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EFFECTS OF HYPERGLYCEMIA ON RAT CAVERNOUS NERVE AXONS: A FUNCTIONAL AND ULTRASTRUCTURAL STUDY

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Abstract

The present study explored parallel changes in the physiology and structure of myelinated (A δ) and unmyelinated (C) small diameter axons in the cavernous nerve of rats associated with streptozotocin-induced hyperglycemia. Damage to these axons is thought to play a key role in diabetic autonomic neuropathy and erectile dysfunction, but their pathophysiology has been poorly studied. Velocities in slow conducting fibers were measured by applying multiple unit procedures; histopathology was evaluated with both light and electron microscopy. To our knowledge, these are the initial studies of slow nerve conduction velocities in the distal segments of the cavernous nerve. We report that hyperglycemia is associated with a substantial reduction in the amplitude of the slow conducting response, as well as a slowing of velocities within this very slow range (<2.5 m/sec). Even with prolonged hyperglycemia (> 4 months), histopathological abnormalities were mild and limited to the distal segments of the cavernous nerve. Structural findings included dystrophic changes in nerve terminals, abnormal accumulations of glycogen granules in unmyelinated and preterminal axons, and necrosis of scattered smooth muscle fibers. The onset of slowing of velocity in the distal cavernous nerve occurred subsequent to slowing in somatic nerves in the same rats. The functional changes in the cavernous nerve anticipated and exceeded the axonal degeneration detected by morphology. The physiologic techniques outlined in these studies are feasible in most electrophysiologic laboratories and could substantially enhance our sensitivity to the onset and progression of small fiber diabetic neuropathy.

Keywords

Diabetes; Nerve conduction velocity; Small fiber neuropathy; Diabetic autonomic neuropathy; Erectile dysfunction; Neuroaxonal dystrophy; Cavernous nerve; Parasympathetic; Major pelvic ganglia; Cavernous smooth muscles

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INTRODUCTION

Diabetic autonomic neuropathy (DAN) is a critical and often life-threatening complication of diabetes that is generally poorly understood and inadequately studied (Vinik et al., 2003). DAN affects the cardiovascular, respiratory, gastrointestinal, genitourinary, thermoregulatory, papillary, and neuroendocrine systems (Boulton et al., 2005; Low et al., 2003; Vinik and Ziegler, 2007). The clinical onset and progression of DAN generally parallels the course of somatic neuropathy, but structural and functional deficits in autonomic nerves can occur in isolation or be dissociated from their somatic counterparts (Tentolouris et al., 2001; Töyry et al., 1997; Winkler et al., 2000; Young et al., 1986). Although DAN is usually considered a late stage complication of diabetes, sensitive measures can detect autonomic dysfunction at disease onset (Said, 2007). The incidence of DAN increases with age and duration of diabetes, thus this complication will likely gain increasing importance as the mean age of the population increases.

The role of DAN in cystopathy and erectile dysfunction (ED) is especially insidious (Sasaki et al., 2003). ED has an incidence of 35–75% in patients with diabetes (Vinik et al., 2003) and this complication is now considered a prediction tool for DM itself (Sun et al., 2006). ED may stem from deficits in the vascular system, penile smooth muscles and autonomic nerves (Hecht et al., 2001). The integrity of cavernous nerve (CN) is critical for normal erectile function; even minor surgical impairment of this nerve can lead to impotence (Klotz, 2004; Walsh and Donker, 2002). The CN is principally composed of unmyelinated axons with a few scattered myelinated fibers (Schaumburg et al., 2007). The myelinated fibers range in diameter from 1–8 μm , with 2 μm as the most common value; they originate in the dorsal root ganglia (DRG) or sympathetic chain and course through the pelvic or hypogastric nerves prior to entering the CN (Dail et al., 1989; Purinton et al., 1973). The majority of unmyelinated fibers are postganglionic parasympathetic and sympathetic axons originating in the major pelvic ganglia (MPG). A recent ultrastructural study in control rats confirmed the abundance of postganglionic cells in the proximal segment of the CN and described the scarcity of unmyelinated fibers in the distal third of the penis shaft (Schaumburg et al., 2007). In contrast, approximately 40% of the axons in the dorsal nerve of the penis (DNP), a sensory branch of the pudendal nerve, are myelinated (Hulsebosch and Coggeshall, 1982).

