Accelerated diabetic neuropathy in axons without neurofilaments

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Summary
Diabetic neuropathy is characterized by slowing of conduction velocity and axonal atrophy. Both of these cardinal features of neuropathy might be linked to impaired neurofilament investment of axons. Since neurofilaments form the critical structural latticework of axons, their importance in neuropathy is of interest. We tested directly the relationship of neurofilaments to diabetic neuropathy by superimposing streptozotocin-generated diabetes on a unique but viable transgenic mouse described by Eyer and Peterson. These mice express a fusion protein in which the carboxyl terminus of the high molecular weight neurofilament protein (Nf-H) was replaced by β-galactosidase, in turn blocking normal neurofilament export and rendering axons completely lacking neurofilaments. Despite similar levels of hyperglycaemia, diabetic mice lacking neurofilaments developed progressive slowing of conduction velocity in their motor and sensory fibres between 4 and 8 weeks after the onset of diabetes (P < 0.05), unlike diabetic mice with normal neurofilaments, who developed only mild evidence of neuropathy over the same time-frame. Diabetic mice without neurofilaments, but not those with neurofilaments, had a progressive decline in the amplitude of the caudal nerve compound action potential and there were trends toward increased axonal atrophy in diabetics lacking neurofilaments. Single daily doses of insulin that restored normoglycaemia (0.1 IU subcutaneous insulin daily 5 of 7 days weekly for 4 weeks) reversed conduction slowing and restored sensory axon calibre. Our findings indicate that abnormalities in neurofilament export or transport alone cannot account for features of diabetic neuropathy. Instead, neurofilaments may allow axons to better resist the ravages of diabetes. Our findings also confirm the impact of insulin on reversing the phenotype.

Keywords: axonal atrophy; conduction velocity; diabetes mellitus

Abbreviations: ANOVA = analysis of variance; Nf-H = heavy subunit of neurofilament; Nf-L = light subunit of neurofilament; Nf-M = medium subunit of neurofilament; STZ = streptozotocin

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Introduction
Neurofilaments are critical components of axons. As triplet intermediate filament proteins, neurofilaments consist of heavy (Nf-H), medium (Nf-M) and light (Nf-L) subunits, forming the major structural lattice of axons. There are complex consequences of disruption of the triplet protein that include axon loss and atrophy (for a review see Lariviere and Julien, 2004). The relationship between the neurofilament protein and diabetes may also be complex, yet is important to understand in the development of peripheral nerve disease.

A number of abnormalities in neurofilament biology have been identified in models of diabetes. For example, mitogen-activated protein kinase (MAPK)-mediated neurofilament hyperphosphorylation of lysine-serine-proline sites on the carboxyl terminal tail domains of Nf-H and Nf-M appears early (12–14 weeks) in the diabetic rat sural nerve and sensory perikarya (Fernyhough et al., 1999). Normally, such phosphorylation occurs after export of the protein out of the perikarya and into the axon (Sternberger and Sternberger, 1983), where Nf-M (rather than previously considered Nf-H) contributes to the radial diameter of axons (Elder et al., 1999; Rao et al., 2002; Garcia et al., 2003). Sayers et al. (2003) identified accumulation of Nf-H and Nf-M in proximal nerve axon segments with concurrent reductions of their presence in distal sural axons after 14 weeks of diabetes in rats. Such declines
in distal expression of NF-M may be related to the distal axon atrophy and neurofilament loss noted in diabetes models. The impact of heightened, and inappropriately localized, phosphorylated neurofilaments is uncertain, but may contribute to axonal disease. Finally, Schmidt et al. (1997) described neurofilament accumulations, termed axonal dystrophy, in proximal axon segments of diabetic dorsal root ganglia sensory neurons. Whether such changes are markers of disease, or disrupt integrity of the neurofilament protein leading to axon loss, is also uncertain.

