal atrophy and degeneration predominantly affecting peripheral sensory and autonomic neurons. In Thomas, P. K. (eds.): *Peripheral neuropathy*. Philadelphia, WB Saunder, pp. 1065-1093,1993.
20. Dyck, P.J., Winkelmann, R.K. and Bolton, C.F.: Quantitation of Meissner's corpuscles in hereditary neurologic disorders. Charcot-Marie-Tooth disease, Roussy-Levy syndrome, Dejerine-Sottas disease, hereditary sensory neuropathy, spinocerebellar degenerations, and hereditary spastic paraplegia. *Neurology* 16:10,1966.
21. Ekblom, K.: Restless legs. *Acta Med Scand* 158 (suppl):1,1945.
22. Fundin, B.T., Arvidsson, J., Aldskogius, H., Johansson, O., Rice, S.N. and Rice, F.L.: Comprehensive immunofluorescence and lectin binding analysis of intervibrissal fur innervation in the mystacial pad of the rat. *J Comp Neurol* 385:185,1997.
23. Gahan, M.E., Miller, F., Lewin, S.R., Cherry, C.L., Hoy, J.F., Mijch, A., Rosenfeldt, F. and Wesselingh, S.L.: Quantification of mitochondrial DNA in peripheral blood mononuclear cells and subcutaneous fat using real-time polymerase chain reaction. *J Clin Virol* 22:241,2001.

24. Goebel, H.H.: Extracerebral biopsies in neurodegenerative diseases of childhood. *Brain Dev* 21:435,1999.
25. Goebel, H.H., Veit, S. and Dyck, P.J.: Confirmation of virtual unmyelinated fiber absence in hereditary sensory neuropathy type IV. *J Neuropathol Exp Neurol* 39:670,1980.
26. Gold, M.S., Reichling, D.B., Shuster, M.J. and Levine, J.D.: Hyperalgesic agents increase a tetrodotoxin-resistant Na⁺ current in nociceptors. *Proc Natl Acad Sci U S A* 93:1108,1996.
27. Griffin, J.W., McArthur, J.C. and Polydefkis, M.: Assessment of cutaneous innervation by skin biopsies. *Curr Opin Neurol* 14:655,2001.
28. Halata, Z.: Sensory innervation of the hairy skin (light- and electronmicroscopic study). *J Invest Dermatol* 101:75S,1993.
29. He, C.L., Wendelscafer-Crabb, G. and Kennedy, W.R.: Reinnervation of epidermal nerve fibers in pig skin. *Soc Neuroscience Abstracts* 22:760,1996.
30. Helm, T. and Helm, K.F.: CADASIL: blood test versus skin biopsy? *J Am Acad Dermatol* 46:798,2002.

31. Herrmann, D.N., Griffin, J.W., Hauer, P., Cornblath, D.R. and McArthur, J.C.: Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. *Neurology* 53:1634,1999.
32. Hilliges, M. and Johansson, O.: Comparative analysis of numerical estimation methods of epithelial nerve fibers using tissue sections. *J Peripher Nerv Syst* 4:53,1999.
33. Hilz, M.J., Kennedy, W.R., Stemper, B., Wendelschafer-Crabb, G. and Axelrod, F.B.: Cutaneous innervation in familial dysautonomia. *Clin Autonomic Res* 11:182,2001.
34. Holland, N.R., Crawford, T.O., Hauer, P., Cornblath, D.R., Griffin, J.W. and McArthur, J.C.: Small-fiber sensory neuropathies: clinical course and neuropathology of idiopathic cases. *Ann Neurol* 44:47,1998.
35. Holland, N.R., Stocks, A., Hauer, P., Cornblath, D.R., Griffin, J.W. and McArthur, J.C.: Intraepidermal nerve fiber density in patients with painful sensory neuropathy. *Neurology* 48:708,1997.
36. Hsieh, S.T., Choi, S., Lin, W.M., Chang, Y.C., McArthur, J.C. and Griffin, J.W.: Epidermal denervation and its effects on keratinocytes and Langerhans cells. *J Neurocytol* 25:513,1996.

37. Iannaccone, S., Zucconi, M., Marchettini, P., Ferini-Strambi, L., Nemni, R., Quattrini, A., Palazzi, S., Lacerenza, M., Formaglio, F. and Smirne, S.: Evidence of peripheral axonal neuropathy in primary restless legs syndrome. *Mov Disord* 10:2,1995.
38. Indo, Y., Tsuruta, M., Hayashida, Y., Karim, M.A., Ohta, K., Kawano, T., Mitsubichi, H., Tonoki, H., Awaya, Y. and Matsuda, I.: Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat Genet* 13:485,1996.
39. Johansson, O., Han, S.W. and Enhamre, A.: Altered cutaneous innervation in psoriatic skin as revealed by PGP 9.5 immunohistochemistry. *Arch Dermatol Res* 283:519,1991.
40. Johansson, O., Wang, L., Hilliges, M. and Liang, Y.: Intraepidermal nerves in human skin: PGP 9.5 immunohistochemistry with special reference to the nerve density in skin from different body regions. *J Peripher Nerv Syst* 4:43,1999.
41. Karanth, S.S., Springall, D.R., Lucas, S., Levy, D., Ashby, P., Levene, M.M. and Polak, J.M.: Changes in nerves and neuropeptides in skin from 100 leprosy patients investigated by immunocytochemistry. *J Pathol* 157:15,1989.
42. Kennedy, W.R., Brown, J. and Wendelschafer-Crabb, G.: Sensory reinnervation in diabetic neuropathy. *J Peripher Nerv Syst* 6:150,2001.