Functional and structural deficits in small diameter, myelinated ($A\delta$) and unmyelinated (C) axons are key components of DAN, ED and painful somatic neuropathies, but deterioration or improvement in activity of these axons is virtually undetected by standard nerve conduction velocity (NCV) procedures (Arezzo and Zotova, 2002). This limitation of the NCV technique is generally true for both clinical and pre-clinical studies and it diminishes the value of this important technique. The assessment of NCV in myelinated and unmyelinated small diameter fibers is a formidable task due to temporal dispersion, asynchronous signals, small amplitudes and phase cancellation (Arezzo and Zotova, 2002).

The present study examined parallel changes in the physiology and structure of small diameter axons in the CN of the streptozotocin-treated diabetic rat. Velocities in slow conducting fibers were measured by applying procedures originally designed to assess the relatively slow and often unorganized transmission of neural signals in the brain (Arezzo et al., 1986; Legatt et al., 1980; Steinschneider et al., 2008). Histopathology assessment examined step sections of the CN, MPG, and cavernous smooth muscle.

MATERIALS AND METHODS

Animals

Electrophysiology studies were performed on 10 Fischer rats (F344, Charles River Lab., Wilmington, MA); 7 were hyperglycemic and 3 were controls. An additional 8 Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, Indiana) were used to optimize recording procedures and compare the NCV measure across strains. Histopathological studies were completed in two of the Fischer rats employed in the NCV studies, an additional 6 hyperglycemic Fischer rats and 5 age-matched controls. Histopathology was assessed at either 2, 4, 6, 8, or 10 months following the onset of hyperglycemia (HG). HG was induced by a single 35 mg/kg i.p. injection of streptozotocin (STZ). Prior to injection, STZ was dissolved in citrate buffer (60 mL of 0.1 M citric acid and 40 mL of 0.2 M Na₂HPO₄, pH 4.6). HG was confirmed by blood glucose levels above 300 mg/dL. The mean blood glucose level in the STZ-treated rats was 394.1 mg/dL (range 323 to 567 mg/dL). All animals were given free access to standard laboratory chow and water throughout the study. CN electrophysiology is an invasive procedure so data were recorded at a single time point in each rat at a period ranging from 2–10 months following the induction of HG. All procedures were approved by the Animal Institute Care and Use Committee at Albert Einstein College of Medicine.

Electrophysiological procedures

Data were collected from the CN, the DNP and the caudal nerve under isoflurane anesthesia (2.5–3.5 % /O₂). For the CN, the lower part of the abdomen was opened by surgical incision and the ventral prostate was moved aside to expose the area around the MPG. The opening was kept narrow to diminish bleeding and drying or cooling of the exposed area. Rectal and near nerve temperature was monitored and maintained within 35–37°C using a circulating water heating unit. The main CN was located, and the recording electrodes were placed 2–3 mm below its exit from MPG; the nerve was stimulated further distally at the base of the shaft of the penis (Fig. 1A).

Activity in the DNP was recorded antidromically using electrodes positioned along the proximal and distal portions of the penile shaft (Fig. 1A) and evoked by stimulation at the same site as employed for the CN. A small incision was made in the scrotum to expose the crura and the stimulating cathode was inserted into the corpus tissue to a depth of approximately 1–2 mm at the base of the penis. At this location, both the CN and DNP run dorsal to urethra on their way to innervate corpus cavernosum (Walsh and Donker, 2002). Appropriate stimulation at this site can simultaneously trigger a response in both nerves. However, in some cases, the stimulating electrodes had to be moved distally along the penile shaft for optimal stimulation of the DNP, and to a point 2–3 mm below the base of the penis for isolation of CN activity. Caudal nerve activity was measured on the dorsal surface of the tail 10 mm below the hairline and 80 mm proximal to the site of stimulation.

