Impaired sensory nerve function and axon morphology in mice with diabetic neuropathy

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Abbreviated Title: Sensory nerve function & morphology in diabetic mice

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Figures: 7
Tables: 2
Supplemental Figures: 2

Word count
Abstract: 238
Body: 4892
Abstract

Diabetes is the most prevalent metabolic disorder in the US and between 50-70% of diabetic patients suffer from diabetes-induced neuropathy. Yet our current knowledge of the functional changes in sensory nerves and their distal terminals caused by diabetes is limited. Here we set out to investigate the functional and morphological consequences of diabetes on specific subtypes of cutaneous sensory nerves in mice. Diabetes was induced in C57Bl/6 mice by a single i.p. injection of streptozotocin. After 6-8 weeks, mice were characterized for behavioral sensitivity to mechanical and heat stimuli, followed by analysis of sensory function using teased nerve fiber recordings and histological assessment of nerve fiber morphology. Diabetes produced severe functional impairment of C fibers and Rapidly Adapting Aβ fibers, leading to behavioral hyposensitivity to both mechanical and heat stimuli. Electron microscopy images show that diabetic nerves have axoplasm with more concentrated organelles and frequent axon-myelin separations compared to control nerves. These changes were restricted to the distal nerve segments nearing their innervation territory. Furthermore, the relative proportion of Aβ fibers was reduced in diabetic skin-nerve preparations compared to nondiabetic control mice. These data identify significant deficits in sensory nerve terminal function that are associated with distal fiber loss, morphological damage and behavioral hyposensitivity in diabetic C57Bl/6 mice. These findings suggest that diabetes damages sensory nerves, leading to functional deficits in sensory signaling that underlie the loss of tactile acuity and pain sensation associated with insensate diabetic neuropathy.

Key Words: sensory nerve, C fiber, skin-nerve, electron microscopy, mechanotransduction
Introduction

Diabetes is the most prevalent metabolic disorder in the US and is projected to increase by 3-fold by the year 2050 (Boyle et al., 2010). Approximately 50-70% of diabetic patients exhibit diabetic neuropathy (DN), which first emerges in the hands and feet as distal nerve fibers degenerate in a “glove and stocking pattern” (Harati, 2007). Patients may experience painful burning and tingling sensations, but all gradually lose normal touch and temperature sensation, leading to accidental personal injuries and impaired quality of life. Despite the prevalence and severe sequelae of DN, the biological mechanisms underlying this devastating disorder remain elusive (Zochodne, 1996; Feldman et al., 1999). Hyperglycemia has been identified as the fundamental metabolic disturbance; however, the relationship between early metabolic events, vascular damage and structural and functional changes in nerves is unclear. Although controlling blood glucose and glycated hemoglobin (HbA1C) levels with insulin remains the most effective method to prevent DN, this approach fails for many patients as it only reduces the incidence of DN by 34% over a 9 year period (DCCT Research Group., 1993). Also, insulin treatment does little for the 7% of patients who already exhibit neuropathy when diagnosed with diabetes (Pirart, 1977). Tricyclic antidepressants, duloxetine, gabapentin, aldose-reductase inhibitors, α-lipoic acid, and antioxidants demonstrate mixed efficacy in treating DN and practitioners cannot predict the medication(s) that will aid a given patient (Fedele and Giugliano, 1997; Ziegler, 2009). The combination of both painful symptoms and sensory loss caused by DN further complicates treatment.

From a morphological standpoint, chronic diabetes leads to the degeneration and regeneration of peripheral axons (Greene et al., 1999). Complicating this process, diabetes attenuates the expression of genes that are important in axon regeneration such as neurofilament,
Tα1-tubulin, and GAP-43 mRNA (Mohiuddin et al., 1995; Maeda et al., 1996; Mohiuddin and Tomlinson, 1997). Collectively, it is postulated that chronic diabetes damages the distal ends of sensory axons and suppresses axon regeneration, ultimately leading to chronic denervation of cutaneous tissues. Multiple studies using quantitative immunohistochemical assessments of cutaneous innervation have established that human diabetics experiencing decreased tactile sensation concomitantly have significantly reduced dermal and epidermal innervation (McCarthy et al., 1995; Kennedy et al., 1996; Shun et al., 2004; Lauria et al., 2005).

The molecular, anatomical and behavioral changes associated with diabetes have been studied in numerous rodent models, including streptozotocin-treated animals, animals on high fat diet, leptin-deficient animals, leptin receptor-deficient animals, insulinopenic animals, hyperinsulinemic animals and others. These different diabetic models have produced either hyper- or hyposensitivity to heat and mechanical stimuli (for review see Obrosova 2009). In some models, the behavioral phenotype appears to depend on the time course of the study. For instance, streptozotocin-treated rats develop thermal and mechanical hyperalgesia after 2-8 weeks (Corteix 1993; Calcutt et. al., 2004; Li et. al., 2005; Talbot 2009), but become hypoalgesic after longer periods of diabetes (Pertovaara et. al, 2001; Calcutt et. al., 2004; Obrosova et. al., 2008). The development of hyper- or hyposensitivity may also be species dependent, as streptozotocin-treated mice develop hyposensitivity to heat and mechanical stimuli (Christianson, 2003; Christianson et. al., 2007; Drel et. al., 2007; Vareniuk et. al., 2008).