The distal nerve loss of neurofilament and atrophy may contribute to retraction of nerve terminals from their end-organs and loss of function, particularly in long-term models (Scott et al., 1999). At least two mechanisms may account for such loss. There is a reduction in the slow axonal transport of neurofilaments (Medori et al., 1985, 1988a, b), likely accounting for proximal accumulations discussed above. Neurons in long-term diabetic models also experience progressive failure to synthesize all three neurofilament subunit proteins, and therefore to export neurofilament triplet proteins (Scott et al., 1999).

All of these findings suggest that neurofilament alterations are central to the development of diabetic neuropathy. This, however, may not be the case. In models of experimental amyotrophic lateral sclerosis (ALS) resulting from mutations in superoxide dismutase (SOD1), for example, the presence of neurofilaments appears to protect motor axons from loss (Williamson et al., 1998). In diabetes, an appropriate way to identify the potential impact of neurofilament alterations in development of neuropathy might be to study diabetes in axons lacking neurofilaments.

By serendipity, as previously described (Eyer and Peterson, 1994), transgenic mice were generated that completely lacked peripheral axonal neurofilaments (Fig. 1). The mice express a fusion protein wherein the carboxyl terminus of the high molecular weight neurofilament protein is replaced by β-galactosidase, which precipitates the neurofilament cytoskeleton in perikarya as dense aggregates. Although axons in this transgenic model failed to develop their normal size calibre, the mice had few ill effects and, except for Purkinje cells, most of their neurons did not degenerate (Tu et al., 1997). If axons lacking neurofilaments resist the cardinal properties of diabetic neuropathy, such as conduction slowing or atrophy, then abnormal neurofilaments might be deemed responsible for them. Alternatively, if neuropathy were to develop irrespective of the presence of neurofilaments, it might instead suggest they are bystanders similarly targeted by abnormal diabetic neurobiology.

**Material and methods**

**Mice, diabetes**

The derivation of the Nf-H–lacZ construct and the production of transgenic mice was carried out as described previously (Eyer and Peterson, 1994). The DNA construct contained 14.9 kb of the mouse Nf-H gene, from –2.9 kb to the middle of exon 4, ligated in-frame to the *Escherichia coli lacZ* gene, followed by an SV40 polyadenylation signal. The fusion protein included the complete Nf-H amino terminal and α-helical rod domains followed by approximately one-half of the Nf-H carboxyl terminus, up to and including the 45th repeated KSP sequence, then joined to the complete β-galactosidase protein. The fusion protein precipitated in perikarya as large filamentous aggregates and axons were not invested with neurofilaments. The background strain of the transgenic mice and littermates was B6C3. Transgenic mice and littermate beta cells were less sensitive to streptozotocin (STZ), requiring 320 mg/kg split in three separate intraperitoneal injections (100 mg/kg random; 100 mg/kg random; then a final dose of 120 mg/kg fasting) over 10 weeks. No deaths or adverse effects from the STZ were encountered, and citrate was given to the controls at each of these times as well. We studied five mice cohorts over 8 weeks: (i) transgenic non-diabetic mice lacking neurofilaments (Nf Dia−); (ii) transgenic diabetic mice lacking neurofilaments (Nf Dia−); (iii) non-transgenic non-diabetic littermates (Nf Dia−); (iv) non-transgenic diabetic littermates (Nf Dia−); and (v) unrelated Swiss mice rendered diabetic with STZ. Only mice with a subsequent blood glucose level of 9.0 mmol/l or higher were studied. Blood glucose measurements were made from the ventral tail vein using a glucometer (AccuChek II; Boehringer Mannheim, Dorval, Quebec, Canada). Swiss mice received only a single injection of STZ (100 mg/kg intraperitoneally; random). Weights and glucose levels are given in Table 1. The protocol was approved by the Animal Care Committees of McGill University and the University of Calgary.