For each nerve, responses were evoked by constant current, square pulses of 0.5–14 mA, duration 0.05–1 ms, applied at a rate of 1–2 Hz. Subcutaneous platinum needle electrodes (Grass Astro-Med, Inc) were used for both stimulation and recording. Stimuli were generated by Grass stimulator (Model 11, Warnick, RI) and isolated from ground by a dedicated isolation unit (Grass Model SIU-7, Warnick, RI). All neuroelectric signals were impedance-matched and differentially amplified with a gain of 20,000 and a frequency band of 20 Hz–3 kHz using a BIOPAC MP100 and AcqKnowledge software version 3.7.2 (BIOPAC Systems Inc., Goleta, CA). Data were scanned for artifacts, digitized at a rate exceeding 20 kHz and averaged across 50–100 stimuli. Single sweeps of activity in CN and DNP were stored in a digital format (MDI DT-1600) for further off-line analysis.

Multiple Unit Activity (MUA)

Measures of MUA were extracted from single sweep recording by first high-pass filtering the response above 450 Hz, then full-wave rectifying the signal and finally averaging the response (Arezzo et al., 1986; Legatt et al., 1980; Steinschneider et al., 2008). Rectification minimizes phase cancellation of the slow and asynchronous signals. MUA data represent composite voltages at specific latencies and at a fixed distance from the site of stimulation; these responses can then be converted into a distribution of conduction velocities. MUA can be measured at unique peaks to indicate specific velocities or integrated across a specific time period (i.e., 8.0 msec), to reflect the signal strength in a certain range of slow velocities (Fig. 1B). Slow NCV (sNCV) was calculated by dividing the distance between the stimulating cathode and the recording site by the latency of responses. The magnitude of the sNCV response was calculated across four bins (i.e., 2–2.5 m/sec, 1.5–2.0 m/sec, 1–1.5 m/sec and 0.5–1.0 m/sec). These bins did not correspond to specific patterns of velocities, but rather provided a means of sampling the distribution of C-fiber related activity across animals and degrees of neuropathy.

Statistics

Comparison of responses in somatic nerves (i.e., caudal and DNP) across conditions utilized standard inferential procedures (i.e., ANOVA, Tukey *post hoc* test); alpha levels were set at 0.05. Pearson coefficient was used to examine correlations across measures. The statistical analysis was severely limited by the small number of animals included in each condition.

Morphology of the Cavernous Nerve

Tissue was harvested under general anesthesia (35 mg/kg of sodium pentobarbital) and aseptic conditions. As previously described (Schaumburg et al., 2007), animals were placed in the supine position, and the bladder, prostate and abdominal aorta exposed through a midline abdominal incision. ICP and status of induced erectile function were monitored in 5 of the hyperglycemic animals immediately prior to tissue harvest using procedures previously described (Christ et al., 2006; Rehman et al., 1997). The penis was denuded of skin, followed by removal of the ischiocavernosus muscle, to expose both crura (corpus cavernosa). Before dissection, the abdominal aorta was cannulated just below the renal arteries, and the lower body perfused with cold 4% paraformaldehyde, followed by 2.5% phosphate-buffered glutaraldehyde. The pelvic ramus was split to enable dissection of the MPG, the entire length of the cavernous (main penile) nerve, and the penile crus, shaft, and glans. Consistent with methods outlined in a previous publication (Schaumburg et al., 2007), cross sections were taken from 7 levels of the cavernous nerve and penis, immersed in cold 1% osmium tetroxide for 1–2 hours, dehydrated in graded alcohols, and embedded in epoxy resin. For light microscopy, one-micron epoxy sections were stained with toluidine blue. For electron microscopy, thin sections were cut, contrasted with lead citrate and uranyl acetate, carbon-coated and examined in a Hitachi H-600 electron microscope (Ibaraki, Japan).

RESULTS

General Status

Weight loss was evident by 4 weeks after confirmed HG. Consistent with the known effects of induced HG, the STZ-treated rats displayed polydipsia, polyuria and relatively poor grooming. Intracavernous pressure measurements documented partial erectile failure at 1 month, which became total at 6 months. At autopsy, the prostate glands of rats with HG for periods of 6 months or greater were smaller than those of control animals.