Here, we used electrophysiological approaches along with histological and behavioral assessments to determine the consequences of diabetes on sensory nerve fiber function in STZ-treated C57Bl/6 mice. Our results demonstrate that diabetes causes morphological changes in the distal saphenous nerve and decreases the proportion of functional Aβ fibers that innervate
diabetic skin. Furthermore, diabetes severely impaired the function of cutaneous C fibers and Rapidly Adapting $A\beta$ fibers. These changes lead to the development of behavioral mechanical hypoalgesia in diabetic mice, which contrasts sharply from the hypersensitivity and pain behavior observed in STZ-treated rats. The neuropathic changes in diabetic mice closely resemble the neuropathic changes in human DN, and these results suggest that impairment of sensory nerve function may play an important role in human insensate neuropathy.
Materials and Methods

Experimentally-induced diabetes: Six week-old male C57BL/6 mice (Charles River) were given a single intraperitoneal (i.p.) injection of 180 mg/kg streptozotocin (Sigma) dissolved in 0.4 ml sodium citrate buffer to pH 4.5 (Wang et al., 1993). This dose was sufficient to induce diabetes in 26/33 of the mice injected, and no mice died as a result of the injection. Nondiabetic control mice were injected with vehicle (0.4 ml sodium citrate buffer). Following injection, mice were monitored for symptoms of diabetes, including polydipsia, polyuria and weight loss. Mice were weighed prior to STZ injection and weekly thereafter to monitor weight loss. Blood glucose levels were monitored every two weeks and at sacrifice using the Accu Chek Active glucometer (Roche). STZ-injected mice with blood glucose levels >350 mg/dl (normal blood glucose < 150 mg/dl) were included in the diabetic groups. Diabetic and nondiabetic control (vehicle-injected) mice were sacrificed after 6-8 weeks for histology or electrophysiology experiments. Every attempt was made to blind the experimenter to the treatment group of the mice throughout the behavioral, electrophysiological and morphological experiments. However, for behavioral assays, true blinding was often not possible due to the affects of diabetes on the appearance and body habitus of the mice. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin and followed the guidelines established by the National Institutes of Health.

Behavioral Testing: For mechanical stimuli, mice were allowed acclimate for at least 30 min in a plexiglass cage on a wire mesh floor before testing. Von Frey monofilaments (0.04, 0.22, 0.27, 0.66, 1.63, 4.0, 6.8, 11.7, 14.6, 20.1, 35.6, 53.9 and 84.8 mN; Smith and Nephew, Inc., Germantown, WI) were applied to the plantar surface of each hindpaw. The mechanical stimulus
that produced a 50% paw withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). Heat hypersensitivity was assessed using a radiant heat paw withdrawal test (Hargreaves et al., 1988). Mice were allowed acclimate for at least 30 min in a plexiglass cage on a glass surface. Radiant noxious heat was applied to the plantar surface of the hind paw and the latency to paw withdrawal was measured. Each hind paw was tested 4 times and the average paw withdrawal latency was recorded for each mouse. Light intensity was adjusted so that baseline paw withdrawal latencies averaged between 8-10 sec.

Skin-saphenous nerve preparation: The saphenous nerve and associated skin from the dorsal hindpaw were dissected free, placed corium side up in an organ bath and superfused with buffer as previously described (Reeh, 1986; Koltzenburg et al., 1997). Thin filaments were teased from the nerve until extracellular recordings were obtained from single fibers. Receptive fields were identified with a mechanical probe. Fibers conducting > 10 m/s were classified as myelinated (Aβ) axons, fibers conducting 1.2-10 m/s were classified as thinly myelinated (Aδ) axons and those conducting < 1.2 m/s were classified as unmyelinated C-fibers (Koltzenburg et al., 1997).

The mechanical threshold for each fiber was determined by calibrated von Frey monofilaments (0.04 to 84.8 mN; Smith and Nephew, Inc., Germantown, WI). The mechanical stimulus-response properties of each fiber were determined by applying constant force (5-200 mN, 10 sec stimulus, 2 min interval between stimuli) via a feed-back controlled, computer driven mechanical probe (Kwan et al., 2009). Aβ fibers that fired throughout a sustained force were classified as Slowly-Adapting. Aβ fibers that fired only at the onset and/or offset of a sustained force were classified as Rapidly-Adapting. Both Slowly- and Rapidly-Adapting Aβ fibers had characteristic small receptive fields (<2 mm). Similarly, Aδ fibers that adapted rapidly to mechanical stimuli were classified as down hair (D-hair) fibers and Aδ fibers that
adapted slowly were classified as A-mechanoreceptor (AM) fibers. As previously reported, D-hair fibers had large receptive fields (4-8 mm) and were sensitive to very low mechanical forces (<1.0 mN; Stucky et. al., 1998). For AM and C fibers, after mechanical stimuli, thermal and chemical stimuli were applied as follows. Receptive fields were superfused with heated buffer, increasing the temperature from 32°C to 52°C over 10 sec. Subsequently, a 2 min baseline was recorded followed by the application of 1 µM capsaicin for 2 min (Lennertz et al., 2010). The heat response threshold and number of action potentials elicited by heat or capsaicin were quantified. All stimulus traces were collected via a Powerlab/4sp multichannel recorder and analyzed via Chart software (AD Instruments).

