

Morphometry of Dorsal Root Ganglion in Chronic Experimental Diabetic Neuropathy

Motoko Kishi, James Tanabe, James D. Schmelzer, and Phillip A. Low

Chronic hyperglycemia results in a predominantly sensory neuropathy. Recent studies suggest that dorsal root ganglion (DRG) neurons comprise a specific target and may be responsible for the important complication of diabetic sensory neuropathy, since hyperglycemia for longer than 6 months results in a vacuolar ganglionopathy with associated radiculopathy and distal sensory neuropathy. We undertook morphometric analysis of L5 DRG neurons in seven diabetic rats and six age- and sex-matched littermates. Nerve conduction studies were also performed, and neuropathy was confirmed. Diabetes was induced with streptozotocin; duration of diabetes was 12 months. The DRG count for control rats was $15,304 \pm 991$ neurons. Two of seven diabetic DRG counts were reduced, but the group mean count at $14,847 \pm 1,524$ was not significantly reduced. The number of small neurons (type B) considerably exceeded that of large neurons (type A), at a ratio of 71:29. The percentage of large cells was significantly reduced in diabetic compared with control rats ($P = 0.01$). The large-diameter population can be subdivided into two groups; with this subdivision, the number of neurons $<50 \mu\text{m}$ was not reduced in samples from diabetic rats, but the neurons of largest size ($\geq 50 \mu\text{m}$) were significantly reduced (by 41%). *Diabetes* 51:819–824, 2002

The precise pathogenesis of diabetic neuropathy is unknown; however, a number of putative pathophysiological mechanisms exist. Hyperglycemia results in nerve hypoxia/ischemia (1), autoxidative stress (1,2), polyol pathway overactivity (3), increased advanced glycation end products (4), deficiency of γ -linolenic acid (5), increase in protein kinase C, especially β -isoform (6), and growth factor deficiency (7). These pathways all result in oxidative stress, with deficits in nerve blood flow and sensory conduction (8). Oxidative stress appears to be more severe at the dorsal root ganglion (DRG) than at the nerve (9), and recent findings—that beyond 6 months of diabetes, diabetic rats develop florid radicular pathology (10) and vacuolar degeneration of DRG neurons (11)—have led to the hypothesis that the primary target of diabetic neural complications is the sensory neuron (1).

In our studies of rats that have been diabetic for 12–18

months, we found DRG ischemia, with prominent vacuolar degeneration of L5 DRG neurons and demyelination of dorsal and ventral roots (11). Using electron microscopy, we demonstrated myelin alteration from mild separation to severe ballooning of myelin with relative axonal sparing. DRG cells showed vacuoles of all sizes with cristae-like residua, suggestive of mitochondria. Nerve conduction studies showed reduced conduction velocities in the distal nerve segments and prolonged F-wave latency and proximal conduction time, despite the shorter conduction pathway in diabetic rats. We suggested that the combination of hyperglycemia and ischemia results in oxidative stress and a predominantly sensory neuropathy.

In this study, we report nerve conduction and morphometric analysis of L5 DRG neurons of diabetic rats (diabetes duration 12 months) and compared these findings to age- and sex-matched littermates to address the questions of whether there is a loss of neurons and what specific populations are involved.

RESEARCH DESIGN AND METHODS

Animals. We used male Sprague-Dawley rats weighing 250 ± 5 g at the beginning of the study. Experimental diabetes was produced by the intraperitoneal injection of streptozotocin (STZ) in 0.05 mol/l citrate buffer, pH 4.5 (65 mg/ml; dose 1.32 ml/kg). The control group received an intraperitoneal injection of citrate buffer alone. Control and diabetic rats had free access to Purina rodent laboratory chow and water. They did not receive insulin treatment. The rats were accepted as diabetic if their fasting blood glucose exceeded 16.7 mmol/l 3 days after injection of STZ and remained >16.7 mmol/l at the time of surgery. Duration of diabetes was 12 months for diabetic rats, and control rats were age- and sex-matched littermates.

Electrophysiology. We used techniques that are standard for our laboratory (11,12). Sensory nerve conduction velocity of digital and caudal nerves was measured using fine stainless steel near-nerve stimulating and recording electrodes. Motor nerve conduction velocity was measured in the sciatic-tibial and caudal nerves. The compound muscle action potentials were recorded from the pair of fine stainless steel electrodes in the dorsum of the hind paw, with stimulation by another pair of electrodes at the level of the sciatic notch and ankle. Recordings were made at 35°C, amplified 1,000 times, stored on computer discs, and analyzed off-line using a Nicolet 4094 digital oscilloscope (Nicolet Instruments, Madison, WI) with associated stimulators and stimulus isolation units.