**Electrophysiological recordings**

Multifibre motor and sensory conduction measurements were carried out using a protocol similar to that used previously in rats (Zochodne et al., 1996), with a near nerve temperature maintained at 37°C using a subcutaneous thermistor probe connected to a heating lamp feedback temperature control unit. Motor conduction was carried out in sciatic-tibial fibres with stimulation at the sciatic notch and recording under the skin of the dorsum of the foot (interosseous foot muscles). Sensory conduction velocity was assessed in mixed caudal fibres stimulated in the distal tail and recorded proximally (the nerve is mixed motor and sensory, but the fastest sensory fibres determine the conduction velocity). After the 8-week end point, four NF Dia− mice were treated with insulin (regular Humulin) for a further 4 weeks (0.1 IU intraperitoneally once daily for 5 days a week), followed by further electrophysiology and harvesting. The treatment was intermittent and was associated with fasting blood glucose levels of <5.0 mmol/l tested on the end point day. No significant impact on body weight was noted.

**Morphometry**

After the final electrophysiological recordings, the mice were euthanized and sural and tibial nerves removed. The nerves were fixed in cacodylate-buffered (0.025 M) glutaraldehyde (2.5%) overnight, washed in cacodylate buffer (0.15 M), dehydrated in graded alcohols, stained with osmium tetroxide and embedded in epon. One micrometre sections were stained with Toluidine Blue for graded alcohols, stained with osmium tetroxide and embedded in epon. One micrometre sections were stained with Toluidine Blue for quantitative morphometric studies (Auer, 1994) to measure fibre numbers and calibre in all myelinated axons of the sural and tibial nerves.
Analysis
Results are reported as means ± SEM. Groups were compared with a one-way analysis of variance (ANOVA) and post-ANOVA comparisons made with an unpaired Student’s t-test (two-tailed unless there was an expected direction of change and as specified). The impact of insulin on sensory conduction was compared between 4 and 8 weeks with a paired Student’s t-test.

Results
The weights and blood glucose levels of the mice are given in Table 1. Expected maturational rises in motor sciatic-tibial and sensory caudal conduction velocities occur in normal mice and rats (Schmelzer and Low, 1987; Zochodne and Ho, 1992a), but did not occur in mice lacking neurofilaments.

Fig. 1 Atrophy of axons and loss of neurofilaments, a serendipitous finding in transgenic mice with a β-galactosidase–Nf-H fusion protein that precipitated neurofilaments in perikarya, as previously reported (Eyer and Peterson, 1994). Upper panels show light microscopy appearance of epon-embedded semi-thin section (×400) of the nerve stained with Toluidine Blue (A is Nf− transgenic mouse; B is Nf+ littermate). Lower panels show high power (×10 000) electron microscopy photomicrographs of myelinated axons in (C) a myelinated axon lacking neurofilaments, Nf−, and (D) in a normal axon, Nf+.
Values are means ± SEM. Nf− are mice lacking neurofilaments; Nf+ are littermates with normal neurofilaments; Dia− are non-diabetic controls; Dia+ are diabetic mice. *ANOVA P = 0.0019 (Nf− Dia+ versus Nf− Dia+ post hoc); †ANOVA P < 0.0001 (P < 0.05 for all diabetic versus non-diabetic; P = not significant among diabetics).

Discussion

The major findings of this work were: (i) mice with a normal starting complement of neurofilaments developed only relatively mild features of neuropathy by 8 weeks of diabetes; (ii) non-diabetic transgenic mice lacking neurofilaments have slowing of motor and sensory conduction velocities; (iii) superimposing diabetes on axons lacking neurofilaments was associated with significant further declines in conduction velocity, declines in nerve action potential amplitudes and trends toward increased axonal atrophy; and (iv) insulin increased conduction velocities and axon calibre.