43. Kennedy, W.R., Nolano, M., Wendelschafer-Crabb, G., Johnson, T.L. and Tamura, E.: A skin blister method to study epidermal nerves in peripheral nerve disease. *Muscle Nerve* 22:360,1999.
44. Kennedy, W.R. and Sakuta, M.: Collateral reinnervation of sweat glands. *Ann Neurol* 15:73,1984.
45. Kennedy, W.R. and Wendelschafer-Crabb, G.: Utility of skin biopsy in diabetic neuropathy. In Younger, D. (eds.): *Seminars in neurology; Diabetic neuropathy*. New York, G. Thieme, pp. 163-171,1996.
46. Kennedy, W.R. and Wendelschafer-Crabb, G.: The innervation of human epidermis. *J Neurol Sci* 115:184,1993.
47. Kennedy, W.R., Wendelschafer-Crabb, G. and Johnson, T.: Quantitation of epidermal nerves in diabetic neuropathy. *Neurology* 47:1042,1996.
48. Kennedy, W.R., Wendelschafer-Crabb, G. and Johnson, T.: Utility of skin biopsy and skin blister in neurological practice. *Journal Clinical Neuromuscular Disease* 1:196,2000.
49. Kennedy, W.R., Wendelschafer-Crabb, G. and Lindall, A.W.: Quantitative epidermal nerve fiber analysis in skin blisters and skin biopsies. *Ann Neurology* 14:573,1999.

50. Khalili, N., Wendelschafer-Crabb, G., Kennedy, W.R. and Simone, D.A.: Influence of thermode size for detecting heat pain dysfunction in a capsaicin model of epidermal nerve fiber loss. *Pain* 91:241,2001.
51. Kuhlbaumer, G., Young, P., Oberwittler, C., Hunermund, G., Schirmacher, A., Domschke, K., Ringelstein, B. and Stogbauer, F.: Giant axonal neuropathy (GAN): case report and two novel mutations in the gigaxonin gene. *Neurology* 58:1273,2002.
52. Lauria, G.: Innervation of the human epidermis. A historical review. *Ital J Neurol Sci* 20:63,1999.
53. Lauria, G., McArthur, J.C., Hauer, P.E., Griffin, J.W. and Cornblath, D.R.: Neuropathological alterations in diabetic truncal neuropathy: evaluation by skin biopsy. *J Neurol Neurosurg Psychiatry* 65:762,1998.
54. Lauria, G., Sghirlanzoni, A., Lombardi, R. and Pareyson, D.: Epidermal nerve fiber density in sensory ganglionopathies: clinical and neurophysiologic correlations. *Muscle Nerve* 24:1034,2001.

55. Levy, D.M., Terenghi, G., Gu, X.H., Abraham, R.R., Springall, D.R. and Polak, J.M.:
Immunohistochemical measurements of nerves and neuropeptides in diabetic skin:
relationship to tests of neurological function. *Diabetologia* 35:889,1992.
56. Li, Y., Hsieh, S.T., Chien, H.F., Zhang, X., McArthur, J.C. and Griffin, J.W.: Sensory and
motor denervation influence epidermal thickness in rat foot glabrous skin. *Exp Neurol*
147:452,1997.
57. Lichtman, J.W., Magrassi, L. and Purves, D.: Visualization of neuromuscular junctions over
periods of several months in living mice. *J Neurosci* 7:1215,1987.
58. Low, P.A., Caskey, P.E., Tuck, R.R., Fealey, R.D. and Dyck, P.J.: Quantitative sudomotor
axon reflex test in normal and neuropathic subjects. *Ann Neurol* 14:573,1983.
59. Mahadevan, A., Santosh, V., Gayatri, N., Ratnavalli, E., NandaGopal, R., Vasanth, A., Roy,
A.K. and Shankar, S.K.: Infantile neuroaxonal dystrophy and giant axonal neuropathy--
overlap diseases of neuronal cytoskeletal elements in childhood? *Clin Neuropathol*
19:221,2000.

60. Martin, J.L., Brown, C.E., Matthews-Davis, N. and Reardon, J.E.: Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. *Antimicrob Agents Chemother* 38:2743,1994.
61. McArthur, J.C., Stocks, E.A., Hauer, P., Cornblath, D.R. and Griffin, J.W.: Epidermal nerve fiber density: normative reference range and diagnostic efficiency. *Arch Neurol* 55:1513,1998.
62. McArthur, J.C., Yiannoutsos, C., Simpson, D.M., Adornato, B.T., Singer, E.J., Hollander, H., Marra, C., Rubin, M., Cohen, B.A., Tucker, T., Navia, B.A., Schifitto, G., Katzenstein, D., Rask, C., Zaborski, L., Smith, M.E., Shriver, S., Millar, L., Clifford, D.B. and Karalnik, I.J.: A phase II trial of nerve growth factor for sensory neuropathy associated with HIV infection. AIDS Clinical Trials Group Team 291. *Neurology* 54:1080,2000.
63. McCarthy, B.G., Hsieh, S.T., Stocks, A., Hauer, P., Macko, C., Cornblath, D.R., Griffin, J.W. and McArthur, J.C.: Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. *Neurology* 45:1848,1995.

64. Molina, F.A., Burrows, N.P., Jones, R.R., Terenghi, G. and Polak, J.M.: Increased sensory neuropeptides in nodular prurigo: a quantitative immunohistochemical analysis. *Br J Dermatol* 127:344,1992.
65. Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q. and Snider, W.D.: IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 19:849,1997.
66. Moore, R.D., Wong, W.M., Keruly, J.C. and McArthur, J.C.: Incidence of neuropathy in HIV-infected patients on monotherapy versus those on combination therapy with didanosine, stavudine and hydroxyurea. *Aids* 14:273,2000.
67. Navarro, X., Verdu, E., Wendelschafer-Crabb, G. and Kennedy, W.R.: Innervation of cutaneous structures in the mouse hind paw: a confocal microscopy immunohistochemical study. *J Neurosci Res* 41:111,1995.
68. Navarro, X., Verdu, E., Wendelschafer-Crabb, G. and Kennedy, W.R.: Immunohistochemical study of skin reinnervation by regenerative axons. *J Comp Neurol* 380:164,1997.

