Published in final edited form as:

Neuroscience. 2007 March 2; 145(1): 303–313.

Neurotrophic Modulation of Myelinated Cutaneous Innervation and Mechanical Sensory Loss in Diabetic Mice

Julie A. Christianson¹, Janelle M. Ryals², Megan S. Johnson², Rick T. Dobrowsky³, and Douglas E. Wright²

¹ University of Pittsburgh Med Center, Department of Medicine, Pittsburgh PA 15261

² Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160

³ University of Kansas, Department of Pharmacology and Toxicology, 5064 Malott Hall, Lawrence KS 66045

Abstract

Human diabetic patients often lose touch and vibratory sensations, but to date, most studies on diabetes-induced sensory nerve degeneration have focused on epidermal C-fibers. Here, we explored the effects of diabetes on cutaneous myelinated fibers in relation to the behavioral responses to tactile stimuli from diabetic mice. Weekly behavioral testing began prior to STZ administration and continued until 8 weeks, at which time myelinated fiber innervation was examined in the footpad by immunohistochemistry using antiserum to NF-H and MBP. Diabetic mice developed reduced behavioral responses to non-noxious (monofilaments) and noxious (pin prick) stimuli. In addition, diabetic mice displayed a 50% reduction in NF-H-positive myelinated innervation of the dermal footpad compared to non-diabetic mice. To test whether two neurotrophins NGF and/or NT-3 known to support myelinated cutaneous fibers could influence myelinated innervation, diabetic mice were treated intrathecally for two weeks with NGF, NT-3, NGF and NT-3. Neurotrophin-treated mice were then compared to diabetic mice treated with insulin for two weeks. NGF and insulin treatment both increased paw withdrawal to mechanical stimulation in diabetic mice, whereas NT-3 or a combination of NGF and NT-3 failed to alter paw withdrawal responses. Surprisingly, all treatments significantly increased myelinated innervation compared to control-treated diabetic mice, demonstrating that myelinated cutaneous fibers damaged by hyperglycemia respond to intrathecal administration of neurotrophins. Moreover, NT-3 treatment increased epidermal Merkel cell numbers associated with nerve fibers, consistent with increased numbers of NT-3-responsive slowly adapting A-fibers. These studies suggest that myelinated fiber loss may contribute as significantly as unmyelinated epidermal loss in diabetic neuropathy, and the contradiction between neurotrophin-induced increases in dermal innervation and behavior emphasize the need for multiple approaches to accurately assess sensory improvements in diabetic neuropathy.

Keywords

Neuropathy; diabetes; dermis; innervation; myelinated axon NGF; NT-3

*Correspondence: Douglas Wright, Ph.D., Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160, Phone: 913-588-2713, Fax: 913-588-2710, email: dwright@kumc.edu.

Section Editor: Donna Hammond

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

As a complication of chronic hyperglycemia, approximately half of all patients with Type I or Type II diabetes eventually develop some form of diabetic neuropathy (Brown and Asbury, 1984). The predominant type of diabetic neuropathy is a distal, symmetrical, sensorimotor polyneuropathy and preferentially affects sensory function in the distal regions, including the feet and the hands. Both small- and large-fiber peripheral axons are affected by diabetic neuropathy and their dysfunction can lead to a myriad of symptoms including loss of cutaneous sensation, chronic numbness, tingling or pain, and deficits in proprioception (Greene et al., 1999).

In diabetic neuropathy, peripheral nerves are compromised in their ability to transport neurotrophins, suggesting that reduced neurotrophin support contributes to peripheral nerve abnormalities (Jakobsen et al., 1981; Hellweg and Hartung, 1990; Schmidt et al., 1985; Fernyhough et al., 1998; Apfel, 1999; Vinik, 1999). Nociceptive sensory neurons are embryonically dependent upon NGF. Approximately half of these neurons remain dependent upon NGF, while the remainder switch responsiveness to another neurotrophin, GDNF (Snider and McMahon, 1998). Subsets of myelinated axons are responsive to either NGF or NT-3 (Airaksinen et al., 1996; Albers et al., 1996; Fundin et al., 1997; Fang et al., 2005). Interest in using exogenous neurotrophins to treat diabetic neuropathy in humans has slowed due to the outcomes in clinical trials, but studies in animal models continue to report beneficial effects. Importantly, these studies in rodents point out the diverse actions of neurotrophins on select populations of sensory neurons and that identifying neurotrophin-induced effects require selective and appropriate assessments (reviewed in Apfel, 2002; Lenninger et al., 2004).

Recent advances in quantitation of epidermal innervation have confirmed that human diabetics have significantly reduced epidermal innervation (McCarthy et al., 1995; Kennedy et al., 1996). Epidermal changes have been correlated with reduced conduction velocity, suggesting that quantitative analysis of cutaneous innervation may be indicative of developing neuropathies (Herrmann et al., 1999; Hirai et al., 2000). However, the vast majority of studies addressing axon loss in diabetes have focused on C-fiber loss in the epidermis and information regarding dermal innervation by myelinated fibers has been largely ignored (see Lauria et al., 2005). This is surprising since ultrastructural damage to myelinated fibers in diabetic neuropathy has been reported in both animals and patients (Yagihashi et al., 1990; Ochodnicka et al., 1995; Weis et al., 1995; Mizisin et al., 1998). Electrophysiological studies in diabetic rats suggest that the increased sensory input from myelinated afferents plays an important role in diabetic neuropathic pain (Khan et al., 2002).

Using a mouse model of STZ-induced diabetic neuropathy, we have demonstrated behavioral and innervation deficits similar to insensate symptoms in human diabetic patients (Christianson et al., 2003a,b). Here, we have investigated dermal innervation by myelinated fibers in diabetic mice. Diabetic mice have a significant reduction in myelinated innervation and significantly diminished behavioral responses to a range of mechanical stimuli. Importantly, we demonstrate that 2 neurotrophins known to affect myelinated fibers (NGF and NT-3) have selective actions on diabetes-induced deficits in dermal innervation and behavioral responses.

Experimental Procedures

Diabetic mice

Forty-two 8-week old male C57BL/6 mice were purchased from Charles River (Wilmington, MA) and housed in the Laboratory Animal Resources Facility at the University of Kansas Medical Center. All research conformed to NIH guidelines in accordance with the guidelines specified by the University of Kansas Medical Center Animal Care and Use Protocol and the National Institute of Health Guide for the Care and Use of Laboratory Animals. All mice received water and mouse chow ad libitum. Diabetes was induced by intraperitoneal injection

of STZ (Sigma, St. Louis, MO, 180 mg/kg; n=35) dissolved in 0.4 ml sodium citrate buffer, pH 4.5 (Akkina et al., 2001). Non-diabetic mice (n=7) received only 0.4 ml sodium citrate buffer. Mice were weighed at weekly intervals and blood glucose levels were measured using the Glucose Diagnostic Kit 510-DA (Sigma, St. Louis, MO). Diabetic mice were monitored closely for symptoms of hyperglycemia that included polydipsia, polyuria, and weight loss. Only STZ-injected mice with reduced body weight and blood glucose levels greater than 300 mg/dL were included in the diabetic group.