Cavernous Nerve Structure

The proximal third of the CN contained a significant number of myelinated and an abundant number of unmyelinated axons, however the myelinated fibers diminished substantially as the nerve descended and entered the crus (Schaumburg et al., 2007). At the approximate level of our stimulating cathode, the nerve branched into 4 to 6 fascicles composed almost exclusively of small unmyelinated axons (Schaumburg et al., 2007). Distal to our stimulation site, the fascicles further divided into small bundles of 3 to 18 axons and were scattered widely among the abundant collagenous septae and smooth muscle fibers.

Cavernous Nerve Electrophysiology

The pattern of neural activity in the CN varied as a function of stimulus intensity. Short duration and low intensity (0.5 mA) stimulation activated only relatively low threshold fibers and evoked a simple, small amplitude response dominated by velocities in the 12–18 m/sec range (top trace, Fig. 1B). This response reflected the activity in myelinated afferents (A δ). The simplicity of the compound signal and its lack of growth with increased stimulation intensity were consistent with the limited number of myelinated axons in the CN (Schaumburg et al., 2007). An increase in stimulation intensity up to the mid-range (6.0 mA) failed to increase the amplitude of this initial response, but it did result in a complex multi-peak potential which included velocities ranging from 1.0–2.5 m/sec. At maximal stimulation intensity (up to 14 mA), no additional velocities were added, but the contribution of fibers conducting at less than 1.5 m/sec were substantially increased (Fig. 1B). High intensity stimulation activated the high threshold fibers and more than tripled the magnitude of the activity conducting at approximately 1.5 m/sec compared to mid-intensity stimulation.

The pattern of sNCV in the CN was similar for both the F344 and the SD strains. In both strains, the fastest conduction was between 12 and 18 m/sec, while activity consistent with sNCV was detected in the range of 0.5 and 3.0 m/sec, with approximately 80% of the signal representing velocities between 1–3 m/sec. Under conditions of maximized response, the pattern, amplitude and duration of the sNCV signal remained consistent over repeat assessments. Maximal NCV of the DNP was also similar in both strains: mean value for the proximal segment was 45.7 $\pm$ 6.1 m/sec for SD and 46.4 $\pm$ 4.2 m/sec for F344, the mean value for the distal segment was 33.3 $\pm$ 7.5 m/sec and 37.5 $\pm$ 3.5 m/sec, respectively.

Changes in sNCV associated with HG

Rats were placed into one of three groups based on the severity of their neuropathy, as determined by standard caudal nerve NCV: “control” group (N=3, mean NCV 42.6 m/sec), a “moderate” group (N=3, mean NCV 29.1 m/sec), or a “severe” group (N=4, mean NCV 11.3 m/sec). The mean duration of HG was 3 months (range of 2–4 months) in the moderate group and 6 months (range of 4–10 months) in the severe group. NCV in both the distal and proximal segments of the DNP were consistent with progression of neuropathy as determined by the caudal nerve (Table 1).

The magnitude, timing and pattern of sNCV in the CN substantially differed in rats as a function of the severity of hyperglycemia-induced neuropathy. Figure 2A illustrates the mean levels of MUA corresponding to the sNCV range (<2.5 m/sec) for each severity group. The signal in rats with moderate neuropathy was similar to that in age-matched controls, while in the rats with severe neuropathy, it was reduced by more than 90%. In addition to the magnitude, the pattern of the sNCV signal was also changed by progressive periods of HG (Fig. 2B). Figure 3A depicts representative MUA of sNCV corresponding to severity of hyperglycemia-induced neuropathy in four rats. At 2 months of HG, the timing of the MUA and the distribution of sNCV was similar to that observed in control rats. By 4 months, some STZ-treated rats showed only mild neuropathy characterized by a normal amplitude and a slight slowing of the sNCV

(third trace, Fig. 3A), while others displayed a profound neuropathy with minimal amplitude and almost no response faster than 1.0 m/sec (bottom trace, Fig. 3A). Even when only activity in the sNCV range is considered, there was a slowing of conduction driven by the progressive neuropathy (Fig. 3B).