Microscopy: Samples for microscopy consisted of four STZ-treated mice and four nondiabetic controls. Eight weeks after injection with STZ, animals were killed under anesthesia. The saphenous nerve (proximal nerve samples) and skin from the dorsal hindpaw (distal nerve samples) were exposed and incubated in primary fixative in-situ (4% glutaraldehyde/1x PBS pH 7.4; 5 min). Samples were dissected, immersion fixed at 4 °C for 1.5 days, post fixed with 1% osmium tetroxide + 1.5% potassium ferricyanide/PBS 2 hrs., dehydrated in ethanol and propylene oxide, and embedded in epon araldite resin which was then polymerized at 60 °C overnight. Semithin sections (0.5 µm) were cut using a RMC Powertome XL, post-stained with toluidine blue, and examined using a Nikon Eclipse light microscope. Light micrographs of entire nerve bundles were analyzed for morphology and axon count. In distal sections, this included sub-dermal nerve bundles surrounded by a perineurial sheath. Ultrathin sections for electron microscopy were cut on a Reichert Ultracut E, post-stained with uranyl acetate and lead citrate, and examined on a JEOL 100CX transmission electron microscope. At least 3 high power
fields were selected at random from the proximal and distal nerve segments of each animal. Axon area, axon perimeter and myelin thickness were measured using ImageJ 1.43 software. Separations between the axoplasm and myelin sheath were counted as “axon-myelin separations” when the area of the separation exceeded 6% of the total cross-sectional area of the axon.

Statistical analyses: For normally distributed data, the mean ± standard deviation were calculated and comparisons were made between 2 groups using a Student’s t-test, or across multiple measurements between 2 groups using a two-way ANOVA followed by Bonferroni post-hoc test (*). For non-normally distributed data, the median ± interquartile range were calculated, and comparisons were made between 2 groups using a Wilcoxon rank-sum test, whereas comparisons between ≥3 groups were made using a Kruskal-Wallace test followed by a post-hoc Dunn’s multiple comparison test among selected groups (†). Analyses between 2 proportions were made using a Fisher’s exact test, and between ≥3 proportions were made using a Chi-square test followed by the standardized residual method to compare the contributions of individual proportions (‡). Comparisons between sample locations were made using a Mantel-Haenszel test of homogeneity (§).
Results

Diabetic C57Bl/6 mice develop mechanical and thermal hypoalgesia

Eight weeks after STZ injection, the serum glucose of diabetic mice was elevated from 151±7 in control mice to an average of 521±16 mg/dL (p < .01; Fig 1A). Whereas non-diabetic controls weighed an average of 28±1 grams, diabetic mice weighed only 17±1 grams on average (p < .01; Fig 1B). Behavioral mechanical threshold and thermal response latency were also measured at this time. Diabetic mice displayed markedly increased mechanical thresholds compared to nondiabetic mice (median of 20 mN vs 6.8 mN respectively, p < .01; Fig 1C). Diabetic mice also displayed significantly longer withdraw latencies to a noxious heat stimulus compared to nondiabetic animals (17±1 sec vs 9±1 sec respectively, p < .01; Fig 1D). Thus, C57Bl/6 diabetic mice exhibit hypoalgesia to both mechanical and heat stimuli.

Nerves from diabetic mice have nearly normal morphology in semithin sections

Next, we determined whether diabetes induces nerve fiber loss, demyelination or changes in axon morphology by using light microscopy. Samples were taken from the saphenous nerve trunk above the knee joint (proximal nerve) and at the level of the hindpaw from small fascicles coursing beneath the dermis in the hindpaw (distal nerve). Proximal nerve semithin sections from nondiabetic mice displayed normal morphology. Myelinated nerve fibers appeared mostly round and without pronounced compression, as can be caused by the hypertonic fixative solutions used to fix peripheral nerves (Fig 2A, top left). Excluding fibers near an internode which naturally appear crenellated, myelinated nerve fibers appeared slightly less round in proximal nerve semithin sections from diabetic mice. While this appearance could have been caused by high blood glucose levels in diabetic mice, we can not rule out the possibility that
diabetic tissue responded differently than nondiabetic tissue to our fixative solution. Otherwise, proximal nerve segments from diabetic mice exhibited normal myelination and morphology (Fig 2A, top right). Further, counts of myelinated axons in the proximal nerve trunk did not reveal any nerve fiber loss from diabetic saphenous nerves (Fig 2B).

Similarly, distal nerve semithin sections from both nondiabetic and diabetic mice exhibited mostly normal structure (Fig 2A, bottom left, bottom right). In particular, distal nerve sections from diabetic animals did not exhibit demyelination, axonal degeneration or atrophy. Counts of myelinated axons did not reveal a loss of nerve fibers from distal nerve fascicles in diabetic mice (Fig 2C). However, unmyelinated axons from diabetic mice consistently appeared more intensely stained by toluidine blue. Also, separations between the axon and myelin sheath were noticeable in many myelinated nerve fibers in diabetic nerve fascicles (Fig 2A, bottom right; highlighted by arrows).