Neurotrophin and Insulin Administration

Six weeks after induction of diabetes, mice were treated intrathecally once daily with NGF (n=6; Sigma, St. Louis, MO), NT-3 (n=6; Chemicon, Temecula, CA), or combination NGF and NT-3 (n=7) for two weeks. NGF and NT-3 were separately dissolved in artificial CSF at a concentration of 20 nM. For combination NGF and NT-3 treatment, they were dissolved in CSF at a total concentration of 40 nM. Intrathecal injections (50 μ L, 1 μ g NGF or NT-3, 2 μ g NGF and NT-3) were performed between the L_{V1} and S_I vertebrae using a 30-gauge needle. As a control for the intrathecal injection paradigm, additional diabetic mice were treated daily with artificial CSF (n=7). These CSF-treated mice were referred to as diabetic mice in experiments that evaluated behavioral responses to cutaneous stimuli. Alternatively, slow-release insulin pellets (Linshin Canada, Inc., Ontario, Canada) were implanted subcutaneously in 6-week diabetic mice (0.2 Units/day; n=5) and remained for two weeks.

Immunocytochemistry

Cutaneous innervation in the footpad was assessed in non-diabetic (n=7) and treated diabetic mice (n=32) eight weeks after STZ administration. Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg body weight) and transcardially perfused with ice cold saline followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For NF-H visualization, the skin was dissected from the footpad and immersion fixed overnight and then cryoprotected using 30% sucrose. Sections were cut on a cryostat at 20 μ m and placed on Superfrost Plus microscope slides (Fisher, Chicago, IL). Immunocytochemistry was performed using previously published protocols (Christianson et al., 2003a). Briefly, sections were blocked for one hour in 1.5% normal goat serum, 0.5% porcine gelatin, and 0.2% Triton X-100 in Superblock Buffer (pH 7.4; Pierce, Rockford, IL). Sections were incubated overnight with primary antisera to NF-H (1:500; Chemicon, Temecula, CA) at 4°C under humidified conditions. This polyclonal antibody (#AB1991) is not affected by the level of neurofilament phosphorylation. The primary antibody was removed by two washes in phosphate-buffered saline (PBS, pH 7.4) and sections were then incubated with secondary antibody (donkey anti-rabbit Rhodamine Red, Jackson ImmunoResearch, West Grove, PA, 1:200 in 0.1 M PBS) for one hour at 4 °C. Sections were rinsed in PBS and coverslipped before viewing.

For MBP visualization, mice were deeply anesthetized and the right and left hindpaws were dissected, post-fixed for 1 hour at 4°C in Zamboni's fixative (4% paraformaldehyde, 14% saturated picric acid, 0.1 M PBS; pH 7.4), then immersed overnight in 30% sucrose at 4°C. Tissue was frozen sectioned on a cryostat at 30 μ m. Sections were mounted on Superfrost Plus slides (Fisher Scientific) and stored at -20°C until immunostaining. Following a 15 min. immersion in 100% methanol at -20°C, the tissue treated with a blocking solution (10% normal goat serum, 0.3% Triton-X100, in PBS) for 15 min. at room temperature. The primary antibody (mouse anti-MBP; 1:500; SMI94, Sternberger Monoclonals, Inc.) was diluted in blocking solution and incubated with sections overnight at 4°C. For visualization, sections were incubated for 1 hour at 4°C with a fluorochrome-conjugated secondary antibody (goat anti-mouse Alexa 488; 1:2000; Molecular Probes) diluted in blocking solution. Slides were then coverslipped following three washes with PBS.

In a separate group of animals, Merkel cells were visualized using a monoclonal antibody to cytokeratin-18 (1:200; Chemicon, Temecula, CA). Since this antibody works only on unfixed tissue, mice were deeply anesthetized and fresh skin was dissected from the footpad in 2mm long segments. Groups included non-diabetic mice, diabetic mice receiving CSF, NGF, or NT-3 intrathecally, and mice implanted with insulin (same concentrations as above, n=4 for each group). The skin was immediately frozen on dry ice, sectioned, and processed using immunocytochemical protocols described above. The cytokeratin-18 antibody was used in conjunction with NF-H antibody in order to ensure that cytokeratin-18-positive cells were associated with NF-H-positive axons.

Quantification of Cutaneous Myelinated Axons

Photomicrographs (10X) of labeled footpad sections (7 images/animal) were captured using a MagnaFire digital camera (Optronics, Goleta, CA) attached to a Nikon Eclipse 800 microscope. Images were then coded to blind the investigator to the animal number, status and treatment. NIH Image J was used to measure the total area of the photographed skin by tracing the entire dermal area. Next, immunopositive bundles and individual axons were traced and the area occupied by myelinated axons was then calculated. The sections were decoded and measurements were assigned to non-diabetic, CSF-treated diabetic, and trophic factor-treated diabetic groups and compared using one-way ANOVA with Fisher's Protected Least Significant Difference (PLSD) post hoc tests (StatView, SAS Institute Inc, Cary, NC).

Merkel cell abundance was calculated using conventional fluorescence microscopy by counting the number of cytokeratin-18-positive cells within the basal layer of the epidermis in 2 mm long skin sections. Cytokeratin-18-positive cells had to be associated with an NF-H-positive axon to be counted. For each treatment group, 10 sections were used to determine the average number of Merkel cells/section. The mean number of Merkel cells was then calculated from non-diabetic, CSF-treated diabetic, and trophic factor-treated diabetic mice and compared using one-way ANOVA with Fisher's PLSD post hoc tests. Adobe Photoshop 6.0 was used to adjust brightness/contrast and to construct all figures. In certain cases, skin sections were photographed using a LSM 510 Laser Scanning Microscope to generate images.

Behavioral Analyses

Mice were tested for behavioral responses to non-noxious mechanical stimuli using calibrated von Frey monofilaments (Stoelting, Wood Dale, IL). In all cases, experimenters were blinded to the glycemic status and treatment groups. Mice were placed under plastic chambers (3 X 8 X 12 cm) on top of a wire mesh-top table and allowed to acclimate for 20 minutes. Responses to 4 separate monofilaments of increasing force (0.16, 0.6, 1.4 and 2 g) were recorded for each animal. A single monofilament was applied to the plantar surface of the foot for one second. Each monofilament was applied to alternating footpads six times and 10 seconds were allowed to elapse between applications to the same foot. The percent response was obtained by determining the number of withdrawals in response to each filament (out of 6 applications). Comparisons were made between groups using 2-way repeated measures ANOVA with Fisher's PLSD post hoc tests.