HG also caused a progressive slowing of conduction in both the distal and proximal segments of the DNP (ANOVA $p < 0.01$), with mean velocity in the STZ-treated rats falling to levels as low as 17.3 m/sec. In the group identified as showing evidence of a severe neuropathy based on conduction velocities in the somatic caudal nerve (Table 1), the mean velocity in the DNP was also substantially slowed compared to both the control and moderate neuropathy group (Tukey *pos hoc* test $p < 0.01$). Deficits in NCV of the caudal nerve and DNP were strongly correlated ($r = 0.76$; $p < 0.05$).

Possible Electrophysiologic Artifact

The simultaneous stimulation of the CN and DNP raises the possibility that an artifact due to evoked activity in the striate muscle of pelvic floor could be mistaken as long latency neural responses or as sNCV (Steers et al., 1988). We were careful to ensure that the response labeled CN sNCV could be dissociated from activity driven by stimulation of the DNP. This control was accomplished by: 1) adjusting the position of the stimulating cathode to only stimulate the CN, 2) adjusting the intensity of stimulation to drive the low threshold DPN axons without evoking a sNCV response in CN, 3) adjusting the position of the recording electrodes over the prostate away from pelvic floor muscle, and 4) intentionally evoking the muscle artifact to document that the evoked signal differed in timing and amplitude from the recorded sNCV responses. Figure 4 (A, B) illustrates an EP and a MUA response in the hyperglycemic rat recorded over CN and evoked by stimulation at the base of the penis in the absence of a response in the DNP. Figure 4 (C,D) illustrates the intentional activation of the pelvic floor muscle artifact by stimulation of the shaft of the penis, which resulted in a MUA signal over the CN location that was substantially earlier and an order of magnitude larger than the true sNCV response.

Histopathological Changes in the Corpus Cavernosum

The frond/stalk architecture of smooth muscle bundles in the crus was well preserved and smooth muscle fibers at this level were intact in all diabetic animals (Fig. 5A). Desmosomal attachments between distal shaft smooth muscle fibers, rarely observed in control animals, appeared especially prominent in diabetic animals (Fig. 5B). In the distal shaft there was a striking increase in collagen bundles, numerous fibroblasts - some containing lipid droplets in the cytoplasm displacing the nuclei laterally (Fig. 5C), and cholesterol clefts in the walls of helical arteries (Fig. 5D). There were fewer smooth muscle fibers in the distal shaft of the >6 month diabetics, and some were undergoing necrosis (Fig. 6A); no nuclei displayed abnormal aggregation of chromatin or other evidence of apoptosis.

Histopathological Changes in the CN, MPG and DNP

Light and electron microscope examination of step serial longitudinal sections of the MPG and extra cavernous proximal segments of the CN in the STZ-treated rats did not differ from controls. Fascicles of unmyelinated fibers in the distal crus of diabetic animals, even after 10 months of HG, contained normal-appearing axons and Schwann cell nuclei. Scattered myelinated and unmyelinated axons in the proximal shaft of rats with > 6 months of HG contained increased numbers of glycogen granules and foci of Schwann cell sequestration of axonal debris. Occasionally, preterminal axons in the distal shafts of these rats displayed dystrophic changes scattered amongst the remaining synaptic vesicles (Fig. 6 B&C). These changes included increased numbers of glycogen granules, dense bodies, and degenerating mitochondria. Many adjacent preterminal axons were unremarkable, even in the 10-month

diabetic animal. Dystrophic changes were not detected in either unmyelinated or myelinated cavernous axons. Light microscope sections of the proximal, mid and most distal portions of the DNP were unremarkable.

DISCUSSION

When considering the development and treatment of hyperglycemia-induced neuropathy, many investigators have failed to distinguish between damage in large diameter myelinated fibers and deficits in the small diameter, largely unmyelinated population of axons. Large and small diameter axons differ in anatomy, spinal pathways, neurogenesis, energy utilization, ion distributions, and relationship to supporting glia cells, sensitivity to differing neurotrophic factors, the modalities that they convey and the signs and symptoms associated with their dysfunction. While it is widely held that transmission in unmyelinated axons is a key component of both normal and pathophysiologic states, the accurate measurement of the velocities associated with these neurons has remained elusive.