69. Nolano, M., Crisci, C., Santoro, L., Barbieri, F., Casale, R., Kennedy, W.R., Wendelschafer-Crabb, G., Provitera, V., Di Lorenzo, N. and Caruso, G.: Absent innervation of skin and sweat glands in congenital insensitivity to pain with anhidrosis. *Clin Neurophysiol* 111:1596,2000.
70. Nolano, M., Provitera, V., Crisci, C., Saltalamacchia, A.M., Wendelschafer-Crabb, G., Kennedy, W.R., Filla, A., Santoro, L. and Caruso, G.: Small fibers involvement in Friedreich's ataxia. *Ann Neurol* 50:17,2001.
71. Nolano, M., Provitera, V., Lullo, F., Saltalamacchia, A.M., Crisci, C., Lanzillo, B. and Santoro, L.: Tactile stimulation and mechanoreceptors in sensory neuropathies. *Neurol Sci* 22 (Suppl 1):31,2001.
72. Nolano, M., Simone, D.A., Wendelschafer-Crabb, G., Johnson, T., Hazen, E. and Kennedy, W.R.: Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. *Pain* 81:135,1999.
73. Novak, V., Freimer, M.L., Kissel, J.T., Sahenk, Z., Periquet, M.I., Nash, S.M., Collins, M.P. and Mendell, J.R.: Autonomic impairment in painful neuropathy. *Neurology* 56(7):861,2001.

74. Novella, S.P., Inzucchi, S.E. and Goldstein, J.M.: The frequency of undiagnosed diabetes and impaired glucose tolerance in patients with idiopathic sensory neuropathy. *Muscle Nerve* 24:1229,2001.
75. Oaklander, A.L.: The density of remaining nerve endings in human skin with and without postherpetic neuralgia after shingles. *Pain* 92:139,2001.
76. Oaklander, A.L., Romans, K., Horasek, S., Stocks, A., Hauer, P. and Meyer, R.A.: Unilateral postherpetic neuralgia is associated with bilateral sensory neuron damage. *Ann Neurol* 44:789,1998.
77. Ochoa, J.L. and Yarnitsky, D.: The triple cold syndrome. Cold hyperalgesia, cold hypoesthesia and cold skin in peripheral nerve disease. *Brain* 117 (Pt 1):185,1994.
78. Periquet, M.I., Novak, V., Collins, M.P., Nagaraja, H.N., Erdem, S., Nash, S.M., Freimer, M.L., Sahenk, Z., Kissel, J.T. and Mendell, J.R.: Painful sensory neuropathy: prospective evaluation using skin biopsy. *Neurology* 53:1641,1999.
79. Polydefkis, M., Allen, R.P., Hauer, P., Earley, C.J., Griffin, J.W. and McArthur, J.C.: Subclinical sensory neuropathy in late-onset restless legs syndrome. *Neurology* 55:1115,2000.

80. Polydefkis, M., Hauer, P., Abraham, B., Griffin, J.Q. and McArthur, J.C.: Novel measures of human axonal regeneration. *J Peripher Nerv Syst* 6:170,2001.
81. Polydefkis, M., Hauer, P., Griffin, J.W. and McArthur, J.C.: Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. *Diabetes Technol Ther* 3:23,2001.
82. Polydefkis, M., Yiannoutsos, C.T., Cohen, B.A., Hollander, H., Schifitto, G., Clifford, D.B., Simpson, D.M., Katzenstein, D., Shriver, S., Hauer, P., Brown, A., Haidich, A.B., Moo, L. and McArthur, J.C.: Reduced intraepidermal nerve fiber density in HIV-associated sensory neuropathy. *Neurology* 58:115,2002.
83. Rajan, B.J., Polydefkis, M., Hauer, P.E., Griffin, J.W. and McArthur, J.C.: Epidermal reinnervation after intracutaneous axotomy in man. *J Peripher Nerv Syst In Press*:2003.
84. Ren, K. and Dubner, R.: Descending modulation in persistent pain: an update. *Pain* 100:1,2002.
85. Rice, F.L., Fundin, B.T., Arvidsson, J., Aldskogius, H. and Johansson, O.: Comprehensive immunofluorescence and lectin binding analysis of vibrissal follicle sinus complex innervation in the mystacial pad of the rat. *J Comp Neurol* 385:149,1997.

86. Riley, C.M., Day, R.L., Greely, D. and Langford, W.S.: Central autonomic dysfunction with defective lacrimation. *Pediatrics* 3:468,1949.
87. Rosenberg, S., Nagahashi Marie, S.K. and Kliemann, S.: Congenital insensitivity to pain with anhidrosis (hereditary sensory and autonomic neuropathy type IV). *Pediatr Neurol* 11:50,1994.
88. Rowbotham, M.C., Yosipovitch, G., Connolly, M.K., Finlay, D., Forde, G. and Fields, H.L.: Cutaneous innervation density in the allodynic form of postherpetic neuralgia. *Neurobiol Dis* 3:205,1996.
89. Rydh, M., Malm, M., Jernbeck, J. and Dalsgaard, C.J.: Ectatic blood vessels in port-wine stains lack innervation: possible role in pathogenesis. *Plast Reconstr Surg* 87:419,1991.
90. Schifitto, G., McDermott, M.P., McArthur, J.C., Marder, K., Sacktor, N., Epstein, L. and Kieburtz, K.: Incidence of and risk factors for HIV-associated distal sensory polyneuropathy. *Neurology* 58:1764,2002.
91. Schondorf, R. and Low, P.A.: Idiopathic postural orthostatic tachycardia syndrome: an attenuated form of acute pandysautonomia? *Neurology* 43.1:132,1993.