Mice were evaluated for behavioral responses to noxious mechanical stimuli using a pinprick test prior to sacrifice. Mice were placed under plastic chambers on a mesh-top table and allowed to acclimate for 20 minutes. A 27-gauge needle was gently applied to the plantar surface of the hind paw without breaking the skin. The needle was applied to alternating feet and 30 seconds were allowed to elapse between applications to the same foot. A response was defined as lifting, shaking or licking the hind paw. The number of responses out of five trials was calculated as a percent for each treatment group and compared using one-way ANOVA with Fisher's PLSD post hoc tests.

Results

STZ-induced diabetes in mice

Within 48 hours of STZ injection, mice developed typical signs of diabetes including weight loss, polyuria and polydipsia. Eight weeks following STZ injection, diabetic mice had lost 13% of their original body weight and developed a threefold increase in mean blood glucose levels compared to sham-injected non-diabetic mice (Table 1). Diabetic mice treated with NGF, NT-3 or combination NGF and NT-3 did not have significantly altered weight or glucose levels when compared to CSF-injected diabetic mice (Table 1). In contrast, diabetic mice implanted with sustained-release insulin pellets significantly increased their body weight and decreased their mean blood glucose levels (Table 1).

Reduction in mechanical sensitivity in diabetic mice

Non-diabetic and diabetic mice were evaluated weekly for their behavioral responses to a range of von Frey monofilaments. As early as 4 weeks post-STZ administration, diabetic mice responded significantly fewer times to an application of a 0.6 g, 1.4 g or 2.0 g monofilament to the dorsal surface of the hind paw ($P < 0.05$; Fig. 1A–D). Diabetic mice also displayed significantly reduced responses to a 0.16 g monofilament beginning at 5 weeks post-STZ ($P < 0.05$; Fig. 1A). The mechanical insensitivity displayed by the diabetic mice progressively worsened throughout the testing period.

NGF but not NT-3 increases mechanical sensitivity of diabetic mice

Six weeks after STZ-injection, diabetic mice were divided into five groups and treated for two weeks with CSF ($n=7$), NGF ($n=7$), NT-3 ($n=7$), NGF and NT-3 ($n=7$) or insulin ($n=6$). Intrathecal injections were used to provide the best opportunity to deliver growth factors to the DRG and to avoid problems associated with poor retrograde transport of trophic factors from the periphery (Akkina et al., 2001; Christianson et al., 2003a,b). Insulin treatment was provided via subcutaneous implantation of insulin pellets. Mice continued to be evaluated weekly for their responses to mechanical stimuli.

Mechanical stimuli—Behavioral responses of non-diabetic and treated diabetic mice were evaluated using the percent withdrawal method with von Frey monofilaments of increasing degrees of mechanical force. CSF-treated diabetic mice responded significantly fewer times than non-diabetic mice to all four monofilaments tested ($P < 0.05$; Fig. 2A–D; Table 2). NGF treatment significantly improved withdrawal responses in 3 out of the four monofilaments tested compared to CSF-treated diabetic mice ($P < 0.05$; Fig. 2A; Table 2). Neither NT-3 treatment nor combination NGF and NT-3 treatment was able to significantly alter behavioral responses to any of the monofilaments tested ($P > 0.05$; Fig. 2B,C; Table 2). Insulin treatment significantly restored withdrawal responses to the lowest three forces tested ($P < 0.05$; Fig. 2D; Table 2), but not the 2.0 g monofilament ($P > 0.05$; Table 2).

Eight weeks after diabetes induction, pinprick tests were performed to assess behavioral responses of non-diabetic and diabetic mice treated with CSF, NGF, NT-3, a combination of NGF and NT-3, or insulin when exposed to a noxious mechanical stimulus. CSF-treated diabetic mice responded significantly fewer times than non-diabetic mice to application of a 27-gauge needle to the hind paw ($P < 0.05$, Table 2). Similarly, diabetic mice treated with NT-3 or combination NGF and NT-3 also elicited significantly fewer responses than non-diabetic mice ($P < 0.05$, Table 2). Insulin treatment significantly restored behavioral responses of diabetic mice to pinprick stimulation compared to CSF-treated diabetic mice ($P < 0.05$, Table 2).

Reduced dermal innervation by myelinated axons in diabetic mice

Immunohistochemistry was performed using antiserum against NF-H and MBP to visualize innervation of myelinated axons in footpad skin of non-diabetic and diabetic mice 6–8 weeks following STZ administration. Analysis of non-diabetic skin revealed large MBP- (Fig. 3) and NF-H-positive (Fig. 4) fascicles coursing below and parallel to the epidermis. Individual axons branch perpendicularly from the fascicles and project upward through the dermis and terminate at the dermal-epidermal border (Fig. 3A). Intense axonal staining was also seen surrounding sweat gland complexes. At both 6 (Fig. 3) and 8 weeks (Fig. 4) post STZ injection, MBP- and NF-H- positive fascicles were evident in diabetic mice within the basal layer of the dermis, however they were much less frequent and smaller in area compared to healthy non-diabetic mice. Also, there was a significant reduction in the perpendicular innervation through the dermis (Fig. 4B). Many individual axons appeared truncated and fragmented. Because NF-H staining provided a more consistent staining, NF-H staining was used to quantify myelinated fiber innervation in 8-week diabetic mice. Overall, innervation of NF-H-positive myelinated fibers in diabetic mice was reduced by 53%, when compared to non-diabetic mice ($P < 0.05$; Fig. 4A, B, Fig. 5).

NGF, NT-3 and insulin increase myelinated innervation in diabetic mice

Following the behavioral assessments (8 weeks post STZ injection), neurotrophin and insulin-treated mice were sacrificed to determine whether neurotrophin and/or insulin treatment altered dermal innervation by myelinated fibers. Assessment of mice treated with NGF revealed a substantial increase in the amount of NF-H-positive in the dermis (Fig. 4C). In many cases, large fascicles were evident in deeper portions of the dermis. Quantitation of axon abundance revealed that NGF treatment induced a significant increase in myelinated fibers compared to CSF-treated diabetic mice ($P < 0.05$), and even surpassed the levels in healthy non-diabetic mice (Fig. 5). Similarly, NT-3 treatment also dramatically increased the amount of NF-H-positive axons in the dermis (Fig. 4D). In comparison to NGF-treated mice, NT-3 treated mice qualitatively appeared to have an increase in individual, finer axons, particularly adjacent to the epidermal surface. Quantitation revealed that similar to NGF, NT-3 treatment significantly increased NF-H-positive axons compared to CSF-treated diabetic mice ($P < 0.05$), and also surpassed the levels in non-diabetic mice (Fig. 5).