Histologic studies

Histologic preparation. Control and diabetic rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and tissues were fixed by intracardiac perfusion with a perfusate consisting of 1,000 ml 4% glutaraldehyde in 0.1 mol/l phosphate-buffered cacodylate buffer, pH 7.38. A laminectomy was then performed, and the lumbar (L4–L6) DRG neurons were exposed and fixed in situ for 20 min. L4–L6 DRG neurons were then harvested, postfixed in 4% glutaraldehyde overnight, and separately dehydrated, infiltrated, and embedded in plastic.

Morphometry. These glutaraldehyde-fixed, plastic-embedded 2- μm sections were stained with toluidine blue and studied under oil immersion ($\times 63$ objective; total magnification $\times 630$). To determine sampling frequency, we obtained 2- μm sections of one L5 DRG and compared the counts and diameter distribution derived from sampling intervals of every 5th, 10th, 15th, 20th,

From the Department of Neurology, Mayo Clinic, Rochester, Minnesota.

Address correspondence and reprint requests to Phillip A. Low, Department of Neurology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905. E-mail: low@mayo.edu.

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DRG, dorsal root ganglion; STZ, streptozotocin.

25th, 30th, 35th, 40th, 45th, 50th, and 100th section. No significant differences in counts and diameter distribution were found for these sampling frequencies (ANOVA). Hence, subsequent analysis was done using analysis of every 100th section. For control rats, the mean (\pm SE) number of sections quantitated was 824.67 ± 47.17 . Corresponding values for experimental diabetic neuropathy were 757.86 ± 77.47 sections. Any neuron containing part of a nucleolus was counted and had its area measured. Corrected cell counts were determined by multiplying by a correction factor to account for split nucleoli. Neuron counts were determined from nucleolar counts, with correction for split-cell error (13,14).

In a preliminary analysis, we visually scanned each area histogram and divided the neurons into large and small from the shape of the histogram. For both control and diabetic neurons, the value of $1,500 \mu\text{m}^2$ appeared to best separate these neurons based on the shape of the histogram and the light-microscopic separation into large and small neurons, i.e., neurons $>20 \mu\text{m}$ were large, with clearer-looking cytoplasm; neurons $\leq 20 \mu\text{m}$ were small and tended to have basophilic cytoplasm (15). The value of $1,500 \mu\text{m}^2$ was therefore chosen as the separator.

Statistics. Total cell counts, large (area $\geq 1,500 \mu\text{m}^2$) and small (area $< 1,500 \mu\text{m}^2$), were expressed as means \pm SE. Comparisons of these data between control and diabetic rats were done using a two-tailed *t* test. Significance was accepted at $P < 0.05$.

RESULTS

A total of nine control rats and nine diabetic rats were used for electrophysiological studies; six control and seven diabetic rats were used for morphometric analysis. Duration of diabetes was 12 months, and control rats were age- and sex-matched littermates. Weights for diabetic rats were significantly reduced, at 307 ± 26 g, compared with control rats, at 584 ± 16 g ($P < 0.01$). Fasting glucose for diabetic rats was 16.6 ± 4.4 mmol/l at the time of surgery.

Electrophysiology. Motor nerve conduction velocity was significantly reduced for caudal ($P < 0.05$) but not sciatic-tibial nerve of diabetic rats. In contrast, sensory conduction velocity of both digital nerve ($P < 0.05$) and caudal nerve ($P < 0.001$) was significantly reduced. Sensory action potential amplitude was not reduced—indeed, as previously noted (12), it was higher in diabetic rats. We ascribe that difference to the loss of tissue around the digital nerve, resulting in closer contact of the near-nerve recording needle to nerve.

Qualitative observations. Prominent vacuolation of DRG neurons was evident, as previously documented. There appeared to be an underrepresentation of the largest-diameter clear neurons (type A) and possibly an overrepresentation of small, more basophilic neurons in diabetic animals (type B) (Fig. 1). Vacuoles and pigmentary alterations were also more common in diabetic animals.