92. Scott, L.J., Griffin, J.W., Luciano, C., Barton, N.W., Banerjee, T., Crawford, T., McArthur, J.C., Tournay, A. and Schiffmann, R.: Quantitative analysis of epidermal innervation in Fabry disease. *Neurology* 52:1249,1999.
93. Shelley, W.B. and Arthur, R.P.: The neurohistology and neurophysiology of the itch sensation in man. *Arch Dermatol Res* 76:296,1957.
94. Simone, D.A., Nolano, M., Johnson, T., Wendelschafer-Crabb, G. and Kennedy, W.R.: Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. *J Neurosci* 18:8947,1998.
95. Simone, D.A. and Ochoa, J.: Early and late effects of prolonged topical capsaicin on cutaneous sensibility and neurogenic vasodilatation in humans. *Pain* 47:285,1991.
96. Singer, W., Spies, J.M., Hauer, P., McArthur, J.C., Griffin, J.W. and Low, P.A.: Epidermal nerve fiber density in the neuropathic type of the postural tachycardia syndrome (S35.001). American academy of Neurology, San Diego 2000.
97. Singleton, J.R., Smith, A.G. and Bromberg, M.B.: Increased prevalence of impaired glucose tolerance in patients with painful sensory neuropathy. *Diabetes Care* 24:1448,2001.

98. Smith, A.G., Kroll, R., Ramachandran, P., Hauer, P. and McArthur, J.C.: The reliability of skin biopsy with measurement of intraepidermal nerve fiber density. *Neurology* 58 (Suppl 3):A500.S66.002,2002.
99. Smith, A.G., Ramachandran, P., Tripp, S. and Singleton, J.R.: Epidermal nerve innervation in impaired glucose tolerance and diabetes-associated neuropathy. *Neurology* 57:1701,2001.
100. Springall, D.R., Karanth, S.S., Kirkham, N., Darley, C.R. and Polak, J.M.: Symptoms of notalgia paresthetica may be explained by increased dermal innervation. *J Invest Dermatol* 97:555,1991.
101. Stocks, E.A., McArthur, J.C., Griffen, J.W. and Mouton, P.R.: An unbiased method for estimation of total epidermal nerve fibre length. *J Neurocytol* 25:637,1996.
102. Suarez, G.A., Opfer-Gehrking, T.L., Offord, K.P., Atkinson, E.J., O'Brien, P.C. and Low, P.A.: The Autonomic Symptom Profile: a new instrument to assess autonomic symptoms. *Neurology* 52:523,1999.
103. Sumner, C.J., Sheth, S., Griffin, J.W., Cornblath, D.R. and Polydefkis, M.: The spectrum of neuropathy in diabetes and impaired glucose tolerance. *Neurology* 60:108,2003.

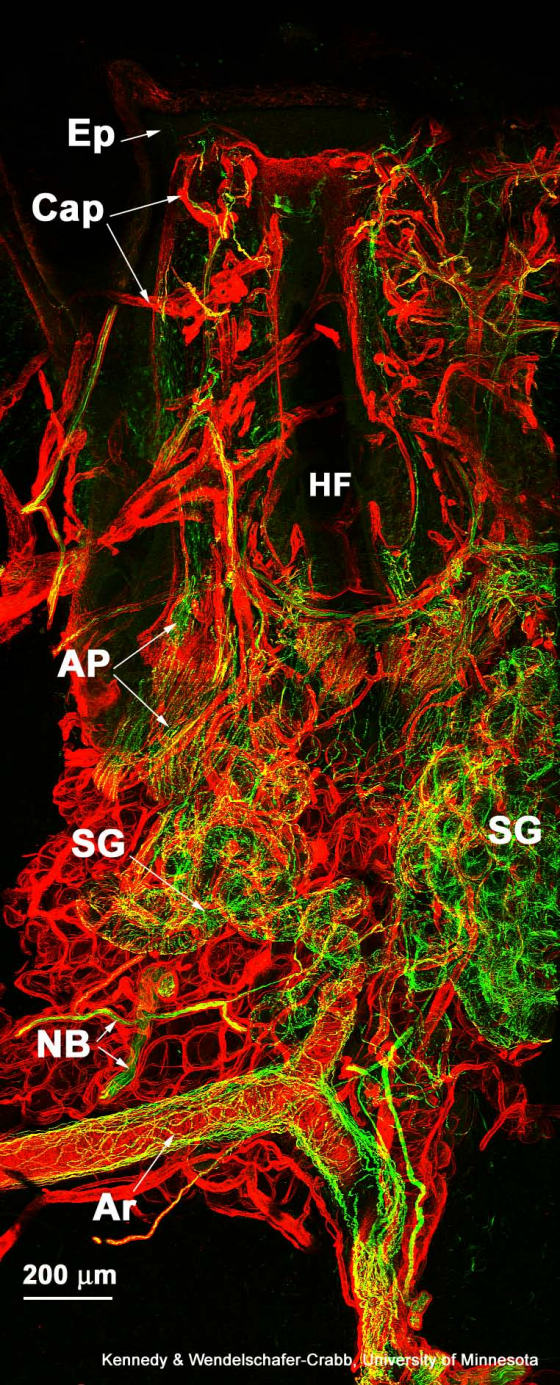
104. Taratuto, A.L., Sevlever, G., Saccoliti, M., Caceres, L. and Schultz, M.: Giant axonal neuropathy (GAN): an immunohistochemical and ultrastructural study report of a Latin American case. *Acta Neuropathol (Berl)* 80:680,1990.
105. Terenghi, G., Bunker, C.B., Liu, Y.F., Springall, D.R., Cowen, T., Dowd, P.M. and Polak, J.M.: Image analysis quantification of peptide-immunoreactive nerves in the skin of patients with Raynaud's phenomenon and systemic sclerosis. *J Pathol* 164:245,1991.
106. Thompson, R.J., Doran, J.F., Jackson, P., Dhillon, A.P. and Rode, J.: PGP 9.5--a new marker for vertebrate neurons and neuroendocrine cells. *Brain Res* 278:224,1983.
107. Underwood, R.A., Gibran, N.S., Muffley, L.A., Usui, M.L. and Olerud, J.E.: Color subtractive-computer-assisted image analysis for quantification of cutaneous nerves in a diabetic mouse model. *J Histochem Cytochem* 49:1285,2001.
108. Urashima, R. and Mihara, M.: Cutaneous nerves in atopic dermatitis. A histological, immunohistochemical and electron microscopic study. *Virchows Arch* 432:363,1998.
109. Walsh, J.S., Perniciaro, C. and Meschia, J.F.: CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy): diagnostic skin biopsy changes determined by electron microscopy. *J Am Acad Dermatol* 43:1125,2000.