**Electron micrographs reveal altered morphology in distal diabetic nerves**

Changes in the morphology of diabetic nerves became increasingly noticeable in higher-magnification electron micrographs of distal nerve segments. Nondiabetic axons exhibit a fine, lightly-stained filament network in the axoplasm with some more darkly stained microtubules and few mitochondria (Fig 3A, arrows). In comparison, the axoplasm of diabetic axons appears quite dense in neurofilaments and contains numerous mitochondria (Fig 3B, arrows). The left axon in Fig 3B displays a small amount of Schwann cell cytoplasm at the axon-myelin junction (arrowheads). The right axon in Fig 3B shows increasing separation between the axoplasm and myelin, with a larger amount of Schwann cell cytoplasm and a partially extracted area. In many axons, this space became progressively larger near the internode (Supplemental Fig 2). It was
noted that the density of axoplasm components was greater near the internode in both nondiabetic and diabetic axons, but nonetheless the density of the axoplasm appeared more dense in diabetic axons than in nondiabetic axons. Although the axoplasm appeared to be more dense in diabetic axons, there was no difference in the cross sectional area of myelinated nondiabetic axons compared diabetic axons (Supplemental Fig 1A).

Unmyelinated axons from diabetic mice stained more intensely than unmyelinated axons from nondiabetic mice (Fig 4A, arrowheads). This made individual components of the axoplasm in unmyelinated axons more difficult to discern in diabetic mice. This effect was not due to a decrease in the size of unmyelinated axons. Rather, unmyelinated axons were slightly larger in proximal nerve segments from diabetic mice (p<0.001, Supplemental Fig 1B). However, as the size distribution of unmyelinated axons overlaps almost completely between nondiabetic and diabetic samples, it is not clear that this represents a meaningful change in the caliber of unmyelinated axons.

Separations between the axoplasm and the myelin sheath were prevalent in distal diabetic nerves, occurring in 47% of myelinated axons and contained varying amounts of material in the developing space (Fig 4A, lower panel, arrows). In comparison, separations between the axon and myelin sheath were smaller in size and were present in only 10% of distal nondiabetic nerve axons (p < 0.001; Fig 4B). Axon-myelin separations were also infrequent in the proximal segments of nondiabetic and diabetic nerves (5% and 8%, respectively). Thus, axon-myelin separations were restricted to distal segments of diabetic nerves (p < 0.05, Fig 4B).

In addition, we examined distal myelinated axons according to their size distribution (μm²). Since Aβ fiber axons are generally larger than Aδ fiber axons, we reasoned that the upper quartile of the distribution would contain mostly Aβ fiber axons and the lower quartile would
contain mostly A\(\delta\) fiber axons. In diabetic mice, the frequency of axon-myelin separations was strikingly high (66%) among axons in the upper quartile compared to axons in the lower quartile of the size distribution (17%, p<0.001, Fig 4C, right). In contrast, the frequency of these changes did not change between the upper and lower quartiles in nondiabetic mice (16% and 10% respectively, Fig 4C, left). These data suggest that A\(\beta\) fiber axons exhibit axon-myelin separations more frequently than A\(\delta\) fiber axons in diabetic mice. These morphological findings support electrophysiological data (see below) indicating that A\(\beta\) myelinated fibers are particularly affected during diabetes in C57Bl/6 mice (Fig 5).

Diabetic mice lose functional A\(\beta\) fiber innervation

To examine functional cutaneous innervation, diabetic and nondiabetic mice were sacrificed for ex-vivo teased fiber recordings from the hindpaw skin 1-2 days after the behavioral measurements were made. Mechanical search stimuli were used to identify isolated receptive fields of nerve fibers. Each fiber encountered was characterized according to its conduction velocity and mechanical adaptation. A\(\beta\) fibers (> 10 m/s) accounted for 44% of all nerve fibers classified from nondiabetic skin, but only 21% of nerve fibers classified from diabetic skin (p < .001; Fig 5A). A reciprocal trend was observed in the prevalence of A\(\delta\) fibers and C fibers, as would be the expected result of a relative loss of A\(\beta\) fibers (1.2 to 10 m/s and less than 1.2 m/s, respectively). No changes were observed in the ratio of Rapidly Adapting versus Slowly Adapting A\(\beta\) fibers in diabetic versus nondiabetic preparations (Fig 5B). Similarly, no significant changes were observed in the ratio of rapidly adapting A\(\delta\) (D hair) versus slowly adapting A\(\delta\) (AM) fibers (Fig 5C). The loss in A\(\beta\) fibers is consistent with the observed increase...
in behavioral mechanical thresholds among diabetic mice, as Slowly-Adapting Aβ and Rapidly-Adapting Aβ fibers mediate light touch sensation.