Based on the idea that NGF and NT-3 affect different populations of myelinated fibers and that NGF- and NT-3-treated mice had slightly different patterns of NF-H-positive axons, mice were treated with a combination of NGF and NT-3 in hopes of further increasing the abundance of NF-H-positive axons. Assessment of these mice revealed that NGF and NT-3 treatment significantly increased NF-H-positive axons compared to CSF-treated mice, but was unable to supercede levels of mice treated with NGF or NT-3 individually ($P < 0.05$, Fig. 5E, Fig. 5). Finally, analysis of NF-H-positive axons in insulin-treated mice revealed a significant increase in axonal abundance compared to CSF-treated diabetic mice ($P < 0.05$, Fig. 4F). Levels of NF-H-positive axons in insulin-treated mice were comparable to mice treated with NGF and/or NT-3 (Fig. 5).

NT-3 increases Merkel cells in diabetic mice

Merkel cells are sensory receptors located within the basal epidermal layer and are innervated by NT-3 responsive slowly adapting type 1 myelinated fibers. To test whether the diabetes-induced reduction in dermal innervation by myelinated fibers affected Merkel cell abundance in the mouse footpad, immunohistochemistry was performed using antiserum to cytokeratin-18, a marker of Merkel cells. As shown in Fig. 6 (arrows), Merkel cells were easily observed in both non-diabetic and diabetic mice beneath the epidermis. In many cases, Merkel cells were aggregated in small clusters with 2–4 Merkel cells in close proximity.

Quantification of the number of Merkel cells associated with NF-H-positive axons was performed in non-diabetic and diabetic mice treated with CSF, NGF-, NT-3 or insulin (Fig. 6D). In all cases, Merkel cells were only counted if they were associated with a NF-H-positive axon to ensure that cells counted were Merkel cells and not some other dermal cell type expressing cytokeratin-18 (arrows, Fig. 6C). Compared to non-diabetic mice, CSF-treated diabetic mice had comparable numbers of Merkel cells, suggesting that Merkel cell numbers are not dramatically reduced by chronic hyperglycemia. NGF-treated diabetic mice had increased numbers of Merkel cells, but this increase was not different from CSF-treated diabetic mice ($P < 0.05$). However, analysis of diabetic mice treated with NT-3 revealed a significant increase in Merkel cells associated with axons compared to CSF-treated diabetic mice ($P < 0.05$). Interestingly, insulin-treated diabetic mice were not different from CSF-treated diabetic mice despite displaying an increase in NF-H positive axons (Fig. 6).

Discussion

Distal sensory axon loss is a significant problem in diabetes and despite our increasing understanding of epidermal C-fibers, comparatively little is known about myelinated fibers that innervate the skin. Using a murine model of Type I diabetes, we demonstrate that diabetic mice develop a slow and progressive deficit in noxious and non-noxious mechanical stimulation. Moreover, these diabetic mice display substantial losses of NF-H- and MBP-positive myelinated sensory fibers that innervate the skin, which are the sensory fibers principally responsible for conveying mechanical information. In addition, we demonstrate that two neurotrophins, NGF and NT-3, can increase the abundance of myelinated fibers in the dermis. However, only NGF was capable of increasing paw withdrawal to mechanical stimuli in diabetic mice. These results demonstrate that dermal myelinated fibers are sensitive to chronic hyperglycemia and illustrate that increased axon innervation does not always associate with behavioral responses to sensory stimuli.

Mechanical hypoalgesia in diabetic mice

Human diabetic patients develop a compromised ability to perceive tactile sensations, particularly in the distal limbs. This insensate neuropathy is attributed to damage of large fibers and along with nerve conduction velocity and vibration sensitivity, monofilament testing is used clinically to assess large-fiber loss. Generally, monofilaments of significantly higher force are required to elicit a response from diabetic patients compared to non-diabetic patients (Mueller, 1996; Norrsell et al., 2001). Consistent with these deficits, diabetic C57Bl/6 mice display reduced behavioral responses to a range of von Frey monofilaments 4 weeks after STZ administration (Christianson et al., 2003a, Wright et al., 2004). Interestingly, diabetic mice displayed similar reduced responses to a noxious mechanical stimulus, suggesting that afferent fibers that convey noxious and non-noxious stimuli are affected. The majority of sensory axons in mouse glabrous skin are myelinated A β -fibers, while the remaining sensory fibers are split between A δ and C-fibers. Within the A β -fiber population, nearly 70% are rapidly adapting, while the remaining are slowly adapting (Cain et al., 2001). Although our study did not discriminate between fiber types, it is clear that the mouse glabrous skin is richly innervated by myelinated A-fibers that transduce a wide range of low-threshold mechanical signals and these myelinated fibers are strongly affected by chronic hyperglycemia. Noxious mechanical stimuli are mediated primarily through high threshold A δ - and C-fibers (Koltzenburg et al., 1997; Cain et al., 2001), and the reduced response of diabetic mice to pinprick stimuli are consistent with the significant C-fiber loss in these diabetic mice (Christianson et al., 2003b).

It is important to point out that mechanical hypoalgesia in STZ-induced diabetic mice contrasts significantly with STZ-induced diabetic rats. Diabetic rats display tactile allodynia and the increased sensitivity of rats has been difficult to relate to the insensate neuropathy that occurs

in the majority of humans with diabetic neuropathy. Moreover, the rapid appearance of the hypersensitivity in rats has not been easily attributable to diabetes-induced abnormalities in peripheral nerve (Brown et al., 1980; Courteix et al., 1993; Calcutt et al., 1996; Sima et al., 1998; Fox et al., 1999; Khan et al., 2002). It is not clear why STZ-induced diabetic rats and mice develop such divergent responses to mechanical stimuli. However, our analysis of 4 different strains of STZ-induced diabetic mice reveals considerable variability in the severity of mechanical hypoalgesia among strains, and C57Bl/6 mice appear to develop the most severe mechanical loss (Wright, unpublished observations). Together, these differences indicate the importance of species and genetic strains in addressing mechanisms of sensory dysfunction. Interestingly, depletion of capsaicin-sensitive C-fibers in STZ-induced diabetic rats does not alter the development of tactile allodynia, suggesting that A-fibers play an important role in increased mechanical sensitivity (Khan et al., 2002).