110. Wang, L., Hilliges, M., Jernberg, T., Wiegleb-Edstrom, D. and Johansson, O.: Protein gene product 9.5-immunoreactive nerve fibres and cells in human skin. *Cell Tissue Res* 261:25,1990.
111. Watkins, L.R. and Maier, S.F.: Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. *Physiol Rev* 82:981,2002.
112. Wendelschafer-Crabb, G., Nolano, M. and Kennedy, W.R.: Sensory reinnervation of a skin blister wound. *J Peripher Nerv Syst* 6:150,2001.
113. Wood, J.N., Winter, J., James, I.F., Rang, H.P., Yeats, J. and Bevan, S.: Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J Neurosci* 8:3208,1988.
114. Woolf, C.J., Shortland, P. and Coggeshall, R.E.: Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355:75,1992.
115. Wu, G., Ringkamp, M., Hartke, T.V., Murinson, B.B., Campbell, J.N., Griffin, J.W. and Meyer, R.A.: Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. *J Neurosci* 21:RC140,2001.
116. Wu, G., Ringkamp, M., Murinson, B.B., Pogatzki, E.M., Hartke, T.V., Weerahandi, H.M., Campbell, J.N., Griffin, J.W. and Meyer, R.A.: Degeneration of myelinated efferent

fibers induces spontaneous activity in uninjured C-fiber afferents. *J Neurosci* 22:7746,2002.

117. Zelickson, B.D., Kilmer, S.L., Bernstein, E., Chotzen, V.A., Dock, J., Mehregan, D. and Coles, C.: Pulsed dye laser therapy for sun damaged skin. *Lasers Surg Med* 25:229,1999.
118. Zelickson, B.D., Mehregan, D.A., Wendelschfer-Crabb, G., Ruppman, D., Cook, A., O'Connell, P. and Kennedy, W.R.: Clinical and histologic evaluation of psoriatic plaques treated with a flashlamp pulsed dye laser. *J Am Acad Dermatol* 35:64,1996.

hand, proximal volar forearm and the paraspinal area of the back at T 4 to 5.