C fiber mechanical responses are markedly impaired in diabetic mice

Although we observed few examples of overt axon degeneration, the majority of axons in the diabetic nerve fascicles exhibited separations from the myelin sheath and a density of axoplasm components that may indicate compromised axon function. Therefore, we tested the mechanical threshold and suprathreshold responsiveness of individual nerve fibers. Mechanical thresholds were assessed by applying calibrated von Frey filaments to the cutaneous receptive field of nerve fibers in the skin-nerve preparation. There was a nonsignificant trend toward increased mechanical thresholds in C fibers from diabetic mice (p = 0.08, Table 1). However, no significant differences were detected in mechanical thresholds among the subtypes of nerve fibers in diabetic and nondiabetic mice (Table 1).

Subsequently, we assessed the mechanical responsiveness of nerve fibers to suprathreshold force. Increasing mechanical forces between 5 and 200 mN were applied to individual receptive fields and the number of action potentials elicited at each force was compared between diabetic and nondiabetic mice. Diabetes had the greatest effect on unmyelinated C fibers. C fibers from diabetic mice exhibited tepid action potential firing to mechanical stimuli at forces greater than 20 mN (p < 0.01, Fig 6A). Normally, C fibers faithfully encode the intensity of mechanical stimuli by firing action potentials in direct relation to the stimulus intensity (Stucky et al., 1999). However, C fibers from diabetic animals failed to encode the intensity of the stimulus as they responded weakly to mechanical stimuli, especially at high intensity forces. Interestingly, other subtypes of neurons with slowly adapting response
properties were essentially normal in diabetic mice. For example, AM nociceptors and Slowly Adapting Aβ fibers were essentially normal in their responsiveness to mechanical stimuli (Fig 6B,D). Rapidly Adapting Aβ fibers from diabetic mice also responded significantly less to mechanical force than fibers from nondiabetic mice (p < 0.05, Fig 6C). In fact, forces above 5 mN elicited only half as many action potentials in diabetic nerve fibers. In contrast, the mechanical response properties of rapidly adapting D-hair afferents were unaffected in diabetic animals (Fig 6E). Thus, in addition to distal cutaneous denervation, these functional changes in mechanical responsiveness at in the sensory terminal of mechanoreceptors would further contribute to the hypoalgesia observed in diabetic C57Bl/6 mice.

C fibers exhibit an increased threshold to heat stimuli in diabetic mice

As diabetic mice exhibit significantly delayed behavioral responses to heat, we also examined the response of C fibers to heat stimuli. Characterized C fibers were superfused with heated buffer, warming the receptive field from 32 to 52°C over 10 sec. We found no difference in the proportion of C fibers that respond to the heat ramp (54% of nondiabetic vs. 61% of diabetic C fibers; p = 0.39; Fig 7A). Also, the heat stimulus evoked similar numbers of action potentials from heat-sensitive C fibers in nondiabetic and diabetic mice (mean of 35 vs. 29 action potentials, respectively; p = 0.55; Fig 7B). However, C fibers from diabetic mice exhibited significantly elevated heat response thresholds compared to nondiabetic mice (median 41.3 vs. 37.1°C, respectively; p < 0.05; Fig 7C). Some AM fibers are sensitive to heat stimuli and contribute to behavioral heat responses. However, we did not record from enough heat-sensitive AM fibers to compare heat responses between diabetic and nondiabetic animals (n = 2 for nondiabetic and n = 3 for diabetic mice). Thus, diabetes increases the threshold at which C fibers
respond to heat stimuli. However, the proportion of heat-sensitive C fibers and the magnitude of
responses to heat stimuli were unaffected.

Following the heat stimuli, C fibers were exposed to the TRPV1 agonist, capsaicin. TRPV1 is sensitive to temperatures above 43°C and is important for normal behavioral responses to heat stimuli. As with heat responses, similar proportions of C fibers responded to capsaicin in nondiabetic and diabetic animals (62% vs. 61%, respectively; Fig 7D). Further, the number of action potentials elicited by capsaicin was similar between nondiabetic and diabetic animals (median 41 vs 39 action potentials, respectively; Fig 7E). These data suggest that neither TRPV1 expression or function are altered considerably in diabetic mice.

Nerve fiber conduction velocity is maintained in diabetic mice

The in vitro skin-nerve preparation allowed the assessment of conduction velocities in individual sensory nerve axons. Here, the conduction velocities of subtypes of sensory nerve fibers in diabetic and nondiabetic animals were quantified. No significant changes in conduction velocity were evident among the various subtypes of sensory nerve fibers in diabetic animals (Table 2). It is plausible to suggest that no differences were observed because conduction velocity itself was one of the parameters used to classify nerve fibers and thus slow-conducting fibers may have been misclassified as a different fiber subtype. While this is possible, we observed no examples of oddly-characterized fibers. For instance, we observed no “D hairs with abnormally small receptive fields” that could be slow-conducting Rapidly Adapting Aβ fibers. Of note, an overall decrease in conduction velocity would have been reported in diabetic mice if sensory nerve fibers had not been classified into subtypes. This resulted from the loss of functional Aβ fibers in diabetic skin nerve preparations.
Discussion

The goal of this study was to identify the effects of diabetes on peripheral nerves of diabetic C57Bl/6 mice in the setting of insensate neuropathy. Behavioral, histological and electrophysiological approaches revealed marked morphological and functional changes in peripheral nerve fibers 6-8 weeks following induction of diabetes. Notably, the mechanical responses of C fibers and rapidly adapting Aβ light touch mechanoreceptors were markedly impaired in diabetic mice.