Neurotrophic modulation of mechanical insensitivity and myelinated innervation

NGF has powerful actions on a range of sensory fibers in adulthood, including A δ fibers and subpopulations of C- and A β fibers (Petruska and Mendell, 2004; Fang et al., 2005). We previously demonstrated that NGF could increase the mechanical sensitivity of diabetic mice in the absence of increased C-fiber innervation, suggesting that perhaps NGF's actions were mediated by sensitizing remaining axons (Christianson et al., 2003a). Here, NGF produced similar increases in paw withdrawals in addition to increasing myelinated fiber content within the dermis. Together, these findings suggest that in addition to sensitization, NGF may alter behavioral responsiveness via myelinated fibers in the skin. NGF overexpression in the skin significantly increases the percentage of A δ fibers in the skin and also increases their mechanical responsiveness (Stucky et al., 1999). Recent studies report that a significant percentage of nociceptive A-fibers express trkA and respond to NGF (Fang et al., 2005). Together, our findings that NGF can significantly increase paw withdrawals to mechanical stimuli may be best explained by stimulation of A δ mechanosensitive afferent fibers growth in addition to decreased thresholds, leading to increases paw sensitivity. That NGF can increase myelinated, but not unmyelinated fibers in the skin of diabetic mice suggest differential actions of NGF on these subpopulations, or alternatively, a differential ability to recover from the effects of chronic hyperglycemia (Christianson et al., 2003b).

In contrast to NGF, NT-3 was ineffective in increasing mechanical sensitivity in diabetic mice, despite having robust effects on myelinated innervation. NGF and NT-3 both support A β -fiber populations but the precise overlap is not clear. A subpopulation of large DRG neurons coexpresses trkA and trkC, suggesting that certain neurons respond to both neurotrophins (Wright and Snider, 1995). Evidence from immunohistochemical studies in the skin and vibrissa suggest that this population may be A δ -fibers (Fundin et al., 1997; Funfschilling et al., 2004). In addition, NT-3 regulates slowly adapting type 1 fibers that innervate Merkel cells during postnatal development (Airaksinen et al., 1996; Albers et al., 1996). Consistent with our findings, NT-3 overexpression in the skin leads to increased sensory innervation particularly related to Merkel cell innervation, suggesting that NT-3 has powerful actions on slowly-adapting type 1 sensory axon growth and innervation (Krimm et al., 2004). It is plausible to suggest that NT-3-sensitive cutaneous afferent fibers are not involved in reflexive paw withdrawal and the effects of NGF could be attributed to sensitizing fibers involved in the withdrawal reflex. This is an important consideration since many rodent studies rely on withdrawal reflexes to assess mechanical sensation and pain, and also emphasizes the importance in both rodent and human studies to design outcome measures that adequately assess therapeutic treatments. Indeed, the failure of NGF in Phase III clinical trials may be partially related to outcome measures that were not suitable for NGF's actions (Apfel, 2002).

Skin biopsy has become a popular approach to investigate the density of small unmyelinated IENF in peripheral neuropathies. Deficits in IENF density correlate strongly with the presence of neuropathy and provide a noninvasive approach to diagnose a variety of neuropathies (Hermann et al., 1995; Hirai et al., 2000). Recent guidelines have been established that aid in standardizing quantification of intraepidermal C-fibers (Lauria et al., 2005). Assessment of individual C-fibers is possible due to the spaced distribution within the epidermis. Here, our estimates of myelinated fiber content in diabetic mice included measurements of individual axons as well as nerve fascicles. Thus, the resolution of our measurements could not approach those of current IENF C-fiber measurements, including those of our own laboratory (Christianson et al., 2003b). It is possible that neurotrophin-induced increases in myelinated fibers were affected by increased axonal size. However, the robust increases in fiber content cannot be explained simply by increased fiber size and we are confident that the increases observed were primarily due to growth of sensory axons.

Diabetic mice treated with insulin also demonstrated robust improvements in mechanical sensitivity and myelinated fiber content. These results are consistent with the view that proper glycemic control is the best defense against diabetic neuropathy, and early in the development of neuropathy, sensory fibers respond well to insulin treatment (DCCT Research Group, 1993). It remains to be determined whether insulin's actions are due to lowered glycemic levels or direct actions on DRG neurons (Russell et al., 1998; Huang et al., 2003; Romanovsky et al., 2004). Moreover, future studies should focus on whether insulin has similar actions on axons exposed to long-term hyperglycemia (i.e., > 12 weeks).

We hypothesized that NGF and NT-3 in combination may have greater actions by distinct populations of myelinated fibers. Although NGF and NT-3 increased myelinated fiber abundance, the combination failed to increase mechanical sensitivity similar to NGF alone. It is possible that NT-3 masked sensitization effects of NGF that increase the paw withdrawal reflex. In support of this, recent studies suggest that NT-3 has antihyperalgesic actions by modifying the phenotype of nociceptive neurons (Gratto et al., 2003; Wilson-Gerwing et al., 2005). In our own studies, NT-3 has anti-nociceptive actions in a model of chronic pain induced by acid injection (Gandhi et al., 2004). Together, these studies illustrate the complicated interactions of NGF and NT-3 on nociceptive populations.

NT-3 has been investigated in relation to impaired axonal transport and delivery of neurofilaments in diabetic neuropathy. These studies reveal that diabetes impairs slow anterograde axonal transport of proteins, accumulates neurofilaments in proximal segments of axons, and increases phosphorylation of neurofilaments. It has been suggested that these deficits contribute significantly to the degradation of distal axons. Complications associated with neurofilament phosphorylation were avoided in this study by choosing an NF-H antibody that is not sensitive to neurofilament phosphorylation. Importantly, NT-3 treatment reduces these deficits, consistent with our findings regarding NT-3 treatment (Mizisin et al., 1999; Mizisin et al., 1998; Fernyhough et al., 1999; Sayers et al., 2003). Interestingly, we also demonstrate that mice treated with NT-3 displayed a significant increase in Merkel cells associated with nerve fibers, suggesting that NT-3 treatment stimulated formation and innervation of sensory receptors *de novo*. Previous studies have demonstrated that Merkel cells require neural innervation and that developmental overexpression of NT-3 in the skin leads to increased sensory neuron survival, which in turn increases Merkel cell numbers (Krimm et al., 2004). Our findings of elevated Merkel cells associated with nerve fibers in NT-3-treated diabetic mice are consistent with data that NT-3-responsive axons modulate axon innervation of Merkel cells.

Acknowledgements

Supported by NIH grant R01NS343314 (DEW). The authors thank the members of the Wright laboratory for helpful comments on the manuscript.