Ep →

Cap →

HF

AP →

SG →

SG

NB →

Ar →

200 μm

100 μ m

Normal Skin

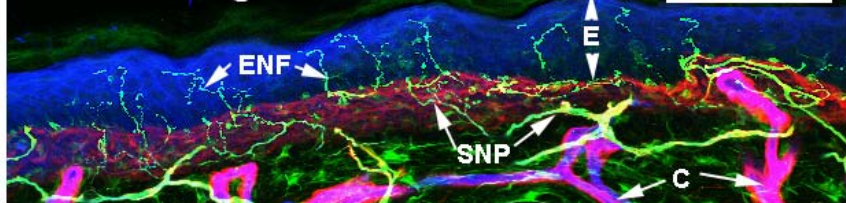
Epidermis



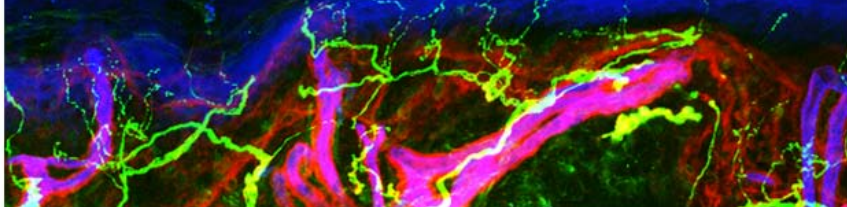
Basement Membrane

Nerves

3A Normal Thigh



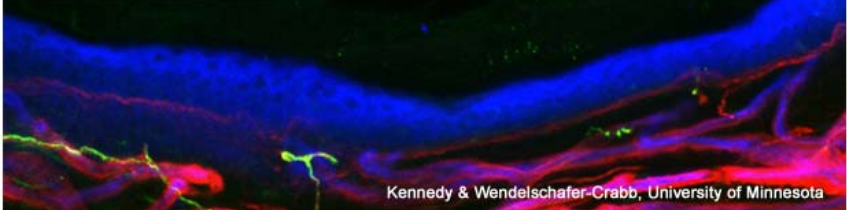
3B Normal Calf



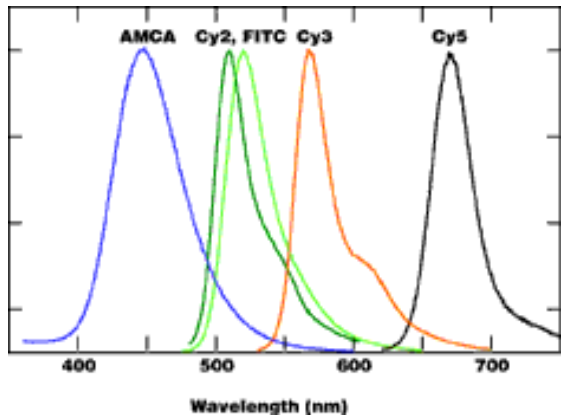
3C Diabetic Thigh

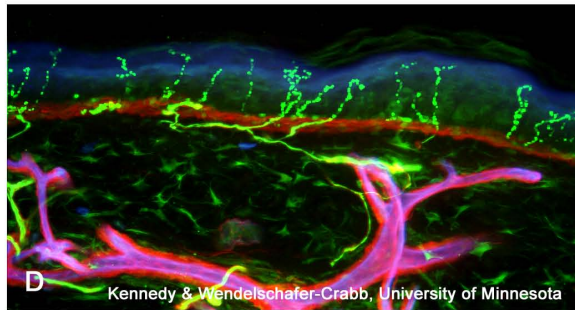
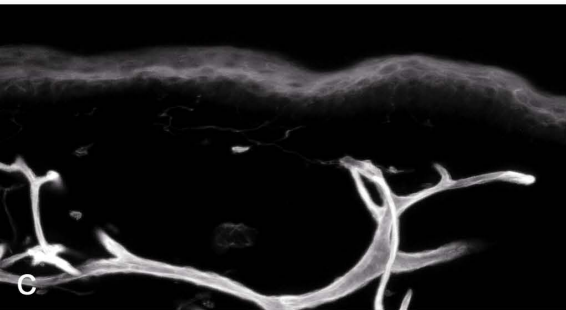
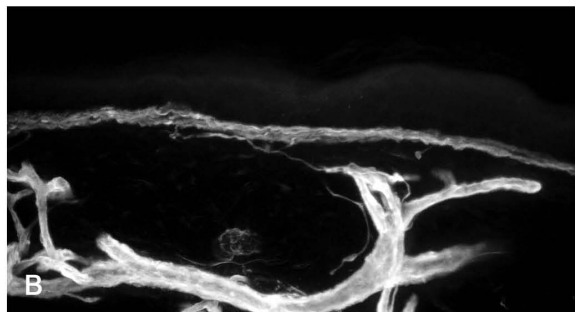
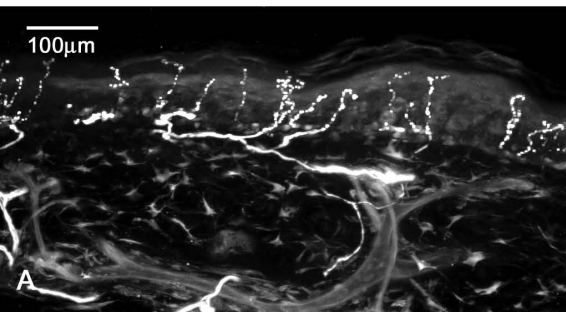


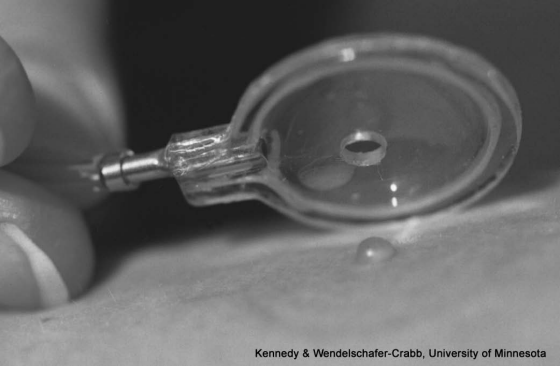
3D Diabetic Calf











100 μm

Confocal

Non Confocal

NERVE COUNTING RULES

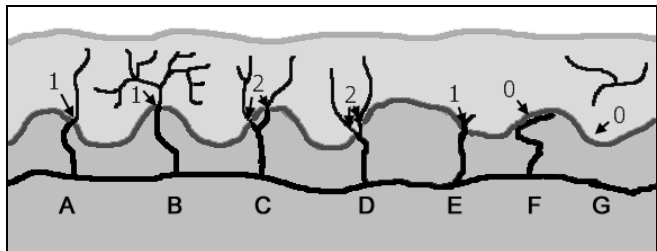


Diagram of skin innervation:

Nerves – black

Basement membrane – dark gray

Dermis – medium gray

Epidermis – light gray

A. Count nerve as it crosses the basement membrane of the epidermis.

B. Nerves that branch after crossing the basement membrane are counted as a single unit.

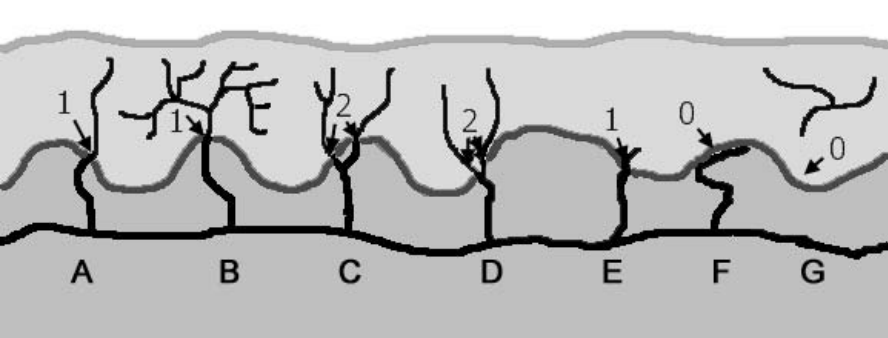
C. Nerves that split below the basement membrane are counted as two units.

D. Nerves that appear to branch within the basement membrane are counted as two units.

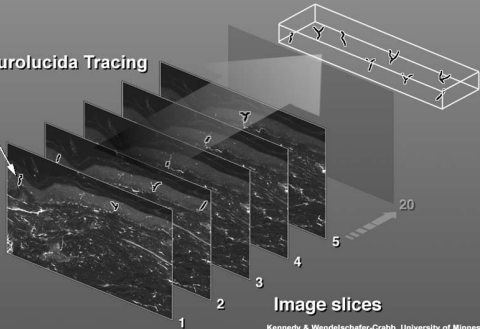
E. Nerve fragments that do cross the basement membrane are counted.

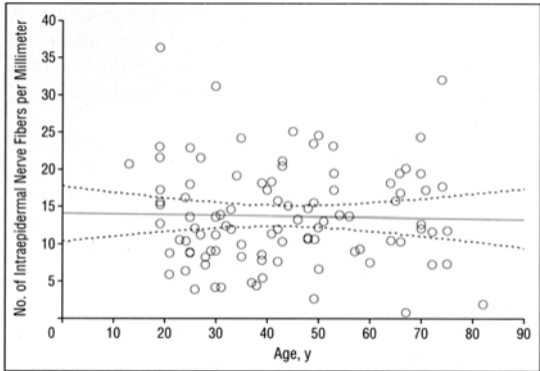
F. Nerve fibers that approach the basement membrane but do not cross it are not counted.