Our electrophysiological results provide novel insight into the function of sensory nerve fibers in mice with diabetic neuropathy. Unmyelinated C fibers from diabetic mice responded poorly to mechanical stimuli across a range of forces (40-200 mN) and failed to increase their response to more intense stimuli. Further, heat response thresholds were significantly increased in C fibers from diabetic mice. These results differ from teased fiber recordings in streptozotocin-treated rats, in which C fibers exhibit decreased mechanical thresholds and increased responsiveness to suprathreshold stimuli, reflecting sensitization of nociceptors in rat. Of note, these rat recordings were performed at a time point when the animals exhibited behavioral hypersensitivity to mechanical stimuli (Chen and Levine, 2001; Suzuki et. al., 2002).

C fibers normally encode a variety of noxious and innocuous stimuli, encode the stimulus intensity, and play an important role in avoiding tissue injury. C fibers in diabetic mice no longer fulfill this role, at least for mechanical stimuli, as noxious forces elicited similar numbers of action potentials as innocuous forces. Although C fibers in diabetic animals respond to heat with similar numbers of action potentials, the increase in heat response threshold coincides with a clear behavioral hyposensitivity to heat. Interestingly, not all nociceptor-type populations were affected by diabetes as myelinated AM fibers exhibited normal mechanical responsiveness in
diabetic animals. Nonetheless, C fibers account for 60-75% of all axons in peripheral cutaneous nerves and many C fibers summate on second-order spinal cord neurons. Therefore, the deficit in C fiber function could greatly affect detection of acute noxious and innocuous force (Griffin et al., 2001).

Diabetes also reduced the mechanical firing of rapidly adapting Aβ fibers. Rapidly Adapting Aβ fibers are light touch mechanoreceptors that innervate guard hair follicles in hairy skin and Meissner corpuscles in glabrous skin. The exquisite sensitivity of Rapidly Adapting Aβ fibers to dynamic stimuli greatly enhances our perception of object texture and edges. Thus, deficits in Rapidly Adapting Aβ function could lead to a considerable loss of normal tactile acuity in diabetic animals.

Significant changes to the structure of distal myelinated nerve fibers occurred in diabetic mice. Myelinated axons contained numerous organelles, possibly reflecting impaired transport along nerve fibers or an increase in the number of mitochondria within the axoplasm. Also, myelinated axons were frequently separated from their myelin sheath. These separations did not appear to result from axonal atrophy, as axons from diabetic mice were not significantly smaller than axons from nondiabetic mice (Supplemental Fig 1). Several scorpion venom toxins have been shown to induce periaxonal edema, presumably due to an osmotic imbalance due to sodium channel dysfunction (Love et al., 1986). Further, high plasma mannitol concentrations produce a hyperosmolar perineurial environment, increase the amount of glycogen present in Schwann cells and lead to axon-myelin separation in rat studies (Myers and Powell, 1983; Myers and Powell, 1984). Thus, axon-myelin separations in diabetic mouse axons may reflect an osmotic imbalance due to hyperglycemia, accumulation of glycogen in the Schwann cell, or secondarily, membrane channel dysfunction in diabetic mice. Separations between the axon and myelin
sheath could also reflect axoglial disjunction in the paranodal region of diabetic nerve fibers, as previously described in diabetic rats (Sima et al., 1986). Importantly, changes in plasma osmolarity due to elevated glucose concentration have been demonstrated to directly affect sensory nerve fiber function in diabetic rats (Suzuki et al., 2002).

Morphological analyses highlighted two important features of neuropathy in streptozotocin-treated mice. First, axon-myelin separations were restricted to distal nerve segments nearing their innervation territory in the skin of diabetic mice. This pattern is consistent with the pattern of peripheral neuropathy observed in human diabetic patients where the distal aspects of long peripheral nerve fibers are affected first and most severely. Second, the axon-myelin separations were significantly more common among large-caliber axons in diabetic mice. This finding suggests that Aβ fiber axons are most susceptible to damage after 6-8 weeks of diabetes and supports electrophysiological data that demonstrates a preferential loss of functional Aβ fiber innervation in the skin.

Morphological changes coincided with a shift in the relative abundance of functional cutaneous nerve fibers. A lesser proportion of heavily myelinated Aβ fibers was encountered in diabetic skin-nerve preparations than in control preparations. However, there was no change in the proportion of rapidly adapting versus slowly adapting subtypes among either the Aβ or Aδ fibers. Thus, diabetes equally affected both Rapidly Adapting Aβ fibers that predominately innervate hair follicles (in hairy skin) or Meissner corpuscles (in glabrous skin) and Slowly Adapting Aβ fibers that terminate either on Merkel cells (SAI) or as free nerve endings (SAII) at the dermal-epidermal border (Lumpkin and Caterina, 2007; Maricich et al., 2009). Previous studies have reported losses of both myelinated and unmyelinated nerve fibers in diabetic skin.
Although conduction abnormalities have been described in human diabetics and in animal models of diabetes, we did not observe significant changes to the conduction velocity of diabetic nerve fibers in mice. Human and rat studies report nerve conduction slowing after several months to several years of diabetes and in the context of severe nerve fiber pathology (Sima et al., 1982). In contrast, we observe only modest nerve fiber pathology after a relatively short (6-8 week) duration of diabetes. In particular, there was no evidence of demyelination in diabetic mice. Thus, the absence of conduction velocity abnormalities may reflect the absence of segmental demyelination and remyelination in diabetic mice during the initial eight weeks of diabetes. A longer time course of diabetes may be necessary for diabetic mice to develop pronounced nerve fiber pathology and to observe a change in nerve fiber conduction velocity.