References

- Airaksinen MS, Koltzenburg M, Lewin GR, Masu Y, Helbig C, Wolf E, Brem G, Toyka KV, Thoenen H, Meyer M. Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* 1996;16:287–295. [PubMed: 8789944]
- Akkina SK, Patterson CL, Wright DE. GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice. *Exp Neurol* 2001;167:173–182. [PubMed: 11161605]
- Albers KM, Perrone TN, Goodness TP, Jones ME, Green MA, Davis BM. Cutaneous overexpression of NT-3 increases sensory and sympathetic neuron number and enhances touch dome and hair follicle innervation. *J Cell Biol* 1996;134:487–497. [PubMed: 8707832]
- Apfel SC. Neurotrophic factors in peripheral neuropathies: Therapeutic implications. *Brain Path* 1999;9:393–413. [PubMed: 10219753]
- Apfel SC. Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? *Int Rev Neurobiol* 2002;393–413. [PubMed: 12198818]
- Brown MJ, Sumner AJ, Greene DA, Diamond SM, Asbury AK. Distal neuropathy in experimental diabetes mellitus. *Ann Neurol* 1980;8:168–178. [PubMed: 7425570]
- Brown MJ, Asbury AK. Diabetic neuropathy. *Ann Neurol* 1984;15:2–12. [PubMed: 6370098]
- Cain DM, Khasabov SG, Simone DA. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J Neurophysiol* 2001;85:1561–74. [PubMed: 11287480]
- Calcutt NA, Jorge MC, Yaksh TL, Chaplan SR. Tactile allodynia and formalin hyperalgesia in streptozotocin-diabetic rats: effects of insulin, aldose reductase inhibition and lidocaine. *Pain* 1996;68:293–299. [PubMed: 9121817]
- Christianson JA, Ryals JM, McCaeson KE, Wright DE. Beneficial actions of neurotrophin treatment on diabetes-induced hypoalgesia in mice. *J Pain* 2003a;4:493–504. [PubMed: 14636817]
- Christianson JA, Riekhof JT, Wright DE. Restorative effects of neurotrophin treatment on diabetes-induced cutaneous axon loss in mice. *Exp Neurol* 2003b;179:188–199. [PubMed: 12618126]
- Courteix C, Eschalir A, Lavarenne J. Streptozotocin-induced diabetic rats: behavioural evidence for a model of chronic pain. *Pain* 1993;53:81–88. [PubMed: 8316394]
- DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993;329:977–986. [PubMed: 8366922]
- Fang X, Djouhri L, McMullan S, Berry C, Okuse K, Waxman SG, Lawson SN. TrkA is expressed in nociceptive neurons and influences electrophysiological properties via Nav1.8 expression in rapidly conducting nociceptors. *J Neurosci* 2005;25:4868–4878. [PubMed: 15888662]
- Fernyhough P, Gallagher A, Averill SA, Priestley JV, Hounsom L, Patel J, Tomlinson DR. Aberrant neurofilament phosphorylation in sensory neurons of rats with diabetic neuropathy. *Diabetes* 1999;48:881–889. [PubMed: 10102707]
- Fernyhough P, Diemel LT, Tomlinson DR. Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin-diabetic rats. *Diabetologia* 1998;41:300–306. [PubMed: 9541170]
- Fundin BT, Silos-Santiago I, Ernfors P, Fagan AM, Aldskogius H, DeChiara TM, Phillips HS, Barbacid M, Yancopoulos GD, Rice FL. Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. *Dev Biol* 1997;190:194–216.
- Fox A, Eastwood C, Gentry C, Manning D, Urban L. Critical evaluation of the streptozotocin model of painful diabetic neuropathy in the rat. *Pain* 1999;81:307–316. [PubMed: 10431718]
- Fundin BT, Silos-Santiago I, Ernfors P, Fagan AM, Aldskogius H, DeChiara TM, Phillips HS, Barbacid M, Yancopoulos GD, Rice FL. Differential dependency of cutaneous mechanoreceptors on neurotrophins, trk receptors, and P75 LNGFR. *Dev Biol* 1997;190:94–116. [PubMed: 9331334]

- Funfschilling U, Ng YG, Zang K, Miyazaki J, Reichardt LF, Rice FL. TrkC kinase expression in distinct subsets of cutaneous trigeminal innervation and nonneuronal cells. *J Comp Neurol* 2004;480:392–414. [PubMed: 15558783]
- Gandhi R, Ryals JM, Wright DE. Neurotrophin-3 reverses chronic mechanical hyperalgesia induced by intramuscular acid injection. *J Neurosci* 2004;24:9405–9413. [PubMed: 15496676]
- Gratto KA, Verge VM. Neurotrophin-3 down-regulates trkA mRNA, NGF high-affinity binding sites, and associated phenotype in adult DRG neurons. *Eur J Neurosci* 2003;18:1535–1548. [PubMed: 14511333]
- Greene DA, Stevens MJ, Feldman EL. Diabetic neuropathy: scope of the syndrome. *Am J Med* 1999;107:2S–8S. [PubMed: 10484039]
- Hellweg R, Hartung HD. Endogenous levels of nerve growth factor (NGF) are altered in experimental diabetes mellitus: a possible role for NGF in the pathogenesis of diabetic neuropathy. *J Neurosci Res* 1990;26:258–267. [PubMed: 2142224]
- Herrmann DN, Griffin JW, Hauer P, Cornblath DR, McArthur JC. Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. *Neurology* 1999;53:1634–1640. [PubMed: 10563605]
- Hirai A, Yasuda H, Joko M, Maeda T, Kikkawa R. Evaluation of diabetic neuropathy through the quantitation of cutaneous nerves. *J Neurol Sci* 2000;172:55–62. [PubMed: 10620661]
- Huang TJ, Price SA, Chilton L, Calcutt NA, Tomlinson DR, Verkhatsky A, Fernyhough P. Insulin prevents depolarization of the mitochondrial inner membrane in sensory neurons of type 1 diabetic rats in the presence of sustained hyperglycemia. *Diabetes* 2003;52:2129–2136. [PubMed: 12882932]
- Jakobsen J, Brimijoin S, Skau K, Sidenius P, Wells D. Retrograde axonal transport of transmitter enzymes, fucose-labeled protein, and nerve growth factor in streptozotocin-diabetic rats. *Diabetes* 1981;30:797–803. [PubMed: 7274587]
- Kennedy WR, Wendelschafer-Crabb G, Johnson T. Quantitation of epidermal nerves in diabetic neuropathy. *Neurology* 1996;47:1042–1048. [PubMed: 8857742]
- Khan GM, Chen SR, Pan HL. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. *Neuroscience* 2002;114(2):291–299. [PubMed: 12204199]
- Koltzenburg M, Stucky CL, Lewin GR. Receptive properties of mouse sensory neurons innervating hairy skin. *J Neurophysiol* 1997;78:1841–1850. [PubMed: 9325353]
- Krimm RF, Davis BM, Woodbury CJ, Albers KM. NT3 expressed in skin causes enhancement of SA1 sensory neurons that leads to postnatal enhancement of Merkel cells. *J Comp Neurol* 2004;471:352–360. [PubMed: 14991566]
- Lauria G, Cornblath DR, Johansson O, McArthur JC, Mellgren SI, Nolano M, Rosenberg N, Sommer C. EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy. *Eur J Neurol* 2005;12:747–758. [PubMed: 16190912]
- Leininger GM, Vincent AM, Feldman EL. The role of growth factors in diabetic peripheral neuropathy. *J Peripher Nerv Syst* 2004;9:26–53. [PubMed: 14871451]
- Lauria G, Cornblath DR, Johansson O, Rosenberg N, Sommer C. EFNS guidelines on the use neuropathy. *European Journal of Neurology* 2005;12:747–758. [PubMed: 16190912]
- McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, Griffin JW, McArthur JC. Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. *Neurology* 1995;45(10):1848–1855. [PubMed: 7477980]
- Mizisin AP, Shelton GD, Wagner S, Rusbridge C, Powell HC. Myelin splitting, Schwann cell injury and demyelination in feline diabetic neuropathy. *Acta Neuropathol (Berl)* 1998;95:171–174. [PubMed: 9498053]
- Mizisin AP, Calcutt NA, Tomlinson DR, Gallagher A, Fernyhough P. Neurotrophin-3 reverses nerve conduction velocity deficits in streptozotocin-diabetic rats. *Peripher Nerv Syst* 1999;4:211–221.
- Mizisin AP, Kalichman MW, Bache M, Dines KC, DiStefano PS. NT-3 attenuates functional and structural disorders in sensory nerves of galactose-fed rats. *J Neuropathol Exp Neurol* 1998;57:803–813. [PubMed: 9737543]
- Mueller MJ. Identifying patients with diabetes mellitus who are at risk for lower-extremity complications: use of Semmes-Weinstein monofilaments. *Phys Ther* 1996;76:68–71. [PubMed: 8545495]