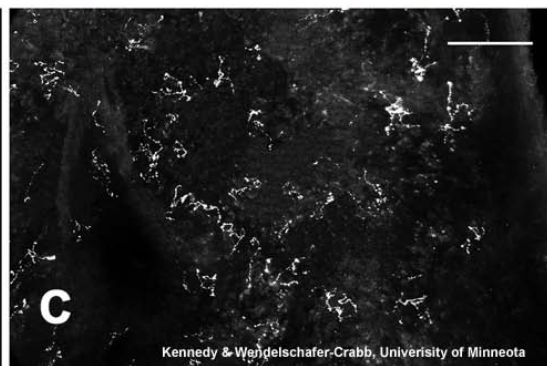
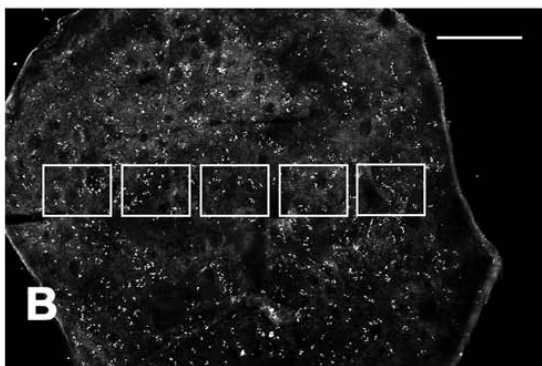
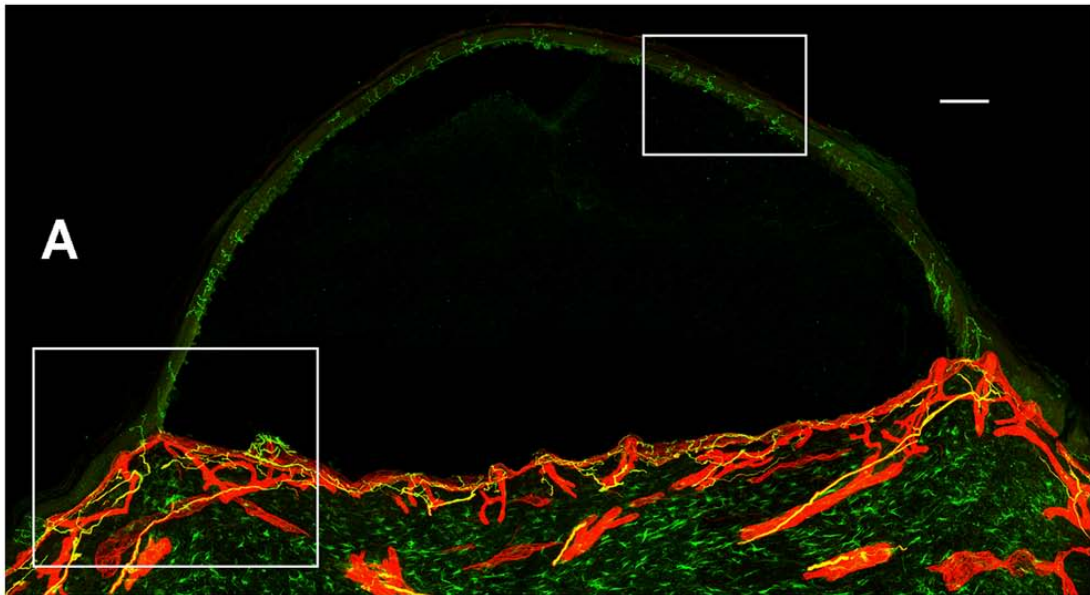
**G. Nerve fragments in epidermis that do not cross the basement membrane in the section are not counted.
(Previously counted at JHU.)**

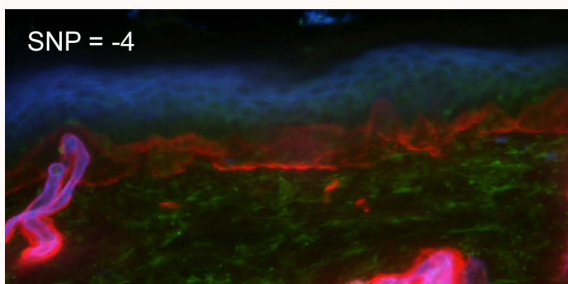
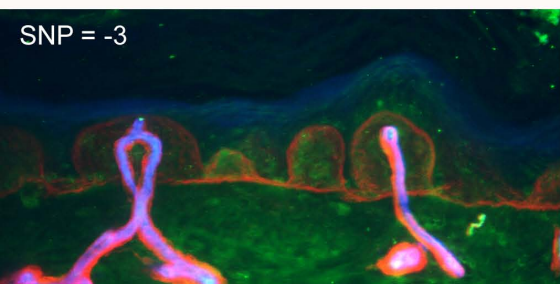
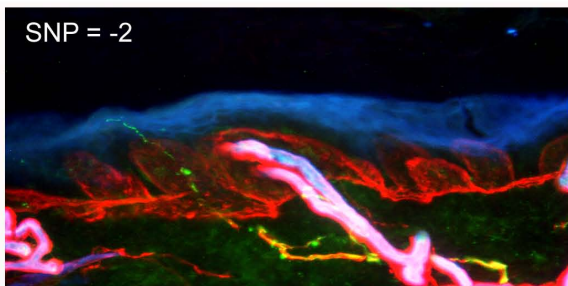
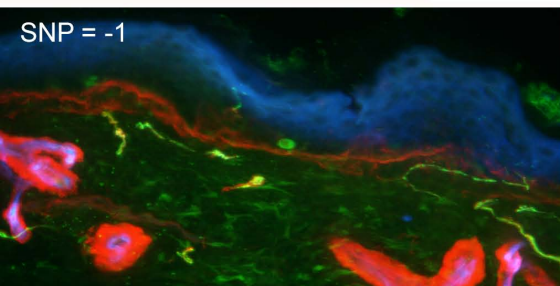
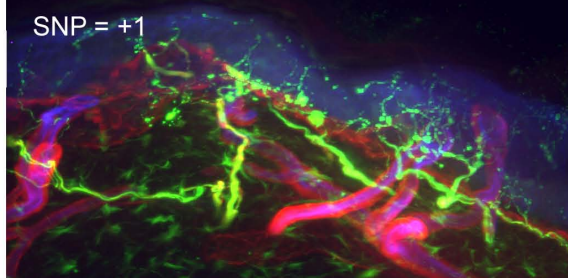
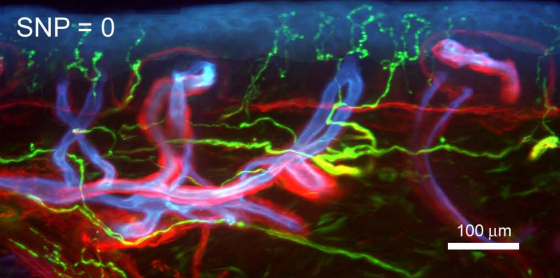