A cardinal conclusion of the present study was that it was possible to reliably record nerve conduction velocities of less than 2.5 m/sec in a rat model of HG and that this activity could be used to trace the onset and progression of DAN. The electrophysiologic procedures needed for the assessment of sNCV were a combination of appropriate stimulation, selective filtering, digital rectification and averaging, which are available in virtually all electrophysiologic laboratories. The procedures outlined in this report could be applied, with little or no modification, to both preclinical and clinical studies of a variety of small fiber neuropathies.

Previous studies of the electrophysiology of the CN were limited to the proximal segment of the nerve in normal (Purinton et al., 1976; Steers et al., 1988) or diabetic rats (McVary et al., 1997). The cytoarchitecture of these segments resembled that of the MPG (Dail et al., 1989; Schaumburg et al., 2007) and was dominated by autonomic and afferent fibers. To our knowledge, our study is the first to examine sNCV and pathophysiology in the distal segments of the CN, which is almost entirely composed of unmyelinated autonomic, primarily parasympathetic, axons critically involved in erectile function.

The fastest activity recorded over the distal CN (i.e., conduction velocities between 12–18 m/sec) most likely corresponds to the subset of myelinated afferents in this region (Purinton et al., 1973; Schaumburg et al., 2007). The low amplitude of this response and lack of an amplitude increase with escalation of the intensity of stimulation is consistent with the small number of these fibers in CN, especially at distal extremes (Schaumburg et al., 2007). A similar range of NCV (10–30 m/sec) was reported by Steers and coauthors (1988) for the early component recorded between pelvic nerve and CN across MPG and over the proximal segment of the CN (8–24 m/sec) by McVary and coauthors (1997). The slightly slower NCV reported in the present study may be due to the thinner diameter and shorter internodal distances of axons in the more distal nerve segments. The authors of the previous studies characterized this component as an activity of the sensory afferent fibers (A δ) passing through pelvic nerve and MPG to CN to innervate urethra, rectum, anus and genital organs. Targets of the afferent fibers recorded in the present study were limited by the position of stimulation to the genital organs and urethra. The range of sNCV recorded in the present study (0.75–3.2 m/sec) was also comparable to those recorded previously in the more proximal nerve segments (McVary et al., 1997; Purinton et al., 1976; Steers et al., 1988). However, in the more proximal recordings, the sNCV signal may have included distal preganglionic axons that pass through MPG and synapse on postganglionic cells in the proximal part of CN, which are absent in the present recordings of the distal segments of CN.

A key difference in the findings of the present versus previous physiologic studies of the CN was the documentation of change associated with HG. McVary and coauthors (1997) examined the compound distribution of conduction velocities, including activity in the proximal CN of BB/WOR rats after 8–9 months of hyperglycemia, and found no evidence of deficits, in spite of the fact that the rats had severely impaired copulatory behavior and half had complete ED. In contrast, our measures of sNCV in the distal CN showed a substantial reduction in response amplitude and a change in the distribution of velocities in the STZ-treated rats. The observed electrophysiologic deficits were first evident at the 2-month time point, with severity increasing as a function of the duration of HG. This pattern is consistent with the progression of small fiber neuropathy over a period of 2–10 months of HG and corresponds to the timing of erectile dysfunction in the diabetic rats as measured by electro-stimulation of CN and DNP (Italiano et al., 1993a; Rehman et al., 1997). If, as strongly suspected, the neuropathy in the CN is a dying-back axonopathy (Schaumburg et al., 1992), it is reasonable that the initial changes in both sNCV and morphology might be limited to the distal segments of the nerve.