Morphometry. Data for individual animals are shown in Table 1, and group data are shown in Figs. 2 and 3. L5 DRG counts for control animals were $15,304 \pm 991$ (range 12,033–17,888) neurons (Table 2). Diabetic group mean count at $14,847 \pm 1,524$ was not significantly reduced, although two values (9,188 and 11,587) fell below the control range. The number of small neurons considerably exceeded that of large neurons, with a ratio of 71:29. The percent of small and large cells, however, was significantly increased and reduced, respectively, for diabetic compared with control samples. As expected, the ratio of large to small cells was significantly reduced in diabetic rats ($P = 0.01$). The ratio of large to small neurons was reduced in diabetic rats, from a normal ratio of 0.42 ± 0.04 to 0.29 ± 0.02 ($P = 0.01$) (Table 2).

The summary histograms of diabetic and control rats

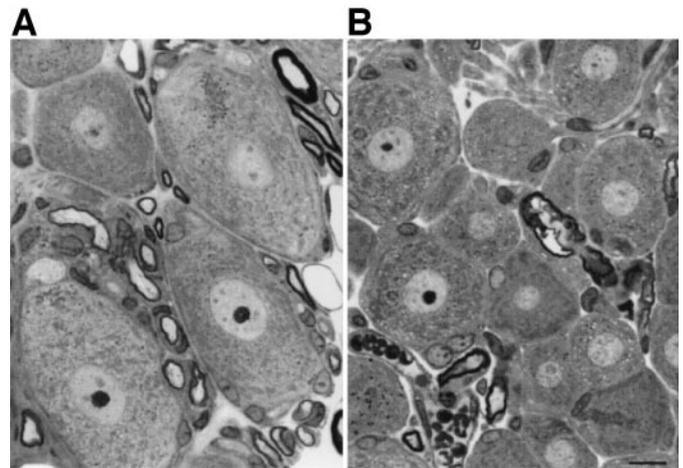


FIG. 1. Dorsal root ganglion neurons of control (A) and diabetic (B) rats. Diabetic neurons tend to be small and more basophilic and have more and larger vacuoles and pigmentary inclusions. Bar represents 25 μm .

are shown in Fig. 2. The absolute counts of the small neurons ($< 1,500 \mu\text{m}^2$) were not significantly different between diabetic and control rats (Figs. 2 and 3). Qualitatively, diabetic small neurons were more clustered, with a sharper peak than that of control neurons, whereas control neurons had a broader distribution area. In contrast, neurons $\geq 1,500 \mu\text{m}^2$ were significantly reduced in experimental diabetic neuropathy (Fig. 3). The large-diameter population can be subdivided into intermediate-large and largest diameter groups (16). With this subdivision, the intermediate-large neurons ($1,500$ – $2,100 \mu\text{m}^2$) were not reduced in diabetic animals, but the largest neurons ($> 2,200 \mu\text{m}^2$) were significantly reduced (by 43%).

DISCUSSION

There is good general agreement between our counts of L5 DRG neurons in control rats and counts reported in the literature. Published values range from 13,000 to 18,000 for rat L5 DRG neurons when high-resolution methods of quantitation are used. Our mean value of $15,304 \pm 991$ is in good general agreement with published results using similar approaches that incorporate stereoscopy or high-resolution imaging, adequate sampling, and correction for split-cell error (17–20). These counts are higher than estimates using paraffin section (e.g., 23% higher than those of Schmalbruch [17]).

Careful correlations have been made between electrophysiological properties of the neurons and their axons and the size of the DRG neurons in the rat and cat (16,21). The small dark neurons (type B) connect mostly to unmyelinated fibers, with a few connected to small myelinated fibers (21). The large clear neurons (type A), which are connected to large myelinated fibers and electrophysiologically are A α and A β fibers, comprise a broader-diameter distribution. The size of the neuron is linearly related to the conduction velocity of the axon (16,21).

We used profile counts instead of the assumption-free stereologic methodology (22). Profile counts are satisfactory if all parts of the anatomical region under study have an equal chance of being sampled (22). Our sampling is appropriate, and the appropriate correction factors have been used. Support for the adequacy of our methodology

TABLE 1
Electrophysiology of 12-month diabetic and age-matched control rats

Group	n	Digital nerve		Sciatic-tibial nerve		Caudal NAP		Caudal CMAP	
		CV (m/s)	Amplitude (μ V)	CV (m/s)	Amplitude (mV)	CV (m/s)	Amplitude (μ V)	CV (m/s)	Amplitude (mV)
Control	9	46.3 \pm 2.1	23.3 \pm 2.6	49.5 \pm 1.6	3.9 \pm 1.1	60 \pm 1.5	101 \pm 7	46 \pm 0.5	4.9 \pm 0.3
Diabetic	9	40.6 \pm 1.7	30.9 \pm 2.7	46.3 \pm 1.0	5.2 \pm 1.2	47 \pm 1.2	103 \pm 8	41.3 \pm 0.2	5.8 \pm 0.9
P		0.0497	0.0590	0.1176	0.4405	0.0000	0.8557	0.0339	0.3818