In summary, diabetes impaired the function of sensory nerve fibers and reduced the abundance of functional Aβ fibers in diabetic skin, resulting in behavioral hyposensitivity to mechanical and heat stimuli. Histological analyses revealed changes that were restricted to distal segments of diabetic nerve fibers. Presumably, these represent early pathological changes associated with murine diabetes. Thus, diabetic mice develop a peripheral, insensate neuropathy that shares physiological and anatomical similarities with insensate diabetic neuropathy in humans. Importantly, our findings suggest that the sensory losses experienced by human diabetic patients may be due to impaired nerve fiber function as well as sensory denervation.
Acknowledgments:

The authors would like to thank Janelle Williams for her help with the experimental procedures.

Grants:

Supported by the JDRF and NINDS RO1NS43314 (DEW), and NS40538 and NS070711 (CLS)


Drel VR, Pacher P, Vareniuk I, Pavlov IA, Ilnytskaya O, Lyzogubov VV, Bell SR, Groves JT, Obrosova IG. Evaluation of the peroxynitrite decomposition catalyst Fe(III) tetra-


Figure 1. Streptozotocin-treated mice develop diabetes, weight loss and sensory hypoalgesia over the course of eight weeks. **A**, Average blood glucose level of diabetic (STZ-injected) and nondiabetic (vehicle-injected) mice. **B**, Average weight of diabetic and nondiabetic mice. **C**, Behavioral mechanical response threshold (von Frey filament applied to plantar hindpaw). **D**, Behavioral thermal response latency (stimulus applied to plantar hindpaw). ** p < 0.01, Student’s t-test; †† p<0.01, Wilcoxon rank sum test.

Figure 2. Diabetic nerves appear mostly similar to nondiabetic nerves at low magnification. **A**, Plastic-embedded .5 µm sections of saphenous nerve stained with toluidine blue and imaged by oil immersion light microscopy (800x magnification); top: proximal nerve segments from nondiabetic (left) and diabetic mice (right) taken from above the knee; bottom: distal, subdermal nerve segments from nondiabetic (left) and diabetic mice (right) taken from the dorsal hind paw. Myelinated axons from diabetic mice appear marginally less circular than axons from nondiabetic mice. Note separations between the axon and myelin sheath in the distal diabetic nerve, highlighted by arrows. **B**, Myelinated axon counts from nondiabetic and diabetic proximal nerve segments (p>0.05, Wilcoxon rank-sum test). **C**, Myelinated axon counts from distal nerve segments (p>0.05, Wilcoxon rank-sum test).

Figure 3. Diabetic nerves exhibit morphologic changes at high magnification. **A**, High magnification image of nondiabetic axons in a distal nerve segment stained with uranyl acetate and lead citrate (magnification 8,710x). The central myelinated axon within the size range of a sensory Aβ fiber (6-12 µm) exhibits a single mitochondrion, a lightly-stained neurofilament network and some more intensely-stained microtubules. Smaller myelinated axons within the
size range of sensory Aδ fibers (1-5 µm) exhibit a few mitochondria and a similar filament network. B, High magnification of diabetic axons in a distal nerve segment (magnification 9260x). Both axons fall within the size range of sensory Aβ fibers. Both axons exhibit an increased number of mitochondria (arrows) and dense neurofilament network in comparison to nondiabetic axons. The upper right of the left axon demonstrates a widened interface between the Schwann cell and the axoplasm (arrowheads). The right axon demonstrates a similar area that has been partly extracted (arrowheads).

Figure 4. Axon-myelin separations are prevalent in distal axons from diabetic mice. A, Electron micrographs of myelinated and unmyelinated axons in distal nerve segments from nondiabetic (top) and diabetic mice (bottom) stained with uranyl acetate and lead citrate (3,430x magnification). Myelinated axons exhibit axon-myelin separations and numerous intracellular organelles compared to nondiabetic axons. Arrows highlight lipid-like material within axon-myelin separations. Arrowheads highlight some unmyelinated axons, which appear more electron-dense in diabetic nerves. B, Frequency of axon-myelin separation in proximal versus distal nerve segments in nondiabetic and diabetic mice. C, Frequency of axon-myelin separation in small diameter axons (lower quartile) versus large diameter axons (upper quartile). Quartiles defined from the size distribution of all myelinated axons in nondiabetic or diabetic samples. ††† p<0.001, Kruskal-Wallis test followed by post-hoc Dunns multiple comparison test between selected groups. § p<0.05, Mantel-Haenszel test of homogeneity.