- Norrrell U, Eliasson B, Frizell M, Wallin BG, Wesslau C, Olausson H. Tactile directional sensibility and diabetic neuropathy. *Muscle Nerve* 2001;24:1496–1502. [PubMed: 11745952]
- Ochodnicka E, Ochodnicki M, Belej K, Fusekova E, Bosel'ova L. Quantitative analysis of myelinated nerve fibers of peripheral nerve in streptozotocin-induced diabetes mellitus. *Mol Chem Neuropathol* 1995;25:225–233. [PubMed: 8534323]
- Petruska JC, Mendell LM. The many functions of nerve growth factor: multiple actions on nociceptors. *Neurosci Lett* 2004;361:168–171. [PubMed: 15135920]
- Romanovsky D, Hastings SL, Stimers JR, Dobretsov M. Relevance of hyperglycemia to early mechanical hyperalgesia in streptozotocin-induced diabetes. *J Peripher Nerv Syst* 2004;9:62–69. [PubMed: 15104693]
- Russell JW, Windebank AJ, Schenone A, Feldman EL. Insulin-like growth factor-I prevents apoptosis in neurons after nerve growth factor withdrawal. *J Neurobiol* 1998;36:455–467. [PubMed: 9740019]
- Sayers NM, Beswick LJ, Middlemas A, Calcutt NA, Mizisin AP, Tomlinson DR, Fernyhough P. Neurotrophin-3 prevents the proximal accumulation of neurofilament proteins in sensory neurons of streptozotocin-induced diabetic rats. *Diabetes* 2003;52:2372–2380. [PubMed: 12941778]
- Schmidt RE, Plurad SB, Saffitz JE, Grabau GG, Yip HK. Retrograde axonal transport of 125I-nerve growth factor in rat ileal mesenteric nerves. Effect of streptozotocin diabetes. *Diabetes* 1985;34:1230–40. [PubMed: 2415414]
- Sima AA, Nathaniel V, Bril V, McEwen TA, Greene DA. Histopathological heterogeneity of neuropathy in insulin-dependent and non-insulin-dependent diabetes, and demonstration of axo-glial dysjunction in human diabetic neuropathy. *J Clin Invest* 1988;81:349–364. [PubMed: 3339124]
- Snider WD, McMahon SB. Tackling pain at the source: new ideas about nociceptors. *Neuron* 1998;20:629–632. [PubMed: 9581756]
- Stucky CL, Koltzenburg M, Schneider M, Engle MG, Albers KM, Davis BM. Overexpression of nerve growth factor in skin selectively affects the survival and functional properties of nociceptors. *J Neurosci* 1999;19:8509–8516. [PubMed: 10493751]
- Vinik AI. Diabetic neuropathy: pathogenesis and therapy. *Am J Med* 1999;107:17S–26S. [PubMed: 10484041]
- Weis J, Dimpfel W, Schroder JM. Nerve conduction changes and fine structural alterations of extra- and intrafusal muscle and nerve fibers in streptozotocin diabetic rats. *Muscle Nerve* 1995;18:175–184. [PubMed: 7823975]
- Wilson-Gerwing TD, Dmyterko MV, Zochodne DW, Johnston JM, Verge VM. Neurotrophin-3 suppresses thermal hyperalgesia associated with neuropathic pain and attenuates transient receptor potential vanilloid receptor-1 expression in adult sensory neurons. *J Neurosci* 2005;25:758–767. [PubMed: 15659614]
- Wright DE, Ryals JM, McCarson KE, Christianson JA. Diabetes-induced expression of activating transcription factor 3 in mouse primary sensory neurons. *J Peripher Nerv Syst* 2004;9:242–254. [PubMed: 15574137]
- Wright DE, Snider WD. Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *J Comp Neurol* 1995;351:329–338. [PubMed: 7706545]
- Yagihashi S, Kamijo M, Watanabe K. Reduced myelinated fiber size correlates with loss of axonal neurofilaments in peripheral nerve of chronically streptozotocin diabetic rats. *Am J Pathol* 1990;136:1365–1373. [PubMed: 2141449]

Abbreviations

STZ	streptozotocin
NF-H	neurofilament heavy chain
MBP	myelin basic protein

NGF	nerve growth factor
NT-3	neurotrophin-3
GDNF	glial cell line-derived neurotrophic factor
CSF	cerebrospinal fluid
PBS	phosphate buffered saline
IENF	intraepidermal nerve fibers

Figure 1. Diabetic mice develop reduced behavioral responses to non-noxious mechanical stimulation

Behavioral responses to mechanical stimuli were evaluated weekly in non-diabetic and diabetic mice using von Frey monofilaments of different strengths. A) Using a 0.16 g monofilament, significant reductions in the number of withdrawals following von Frey application were evident in diabetic mice 5 weeks post-STZ injection. Similar reductions were evident with 0.6 g (B), 1.4 g (C) and 2.0 g (D) monofilaments, although the differences between non-diabetic and diabetic mice were visible at 4 weeks using the larger monofilaments. Data plotted as means $\pm$ SEM. * denotes $P < 0.05$ from non-diabetic values.