Data are means \pm SE. CMAP, compound muscle action potential; CV, conduction velocity; NAP, nerve action potential.

comes from the fact that the total number of DRG neurons is very similar to that reported by other investigators, and that the large-diameter population of 29% is essentially identical to the type A cell population of 28% reported by Tandrup (18) using stereological methods. We chose perfusion fixation and epon embedding to provide us with tissue suitable for detailed fine structural studies.

The shape of the neuronal area distribution curve bears comment. The histogram of the diabetic DRG has a narrower small neuron peak, with a slight left-shift, with the division between large and small neurons remaining unchanged; an area of 1,500 μ m² (diameter 20 μ m) separates large clear (type A) from small dark (type B) neurons qualitatively in both diabetic and control samples. This size of 20 μ m is less than the smallest diameter of type A cells, and neurons below this size are small dark neurons (15) in both control and diabetic rats, arguing against simple atrophy of all neurons in diabetic animals.

These changes are different from the changes previously reported by Sidenius and Jakobsen (22a) in short-duration (4 weeks) STZ-induced diabetes. They reported an 18% reduction in perikaryal volume, affecting neurons of all sizes, and a similar reduction in axonal caliber; these changes have been duplicated by inducing a hyperosmolar

state (23). There are a number of other reasons to support our position. The neuronal counts in 29% of our diabetic DRG neurons fell below the control range. The division between large and small neurons remained unchanged, as did the small-diameter density in diabetic rats. Indeed, the small neurons are qualitatively different from the large neurons. The loss was confined to the largest subpopulation of the large neurons, instead of a uniform reduction as seen in atrophy. There are morphological changes in these neurons (inclusions and vacuolar degeneration [11]). The changes and loss in neurons are associated with dramatic changes in both dorsal and ventral roots, including florid demyelination (11). That these changes are functionally important is supported by the F-wave changes. We, and others (2,24), have demonstrated that these neurons are subject to oxidative damage and caspase-3 expression and undergo terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) positivity at a rate of 12% at 12 months (25).

The present study is similar to previous studies in demonstrating the greater involvement of sensory than motor fibers (12,26), emphasizing the importance of selective involvement of large DRG neurons. The lack of significant slowing of motor conduction emphasizes the