The deficits in sNCV were associated with histopathological evidence of degeneration in the most distal, presynaptic terminals in the penile division of the CN. Abnormalities included, *inter alia*: dystrophic changes in nerve endings manifested by abnormal accumulations of glycogen granules in unmyelinated and preterminal axons in the proximal shaft, necrosis of scattered smooth muscle fibers, fatty infiltration, and deposition of cholesterol in the endothelium of small arteries. Proximal branches of the CN and neurons of the MPG appeared unremarkable. In contrast to previous reports of irreversible apoptotic processes in cells of MPG and loss up to 50% of nerve fibers assessed by the methods of immunohistochemistry (Cellek et al., 2003), we did not detect apoptotic changes or any sign of degeneration in either MPG neurons or fibers in proximal segments of CN, even after 10 months of HG. We also did not detect apoptotic changes in cavernosal smooth muscle fibers, however our sampling of smooth muscle was limited. The one previous ultrastructural study (Italiano et al., 1993b) of the CN in the rat examined only segments immediately adjacent to the MPG, and detected no substantial changes. This same study, and a second (Yagihashi and Sima, 1986), also examined the DNP in STZ-treated rats and reported axonal shrinkage and axonal accumulations of glycogen. Abnormally dilated nerve fibers and engorged endings of autonomic nerves were described in rat penile shaft after 5–8 months of HG (Morrison et al., 2007a,b), corresponding to the time scale of severe neuropathy in our recordings. The lack of change in morphology of MPG and proximal CN, and limited abnormalities in distal segments of CN presented in our findings and previous histological evaluations, suggest the prevalence of functional, but not structural, HG-induced alterations in CN.

The morphologic findings we report herein are concordant with the seminal studies of Schmidt that demonstrated changes in distal portions of unmyelinated sympathetic axons of STZ-treated diabetic rats, with sparing of the parent sympathetic neurons in the mesenteric ganglion. A similar distal distribution of autonomic axonal abnormalities and/or ganglionic axonal dystrophy has now been observed in other murine models of Type 1 diabetes including the BB/W rat, the NOD mouse, and the Chinese hamster (Schmidt et al., 1989; 2003; 2004). A human autopsy study (Schmidt et al., 1993; 1997) demonstrated distal preterminal axonal dystrophy in diabetic sympathetic ganglia. Taken in concert, these studies and our own report suggest that peripheral neuropathy in experimental models of Type 1 diabetes is a distal axonopathy similar to the process present in many metabolic and toxic neuropathies. Schmidt (2002) has suggested that abnormal synthesis, phosphorylation, and delivery of neurofilaments (Fernyhough and Schmidt, 2002), features of many toxic axonopathies, might be key elements in the pathophysiology of the axonal dystrophy in STZ-induced HG.

Although there was evidence of some histopathologic changes in the distal CN, the magnitude and distribution of the structural changes were minimal at the time points examined. As in

other metabolic distal axonopathies, deficits in the sNCV measure (e.g. response amplitude) far exceeded the observed pathology. This dissociation strongly suggests that HG-related deficits in physiology of the CN are substantially related to non-structural factors, such as transmembrane and intra-axonal distribution of ions, and may precede frank axonal dystrophy or other measures of structural pathology. The lack of histopathologic findings may also reflect the limitations of the STZ- model of diabetic peripheral neuropathy, which has been reported to show little frank structural pathology even at prolonged durations of HG (Sima and Sugimoto, 1999; Wright and Nukada, 1994). The effects of hyperglycemia on sNCV should be further explored in genetic models such as the BB/W rat (Sima and Sugimoto, 1999; Kamiya et al., 2004) and in models of type 2 diabetes (eg., Zucker Diabetic Fatty rats, Schmidt et al., 2003; Sima and Kamiya, 2006).

Our data also suggest clear differences in the timing and progression of somatic and autonomic neuropathy in the same rats. For instance, rat with the slowest caudal NCV after 2.5 months of HG (24.2 m/sec) had responses in CN and DNP indistinguishable from the controls. After 7–10 months of HG, caudal nerve response was almost completely absent (amplitude 0–5 μ V), while NCV in the DNP was delayed, but the response was still robust and well formed. This finding supports an earlier onset of somatic neuropathy and is consistent with the absence of changes in a number of myelinated fibers in DNP even after 6 months of HG (Italiano et al., 1993b).