NeuroLucida Tracing







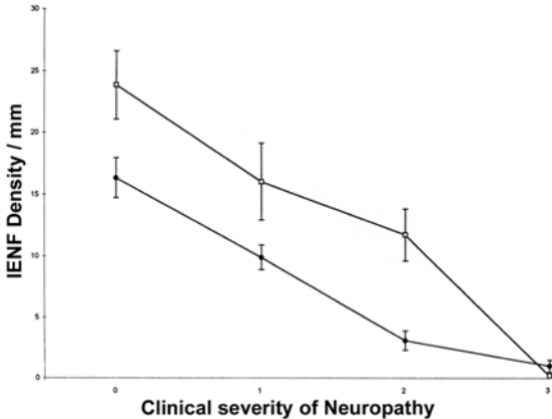


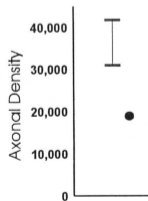
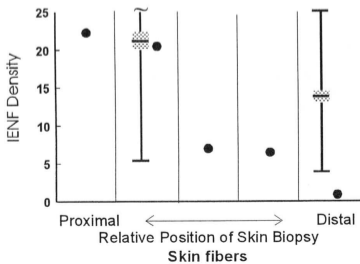
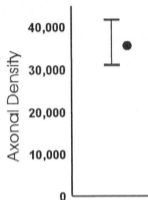
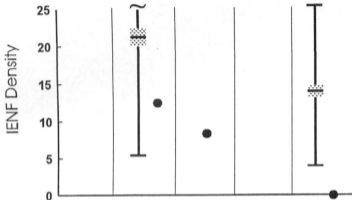
Normal Sweat Gland



Denervated Sweat Gland

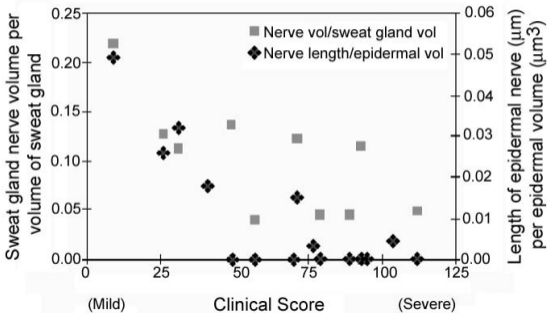






Unmyelinated

Relationship Between Clinical Neurological Evaluation and the Amount of Nerve in Epidermis and Sweat Gland

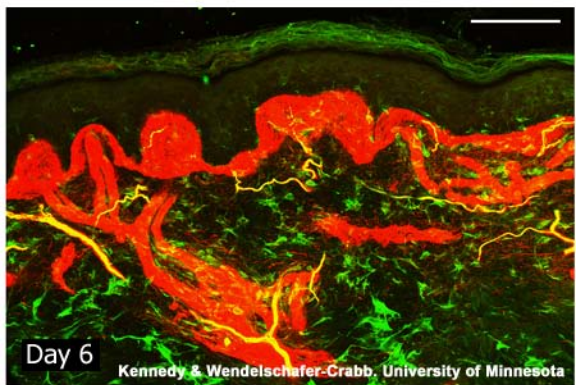
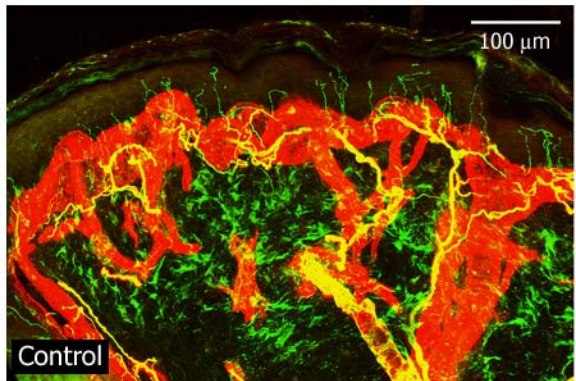


100 μ m

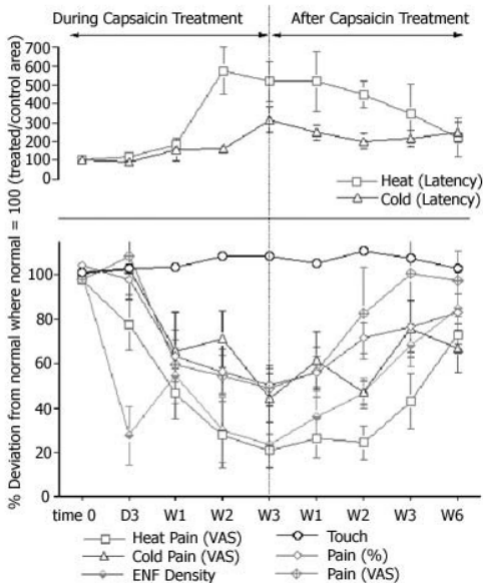
Control

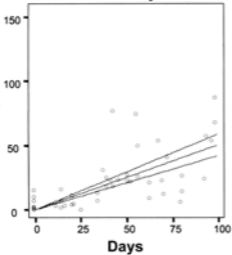
Day 6

Kennedy & Wendelschafer-Crabb. University of Minnesota



Topical Capsaicin



Diabetic subjects**Healthy control subjects**