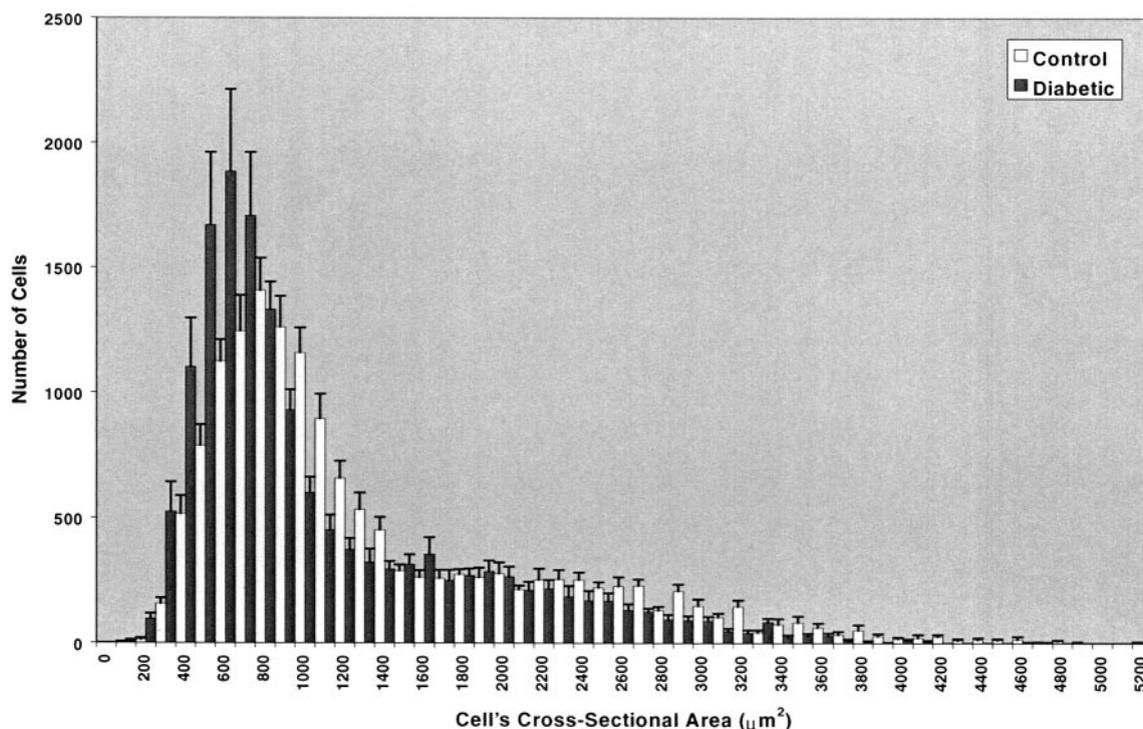


FIG. 2. Composite histogram of L5 dorsal root ganglia of control and diabetic rats.

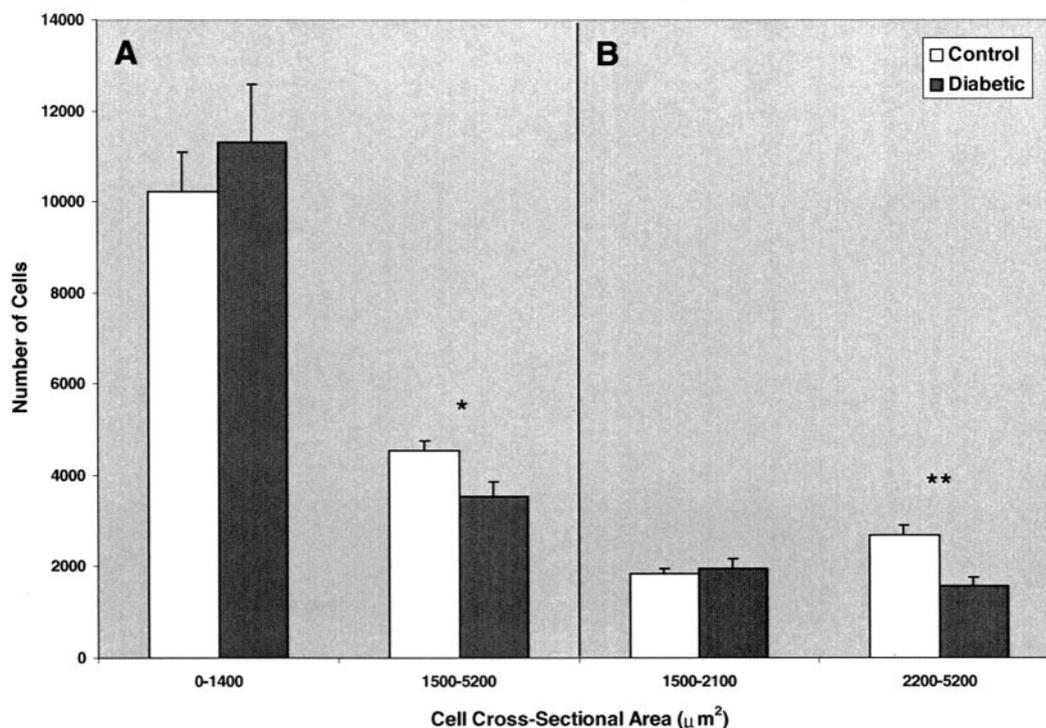


FIG. 3. Diameter histograms of control and diabetic ganglia for all neurons (A) and two groups of large neurons (B). * $P < 0.05$; ** $P < 0.01$.

less constant changes in motor axons. However, motor and autonomic fibers are also clearly affected in experimental diabetes (12), and hence DRG neurons should be considered one target, albeit an important target. Separate involvement of motor, sensory, and autonomic nerve fibers or their parent neurons also needs to be considered.