The analysis of MUA proved a valuable technique to compensate for the temporal dispersion and poorly synchronized signal that characterize sNCV in peripheral nerve. This procedure has a substantial history in measuring the intralaminar flow of cortical activity in the CNS (Arezzo et al., 1986; Legatt et al., 1980; Steinschneider et al., 2008), and the key components of the MUA (i.e., rectification and integration of the raw neural signal) were used previously in microneurographic analysis of sympathetic activity in peroneal nerve of humans (Mano et al., 2006; Wallin, 2004). The application of MUA techniques is feasible in most electrophysiologic laboratories and this procedure could substantially enhance our sensitivity to the onset and progression of diabetic neuropathy in small diameter myelinated and unmyelinated axons.

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Figure 1. Conditions of the recording of cavernous nerve activity

A. Schematic diagram illustrates the sites of recording and stimulation for the cavernous nerve and DNP. MPG-major pelvic ganglia, R1-recording site for cavernous nerve activity, R2-recording site for proximal DNP, R3-recording site used for calculation NCV of the distal segment of DNP between R2 and R3, ** - indicates position of stimulation. **B.** Responses of cavernous nerve are presented as evoked potentials (EP) and multi-unit activity (MUA) at two intensities of stimulation. Open arrows point out initial response of small diameter myelinated fibers ($A\delta$). Vertical dash lines separate the ranges of NCV. Calibration is given in microvolts, and time scale in milliseconds.

Figure 2. Comparison of the changes in the cavernous nerve activity in the control group and in hyperglycemic rats with diverse severity of neuropathy (moderate and severe)

A. indicates total activity above the baseline averaged across animals characterized as control, moderate or severe neuropathy based on caudal nerve velocity (mean, SEM), **B.** represents distribution of cavernous nerve activity within various ranges of NCV as a percent of total MUA and averaged across animals for each of these groups.

Figure 3. Changes in the cavernous nerve response observed after different duration of HG
A. MUA responses of the cavernous nerve in control rat and after 2 and 4 months of HG, the last one - at the greater severity of neuropathy. **B.** Bar graphs on the right illustrate the alterations in responses evaluated by percent of total MUA in various ranges of NCV (marked at the bottom of each graph): disappearance of response in the range of 2–2.5 m/sec and continuous shift of the maximum in response toward the slower NCV. Vertical dash lines - borders of NCV ranges. Calibrations are given in mV; the time scale (in milliseconds) is adapted to fit NCV borders for the activity recorded over different distances.

Figure 4. Comparison of the response in cavernous nerve (A) and possible muscle-twitch related artifact (C) with simultaneous recording evoked activity at DNP (B and D respectively). Calibration is given in μV , and time scale in milliseconds.

Figure 5.

Electron microscopy showing: **A.** A stalk in the crus of a 10-month diabetic. The club-like ultraterminal contains a cluster of normal-appearing smooth muscle cells. (X 3,850). **B.** Desmosome-like attachment between two smooth muscle fibers. The outer intracellular attachment plaque and the intercellular electron-dense leaflet material within the cleft are visible in the insert. (X 25,000, insert X 125,000). **C.** Lipid droplets in fibroblasts with adjacent extracellular collagen bundles. (X 3,000). **D.** Cholesterol clefts in endothelial cells of a helical arteriole. (X 1,950).

Figure 6.

A. An electron micrograph of a degenerating smooth muscle cell (sm) with a dystrophic axon preterminal (arrow, in **C** at the higher magnification, X 62,000) containing dense bodies and degenerating mitochondria. (X 11,500). **B.** Dystrophic axon preterminal in a six-month diabetic rat containing a few synaptic vesicles, glycogen granules, and a membranous body. (X 56,000).

Table 1

Effects of HG on conduction velocity in somatic nerves.

Group	n	Caudal nerve NCV (m/sec)	DNP proximal NCV (m/sec)	DNP distal NCV (m/sec)
Control	3	42.6±6.3	46.4±4.2	37.5±3.5
Moderate	3	29.1±6.9	37.8±2.2	33.1±3.2
Severe	4	11.3±11.6	28.3±4.1	17.3±6.0

HG= hyperglycemia, NCV= nerve conduction velocity, DNP= dorsal nerve of penis, Values=mean±S.D. of n of rats.