Abnormalities of neurofilament synthesis, transport, glycation, phosphorylation and packing have been suggested to account for axonal atrophy and conduction slowing in diabetes (Medori et al., 1985; Yagihashi, 1993; McLean, 1997; Fernyhough et al., 1999; Scott et al., 1999). The relationship between neurofilament transport, neurofilament synthesis, neurofilament investiture of axons and conduction velocity abnormalities in diabetes is not easily untangled. Since slowing of conduction velocity occurs much earlier than loss of neurofilament investment or axonal atrophy in diabetic models, however, there has been skepticism about a direct relationship between them (Scott et al., 1999). Our findings indicate that changes in neurofilament content, export, spacing or their post-translational modifications cannot account for conduction slowing and atrophy. Both slowing, and apparent atrophy developed in axons completely devoid of neurofilaments despite similar degrees of diabetes. Indeed, mice lacking neurofilaments had accelerated electrophysiological and morphological changes, suggesting to the contrary that neurofilament presence may help axons to resist diabetic damage. For example, it may be that relatively slower axonal turnover of neurofilaments than other structural constituents of axons renders them more resistant to disease. The relatively rapid improvement of mice lacking neurofilament in both conduction velocity and calibre after relatively low doses of insulin supports this idea.

Our findings of accelerated neuropathy could not be accounted for by death of inclusion-laden neuron perikarya from diabetes, since total axon numbers were not altered in any of the groups. Actual axon loss is only a late feature of some, but not all diabetic models, and was not expected at the time-points we studied. The deficits we did observe developed
Fig. 2 Multifibre electrophysiological studies of mice with (Dia⁺) or without (Dia⁻) diabetes and having normal neurofilaments (Nf⁺) or lacking neurofilaments in their axons (Nf⁻). (A) Sensory conduction velocity (CV) from caudal fibres. At 14 weeks (8 weeks of diabetes) Nf⁻ Dia⁺ mice had a slower CV than Nf⁻ Dia⁻ (*P < 0.001). Mice lacking neurofilaments had lower CV than mice with normal neurofilaments (**P < 0.001). Diabetics with normal neurofilaments and unrelated diabetic Swiss mice had mild CV slowing (**P = 0.03). (B) Motor conduction velocity (CV) from sciatic-tibial fibres (Nf⁻ versus Nf⁺ P < 0.0001). Between 10 and 14 weeks, diabetes in Nf⁻ mice was associated with a decline in CV, unlike other mice (*P = 0.04, one-tailed paired test, 10 versus 14 weeks). (C) Amplitudes of mixed caudal nerves. Potentials were smaller in Nf⁻ mice, and further reduced by diabetes but only significantly in mice lacking neurofilaments (*P = 0.04, Nf⁻ Dia⁻). (D) Insulin improved caudal sensory conduction velocities in individual mice lacking neurofilaments (P = 0.04, paired t-test); weeks refers to weeks of diabetes.

Table 2A Morphometric studies in tibial fibres

<table>
<thead>
<tr>
<th>Tibial fibres</th>
<th>Nf⁻ Dia⁻</th>
<th>Nf⁺ Dia⁻</th>
<th>Nf⁻ Dia⁺</th>
<th>Nf⁺ Dia⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fibres</td>
<td>669 ± 73</td>
<td>841 ± 55</td>
<td>1032 ± 37</td>
<td>840 ± 27</td>
</tr>
<tr>
<td>Fibre density (no./mm²)</td>
<td>18 452 ± 692</td>
<td>18 285 ± 985</td>
<td>28 859 ± 1286</td>
<td>25 859 ± 1747</td>
</tr>
<tr>
<td>Nerve area (mm²)</td>
<td>0.037 ± 0.005</td>
<td>0.047 ± 0.005</td>
<td>0.041 ± 0.002</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>Fibres ≥ 9.0 μm (no./mm²)</td>
<td>6902 ± 334</td>
<td>8462 ± 131</td>
<td>5010 ± 226</td>
<td>4030 ± 574</td>
</tr>
<tr>
<td>Fibres ≤ 9.0 μm (no./mm²)</td>
<td>11 550 ± 483</td>
<td>9823 ± 1090</td>
<td>20 850 ± 1776</td>
<td>24 830 ± 1689</td>
</tr>
<tr>
<td>Fibre diameter (μm)</td>
<td>8.1 ± 0.1</td>
<td>9.0 ± 0.3</td>
<td>6.9 ± 0.2</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>Axon diameter (μm)</td>
<td>6.6 ± 0.2</td>
<td>7.2 ± 0.4</td>
<td>5.3 ± 0.2</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Axon area (μm²)</td>
<td>43 ± 2</td>
<td>52 ± 6</td>
<td>27 ± 3</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Myelin thickness (μm)</td>
<td>0.8 ± 0.04</td>
<td>0.9 ± 0</td>
<td>0.78 ± 0.04</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Nf⁻ are mice lacking neurofilaments; Nf⁺ are littermates with normal neurofilaments; Dia⁻ are non-diabetic controls; Dia⁺ are diabetic mice. *Nf⁺ Dia⁻ versus Nf⁻ Dia⁻, P = 0.02 (one-tailed Student’s t-test); †ANOVA of Nf⁻ Dia⁻, Nf⁺ Dia⁻, Nf⁻ Dia⁺ and Nf⁺ Dia⁺, P ≤ 0.05.
in a gradual fashion prominent somewhat later, between 4 and 8 weeks of diabetes. The changes in conduction velocity in our non-transgenic diabetic mice were quite modest, and less than that observed in rat models (Zochodne and Ho, 1992b). More gradual evolution of electrophysiological changes in diabetic mice, however, has been described previously (Ng et al., 1998), possibly linked to lower polyol flux in mouse nerve (see below). In human diabetic polyneuropathy,