In our study, the loss of fibers was quite selective, affecting only the largest neurons. Of note is that neurons with areas between 1,500 and 2,100 μm^2 are not reduced. The deficit in the largest neurons (diameter $\geq 50 \mu\text{m}$) is

large, with a loss of 43% of these neurons. This loss of large neurons can explain the slowing of sensory conduction. However, the mechanisms of conduction slowing are quite complicated. Slowing and axonal shrinkage can be induced in hours (23) by hyperosmolar glucose, and such axonal atrophy has been cited as a mechanism of slowing. Reduction in $\text{Na}^+\text{-K}^+\text{-ATPase}$, related to myoinositol deficiency (3), also occurs and could cause slowing (27). These changes occur in short-duration diabetes and are readily reversible. In contrast, changes beyond 6 months

TABLE 2

Morphometric data on L5 DRGs from control and diabetic rats

Identification number	Number of cells			% Small	% Large	Ratio of large to small
	Total	Small	Large			
Control						
671L	17,888	13,305	4,583	74	26	0.344
672L	16,425	12,519	3,906	76	24	0.312
675L	17,700	13,240	4,460	75	25	0.337
676L	12,033	7,811	4,221	65	35	0.540
677L	14,630	9,529	5,101	65	35	0.535
678L	13,146	8,911	4,235	68	32	0.475
Mean \pm SE	15,304 \pm 991	10,886 \pm 988	4,418 \pm 166	71 \pm 2	29 \pm 2	0.424 \pm 0.043
Diabetic						
664L	11,587	9,010	2,577	78	22	0.286
665L	13,937	10,590	3,348	76	24	0.316
667L	18,600	15,288	3,312	82	18	0.217
680L	20,218	16,343	3,875	81	19	0.237
681L	12,870	10,442	2,428	81	19	0.233
682L	17,531	12,999	4,532	74	26	0.349
683L	9,188	6,661	2,527	72	28	0.379
Mean \pm SE	14,847 \pm 1,524	11,619 \pm 1,305	3,229 \pm 297	78 \pm 1	22 \pm 1	0.288 \pm 0.024

Data are means \pm SE. Differences (*t* test) for number of total cells and small cells were not significant ($P = 0.814$ and 0.672 , respectively); differences were significant for number of large cells ($P = 0.007$), % small ($P = 0.014$), % large ($P = 0.014$), and the ratio of large to small ($P = 0.015$).

(10,11) and those seen in the present study presumably are less reversible. This selective reduction likely affects the population of neurons connected to a specific population of myelinated fibers, the A α fibers. Diabetes has some of the characteristics of an exaggerated aging process, and these diabetic rats have significantly lower weights than control age- and sex-matched rats, so the alterations could potentially be related to exaggerated aging and weight alterations. However, morphometric data indicate that these concerns are groundless, since there is no change in the number of L5 DRG sensory neurons with age (19) or weight loss (20).

Earlier pathological studies on spinal roots (10) and dorsal root ganglion showing demyelination, vacuolar degeneration, and pigmentary changes (11) have been supported by recent pathophysiological studies supporting the sensory neuron as a target and, in particular, the mitochondrion as the most relevant organelle. In a series of studies, the Michigan group (2,28–30) has confirmed ballooning of mitochondria and disruption of the internal cristae due to the neurotoxic effects of glucose. They demonstrate apoptosis, with sequential steps including a reduction of mitochondrial membrane potential, leakage of caspase c, and caspase-3 activation. In a recent study (2), the *in vitro* studies were extended to *in vivo* supportive observations of mitochondrial pathology and apoptosis. The concentrations of glucose found by the Michigan researchers were higher than those we found. Some of the differences are more apparent than real. First, our glucose values were fasting glucose. Over a period of 12 months, these rats were likely exposed to much higher levels of glucose some of the time. The Michigan researchers used higher concentrations of glucose but over a shorter duration, in contrast to the more moderate glucose concentrations over many months in our studies. In many toxic agents, there is a dose-duration relationship such that a higher dose over a shorter time can generate changes that will occur with a lower dose over a longer duration. Additionally, similar changes have been found within acute *vivo* studies with lower glucose concentrations (2,24).

There are additional studies that support pathophysiologically important changes in DRG neurons with resultant axonal alterations. DRG blood flow is reduced (11,26), and there is impaired neurotrophic support (31). Fernyhough and colleagues (32) found aberrant neurofilament phosphorylation of dorsal root neurons and sural nerve but not of motor neurons. Scott et al. (33) examined neurofilament and tubulin mRNA expression and demonstrated that a time-dependent decrease in mRNA expression of cytoskeletal proteins in sensory neurons accompanies a reduction in their incorporation into distal axons. These abnormalities build on the observation by Medori et al. (34), who demonstrated axonal dwindling secondary to alterations in the axonal transport of structural proteins, with enlargement proximally and atrophy distally.

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