Figure 2. NGF and Insulin treatments increase mechanical sensitivity

Behavioral responses of non-diabetic and treated diabetic mice to non-noxious mechanical stimuli were evaluated using a 1.4 g von Frey monofilament. Treatments began after testing on the 6th week after diabetes induction (arrows). NGF and insulin treatments both significantly increased behavioral responses of diabetic mice to non-noxious mechanical stimuli. In contrast, treating mice with CSF, NT-3, or combined NGF and NT-3 did not improve the behavioral responses of diabetic mice to von Frey stimulation of the footpad. Data plotted as means $\pm$ SEM. # denotes significant differences between neurotrophin- or insulin-treated diabetic mice and CSF-treated diabetic mice ($P < 0.05$). Non-diabetic mice were significantly different from CSF-treated diabetic mice at all time points.

Figure 3. Reduced MBP-positive fibers in the dermis of diabetic mice

Six weeks after STZ injection, footpad skin from non-diabetic (A) and diabetic mice (B) was processed for visualization of myelin basic protein (MBP)-positive axons. A, B) Images of MBP-positive axons in non-diabetic (A) and diabetic (B) mouse footpad. Arrows denote fascicles of MBP-positive axons, some of which extend up into the dermal papillae. Compared to healthy non-diabetic mice, diabetic mice displayed a significant reduction in MBP-positive fascicles within the dermis. e, epidermis; d, dermis. Scale bar equals 50 μm for each image.

Figure 4. Neurotrophin and insulin restore diabetes-induced reduction of NF-H-positive axons in the footpad

Footpad skin from non-diabetic and treated diabetic mice was processed for visualization of neurofilament heavy chain (NF-H)-positive axons. Diabetic mice treated with CSF exhibited a severe reduction in the innervation pattern of NF-H-positive axons. Following treatment with NGF, NT-3, NGF and NT-3 combined, or insulin for two weeks, the innervation of the footpad skin by NF-H-positive axons appeared markedly improved in diabetic mice. Scale bar = 100 μ m.

Figure 5. Neurotrophin and insulin treatments restore myelinated innervation in the footpad skin of diabetic mice

Area of footpad skin occupied by NF-H-positive axons was measured in non-diabetic and treated diabetic mice. CSF-treated diabetic mice had significantly less myelinated innervation in the footpad than non-diabetic footpad skin. Following treatment with NGF, NT-3, NGF and NT-3, or insulin, myelinated innervation was significantly improved in diabetic footpad skin. Data plotted as means $\pm$ SEM. * denotes significant differences between CSF-treated diabetic mice and all other groups ($P < 0.05$).

Figure 6. NT-3 treatment increases Merkel cell numbers associated with axons in the footpad of diabetic mice

A, B) Photomicrographs of immunocytochemical staining of sections from the mouse footpad for Merkel cells (arrows). Included are sections from a non-diabetic and a diabetic mouse. Diabetic mice had only slightly fewer Merkel cells ($P > 0.05$, see quantification below). C) Photomicrograph of immunocytochemical staining of footpad skin from a C57Bl/6 mouse visualizing both myelinated axons (NF-H-positive, green) and Merkel cells (cytokeratin 18, red). Arrows indicate Merkel cells innervated by myelinated axons. D) Merkel cells were quantified using conventional fluorescence microscopy and were counted if they were adjacent to the epidermis and innervated by NF-H-positive axons. Merkel cell numbers in CSF-treated diabetic mice were not significantly different than non-diabetic mice. NT-3 treatment of diabetic mice significantly increased the number of Merkel cells in the footpad. Data plotted as means $\pm$ SEM. * denotes $P < 0.05$; vs. non-diabetic, CSF-treated diabetic, and insulin-treated diabetic mice. Scale bar = 100 μ m for A–B, and 50 μ m for C.

Table 1
Weight and blood glucose levels of non-diabetic and treated diabetic mice

Treatment	Start Weight	8 wk Weight	% Change	8 wk Glucose
Non-diabetic	23.4 ± 0.30	29.7 ± 0.74	+26.9%	116 ± 11
Diabetic - CSF	24.4 ± 0.20	21.3 ± 0.87 ^{**}	-14.5%	366 ± 2.9 ^{**}
Diabetic - NGF	24.1 ± 0.26	22.1 ± 1.3 ^{**}	-9.0%	362 ± 9.1 ^{**}
Diabetic - NT-3	24.3 ± 0.52	20.3 ± 1.4 ^{**}	-19.7%	337 ± 13 ^{**}
Diabetic - NGF + NT-3	24.1 ± 0.26	21.8 ± 1.5 ^{**}	-10.5%	359 ± 8.6 ^{**}
Diabetic - Insulin	24.5 ± 0.56	27.2 ± 0.54 [#]	+11.0%	245 ± 32 ^{*#}

Weight is in grams, glucose levels are mg/dL. Comparisons were made between test groups using one-way ANOVA with Fisher's PLSD post hoc tests. Data plotted as means ± SEM.

* $P < 0.05$,

** $P < 0.0001$; vs. non-diabetic.

$P < 0.0001$; vs. diabetic -CSF.

Table 2
Responses to Tactile Stimuli after Neurotrophin or Insulin Treatment

Treatment	0.16 g	0.6 g	1.4 g	2.0 g	Pinprick
Non-diabetic	2.00 ± 0.31 *	3.14 ± 0.40 *	3.71 ± 0.42 *	5.00 ± 0.58 *	5.57 ± 0.43 *
Diabetic - CSF	0.71 ± 0.27	1.29 ± 0.42	1.42 ± 0.48	1.29 ± 0.42	3.71 ± 0.64
Diabetic - NGF	2.17 ± 0.60 *	2.17 ± 0.70	3.17 ± 0.83 *	2.66 ± 0.08 *	4.33 ± 0.84
Diabetic - NT-3	1.50 ± 0.43	1.50 ± 0.50	2.17 ± 0.60	2.50 ± 0.50	2.67 ± 0.56
Diabetic - NGF + NT-3	0.57 ± 0.37	1.14 ± 0.40	1.42 ± 0.48	2.86 ± 0.34	4.00 ± 0.58
Diabetic - Insulin	1.83 ± 0.40 *	2.33 ± 0.62 *	3.00 ± 0.63 *	2.33 ± 0.84	5.33 ± 0.33 *

Data represents mean number of withdrawal responses for each treatment group at 8 weeks following STZ administration. Withdrawal responses were measured by determining the number of withdrawal responses out of 6 applications to a single monofilament or 27-gauge needle.

* denotes significant differences from CSF-treated diabetic mice ($P < 0.05$).