Table 2B  Morphometric studies in sural fibres

<table>
<thead>
<tr>
<th>Sural fibres</th>
<th>Nf⁻ Dia⁻</th>
<th>Nf⁺ Dia⁺</th>
<th>Nf⁻ Dia⁺</th>
<th>Nf⁺ Dia⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fibres</td>
<td>202 ± 33</td>
<td>222 ± 14</td>
<td>187 ± 28</td>
<td>217 ± 30</td>
</tr>
</tbody>
</table>
| Fibre density (no./mm²) | 23 702 ± 1182 | 26 191 ± 243 | 32 325 ± 2360 | 31 544 ± 1618<br>
| Nerve area (mm²) | 0.0088 ± 0.001 | 0.0083 ± 0.001 | 0.006 ± 0.001 | 0.0068 ± 0.001 |
| Fibres ≥ 9.0 µm (no./mm²) | 5432 ± 327 | 6920 ± 1284 | 2097 ± 447 | 1056 ± 170<br>
| Fibres ≤ 9.0 µm (no./mm²) | 18 270 ± 1105 | 19 270 ± 1096 | 30 228 ± 2534 | 30 488 ± 1477<br>
| Fibre diameter (µm) | 7.0 ± 0.1 | 7.2 ± 0.3 | 5.6 ± 0.2 | 5.3 ± 0.2<br>
| Axon diameter (µm) | 5.5 ± 0.1 | 5.8 ± 0.3 | 4.3 ± 0.2 | 4.0 ± 0.2<br>
| Axon area (µm²) | 30 ± 1 | 30 ± 4 | 16 ± 2 | 13 ± 2<br>
| Myelin thickness (µm) | 0.73 ± 0.04 | 0.67 ± 0.03 | 0.62 ± 0.03 | 0.68 ± 0.04

Values are means ± SEM. Nf⁻ are mice lacking neurofilaments; Nf⁺ are littermates with normal neurofilaments; Dia⁻ are non-diabetic controls; Dia⁺ are diabetic mice. *Nf⁻ Dia⁻ versus Nf⁺ Dia⁺, P = 0.04 (one-tailed Student’s t-test); †ANOVA of Nf⁺ Dia⁻, Nf⁺ Dia⁺, Nf⁻ Dia⁻ and Nf⁻ Dia⁺, P < 0.05.

Fig. 3  Fibre size histograms of mean (per rat) numbers of axons (y-axis) in (A) tibial and (B) sural nerves of mice with or without neurofilaments and diabetes. Mice lacking neurofilaments have a shift in axon sizes to smaller categories with trends toward a further shift in size by diabetes (fewer large fibres). Values are means ± SEM. Hatched bars = diabetic; solid bars = non-diabetic.
conduction abnormalities generally develop gradually, sometimes over decades of diabetes.