Figure 5. Diabetic mice preferentially lose functional Aβ fiber innervation. A, Proportion of functional Aβ, Aδ and C fibers identified in diabetic and nondiabetic skin-nerve preparations. B,
Proportion of functional Aβ fibers that were rapidly adapting (RA) versus slowly-adapting (SA) in diabetic and nondiabetic skin-nerve preparations. **C**, Proportion of functional Aδ fibers that were rapidly adapting (D hair) versus slowly adapting (AM) in diabetic and nondiabetic skin-nerve preparations. ‡‡‡ p<0.001, Chi-square test followed by analysis of standardized residuals.

**Figure 6.** Diabetes impairs sensory nerve fiber function. A, Force stimulus response curves in C fibers (5-200 mN, 10 sec each, 1 min interstimulus interval), B Slowly-adapting Aβ fiber response, C Rapidly-Adapting Aβ fiber response, D AM fiber response and E D-hair fiber response to increasing mechanical stimuli in diabetic versus nondiabetic mice. * p<0.05 and ** p<0.01, two-way ANOVA followed by a post-hoc Bonferroni t-test.

**Figure 7.** Diabetes increases heat response thresholds in C fibers. A, Proportion of C fibers sensitive to heat, B Numbers of action potentials elicited by heat ramp (32°C to 52°C over 10 sec), C Heat response threshold, D Proportion of C fibers sensitive to 1 µM capsaicin, E Numbers of action potentials elicited by 1 µM capsaicin. † p<0.05, Wilcoxon rank sum test.

**Table 1.** Sensory nerve fiber mechanical thresholds are unaffected in diabetic mice. Median von Frey threshold and interquartile range for nerve fiber subtypes in diabetic and nondiabetic mice. Subtypes were compared using Wilcoxon rank sum tests.

**Table 2.** Sensory nerve fiber conduction velocity is unaffected in diabetic mice. Average conduction velocity for nerve fiber subtypes in diabetic and nondiabetic mice. Subtypes were compared using Student’s t-tests.
Supplementary Figure 1. Cross-sectional area of axons in nondiabetic and diabetic mice. A, Area of myelinated axons in proximal and distal nerve segments, B Area of unmyelinated axons in proximal and distal nerve segments. ††† p<0.01, Kruskal-Wallis test followed by post-hoc Dunns multiple comparison test.

Supplemental Figure 2. Diabetic axon morphology. A, Three large axons, Aβ by size, from distal diabetic nerve segment (magnification 6300x). Right axon does not exhibit axon-myelin separation, but demonstrates a dense neurofilament network and a several mitochondria (arrows). At top, middle, part of a larger axon demonstrates a dense neurofilament network and small axon-myelin separation (arrowhead). At far left, another myelinated axon demonstrates axon-myelin separation (arrowheads) and part of a Schwann cell (S) which has reduced components in the cytosol. Above the left axon is a bundle of unmyelinated axons containing two degenerated axons (UA). Degenerating unmyelinated axons were uncommon in nerve sections. The Schwann cell surrounding these unmyelinated axons stains more intensely than the more normal bundle of unmyelinated axons to the right. B, Larger axons (Aβ by size) and a smaller axon (Aδ by size) display differing degrees of axon-myelin separation (arrowheads; magnification 6,510x). These axons exhibit a dense neurofilament network and numerous mitochondria in their axoplasm. Lower right axon demonstrates a Schwann cell (S) with dense cytosol, dilated vesicles and smooth endoplasmic reticulum network suggestive of increased activity. Upper right axon also demonstrates a small amount of Schwann cell cytoplasm (S) with two large vacuoles.
A. C-fiber
- Nondiabetic
- Diabetic

B. SAAβ

C. RAAβ

D. AM fiber

E. D hair

APs per sec vs. Force (mN)

n = number of fibers

Significance levels:
- * p < 0.05
- ** p < 0.01
- NS = not significant
<table>
<thead>
<tr>
<th>Von Frey Threshold (mN)</th>
<th>Control (n=99)</th>
<th>Diabetic (n=96)</th>
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<tr>
<td>Slowly-Adapting Aβ</td>
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<td>0.7, 0.7 – 1.1 (n=11)</td>
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<td>0.2, 0.04 – 0.3 (n=9)</td>
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<td>4.0, 1.6 – 6.8 (n=43)</td>
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<td>Aδ  Down hair</td>
<td>0.1, 0.04 – 0.2 (n=14)</td>
<td>0.2, 0.04 – 0.2 (n=13)</td>
</tr>
<tr>
<td>C-fiber</td>
<td>4.0, 1.6 – 4.0 (n=11)</td>
<td>6.8, 2.8 – 9.3 (n=20)</td>
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<tr>
<td>Conduction Velocity (m/s)</td>
<td>Control (n=99)</td>
<td>Diabetic (n=96)</td>
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<td>---------------------</td>
</tr>
<tr>
<td>Slowly-Adapting Aβ</td>
<td>16.5 +/- 1.1 (n=24)</td>
<td>16.4 +/- 1.6 (n=11)</td>
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<tr>
<td>Rapidly-Adapting Aβ</td>
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<td>Aδ  A-Mechanoreceptor</td>
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<td>4.6 +/- 0.4 (n=43)</td>
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<td>Aδ  Down hair</td>
<td>5.5 +/- 0.8 (n=14)</td>
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