Several other mechanisms unrelated to neurofilaments have been invoked to explain nerve conduction slowing in human and experimental diabetes. These have included hyperosmolar shrinkage of axons, an event expected to generate immediate declines in velocity, but difficult to explain as a gradual event during tissue equilibration of glucose content. In rats and mice a number of agents reverse conduction slowing without lowering hyperglycaemia. Low-dose intermittent near nerve insulin, for example, reversed slowing unilaterally in diabetic rats without lowering blood glucose levels (Singhal et al., 1997). The polyol hypothesis, based on a series of biochemical events that includes sorbitol accumulation and myo-inositol depletion predicts that conduction slowing occurs through changes in nodal membrane excitability (Sima, 1996). Associated changes include deficits in the sodium-potassium ATPase pump, intra-axonal accumulation of sodium, relative axonal depolarization and impaired action potential ion flux. It is not clear, however, whether increased polyol flux, can account for axon atrophy. Polyol flux in diabetic mice may differ from that of rats (Ng et al., 1998) because of higher activity of sorbitol dehydrogenase, in turn reducing sorbitol accumulation in nerves. Ng et al. (1998), however, have provided evidence that sorbitol accumulation within peripheral nerves alone, does not account alone account for changes in conduction velocity identified in diabetes. It is likely that other consequences of diabetes, developing in parallel with polyol flux and interacting with it, alter membrane excitability and conduction velocity.

Unexplored is what role the lack of insulin itself, a neuronal growth factor, might have in failing to properly support the calibre and electrophysiological properties of axons.

An analysis of insulin’s impact on neuropathy was not the primary goal of this work. Unlike some previous approaches that have examined direct insulin actions in the absence of a glycaemic action (Singhal et al., 1997; Brussee et al., 2001; Huang et al., 2003), insulin did in fact have some impact on hyperglycaemia here, although rigorous control was obviously not sought nor obtained. We were surprised at the rapidity and magnitude of insulin’s action in this model, both on sensory conduction velocity and sensory axons. Insulin and the related insulin-like growth factors (IGFs) are potent neuronal growth factors that can cross-occupy each others receptors (Recio-Pinto and Ishii, 1984; Ishii, 1993, 1995; Brooker et al., 2000; Kurihara et al., 2000; Lackey et al., 2000; O’Kusky et al., 2000). Moreover, insulin receptors (IRs) are expressed both on axons and within perikarya, suggesting a variety of levels at which they can act (Sugimoto et al., 2000, 2002). Insulin and IGF-1 stimulate survival, neuritic outgrowth and other cellular responses within the adult sensory neuron (Recio-Pinto et al., 1986). IGF-1 inhibits apoptotic effects of excessive glucose in cultured DRG neurons (Russell et al., 1999). Low doses of insulin reverse mitochondrial depolarization of sensory neurons in diabetic rats (Huang et al., 2003). Insulin binding leads to a phosphorylation of cellular substrates, including the insulin receptor substrate (IRS) proteins IRS-1 or IRS-2 and Shc (White and Yenush, 1998). Downstream effectors include phosphatidylinositol 3 kinase, Grb2, Nck, Crk, Fyn and SHP2, as well as Grb 2/Sos association with IRS-2 (Patti et al., 1995; White, 2003). In our model, insulin may have had a potent action on non-neurofilament axonal transport, protein synthesis, mitochondrial function or other targets that provided rapid improvements.

Not examined further in this work is the interesting possibility that changes in axons with a more stable lattice of neurofilaments are more difficult to effect. Finally, a very slowly progressive degenerative change at the level of perikarya involving alterations of critical protein synthesis and mitochondrial function, but not reliant on changes in neurofilaments for its progression, may be a fundamental consequence of diabetes. We believe our findings are relevant in understanding this common irreversible disorder in humans.

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References


