Electrodiagnostic Testing and Histopathologic Changes Confirm Peripheral Nervous System Myelin Abnormalities in the Feline Model of Niemann-Pick Disease Type C

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Abstract
Niemann-Pick disease type C (NPC disease) is an incurable, neurodegenerative, autosomal recessive disease caused by mutations in either the NPC1 or the NPC2 gene. These mutations affect the intracellular trafficking of lipids and cholesterol, resulting in the intralysosomal accumulation of unesterified cholesterol and glycosphingolipids. These abnormalities are associated with clinical ataxia and impaired motor and intellectual development, and death frequently occurs in adolescence. The incidence of peripheral neuropathy in NPC patients is not known. We investigated peripheral nerves in the naturally occurring feline model of NPC disease, which has proven to be critical for understanding both disease pathogenesis and for evaluating experimental therapies.

INTRODUCTION
Niemann-Pick disease type C (NPC disease) is caused by mutations in either the NPC1 or the NPC2 gene that result in abnormal cellular lipid and cholesterol trafficking (1). The accumulation of unesterified cholesterol and glycosphingolipids in the endosomal/lysosomal compartments of cells in the CNS and in visceral organs is associated with progressive neurologic dysfunction, hepatosplenomegaly, and early death (1–3). Although the disease manifests in patients at varying ages and with varying deficits, affected individuals typically develop cerebellar ataxia, dysarthria, dysphagia, vertical supranuclear gaze palsy, seizures, and dementia and die in adolescence (1, 3, 4). Brain histology reveals widespread neuronal cytoplasmic vacuolization, neuroaxonal dystrophy, and neuronal loss most severely affecting Purkinje cells, cortical pyramidal neuron meganeurite formation, and ectopic dendritogenesis, gliosis, and inflammation (5–7). Although there is no effective cure, recent experimental work in murine and feline NPC models have shown that administration of cyclohextrins prevents or delays CNS dysfunction and greatly extends the life span (8, 9). These studies have resulted in a clinical trial in affected patients that is scheduled to begin in 2013.

The incidence of clinical or subclinical neuropathy in NPC patients is not known. Peripheral neuropathy has only rarely been described in children with NPC disease, and electrodiagnostic testing is not commonly performed (1, 4, 10, 11). One 3.5-year-old boy presented with CNS signs typical of NPC disease but also with diminished tendon reflexes and sensation (10). A mild demyelinating polyneuropathy was diagnosed after electrodiagnostic testing. In addition, a 3-year-old boy developed muscular atrophy and decreased tendon reflexes in the lower extremities; no electrodiagnostics were described (12). Finally, a 4-year-old girl was diagnosed as having electrophysiologic evidence of a demyelinating motor and sensory neuropathy that was confirmed by nerve biopsy (11).

The purpose of this study was to evaluate peripheral nerve electrodiagnostic findings and histopathology in a cohort of cats with NPC disease versus age-matched unaffected cats. Naturally occurring feline NPC disease is caused by a missense mutation in NPC1 (p.C955S; c.2864G>C), with clinical, neuropathologic, and biochemical abnormalities similar to those in juvenile-onset NPC disease patients, the most common form of the disease in humans (5, 6, 13–17). The feline model has been critical for identifying the late endosomal/lysosomal accumulation of gangliosides (GM2 and GM3) and unesterified cholesterol (7, 18), for evaluating the association of ganglioside storage with meganeurite formation and ectopic dendritogenesis.
correlating neuroaxonal dystrophy with neurologic dysfunction (6), and for evaluating the efficacy of experimental therapies (8, 18–21). Here we describe clinical, electrodiagnostic, and pathologic changes that demonstrate peripheral nerve involvement in the feline model of NPC disease.

**MATERIALS AND METHODS**

**Animals**

Cats were raised in the animal colony of the School of Veterinary Medicine, University of Pennsylvania, under National Institutes of Health and USDA guidelines for the care and use of animals in research. The experimental protocol was approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mature cats from the same line were bred to produce cats with autosomal recessive NPC disease. The cats were housed in 21°C with ad libitum food and water, 12-hour light cycles, with 12 to 15 air changes per hour. Peripheral blood leukocytes from all the cats were tested at 1 day of age for the NPC1 missense mutation using a PCR-based DNA test (16). Cats with no copies of the mutation were classified as control cats. Cats homozygous for the mutant allele were classified as affected. Physical and neurologic examinations were performed on a weekly basis.

**Electrodiagnostic Testing**

Nerve conduction velocity (NCV) and compound muscle action potential amplitude were measured in cats under anesthesia using intravenous propofol (up to 6 mg/kg) for induction, followed by endotracheal intubation and anesthetic maintenance with isoflurane. Data were obtained at 8 and 24 weeks of age using a Nicolet Viking Quest machine (Nicolet Biomedical, Madison, WI). Subdermal 12-mm, 27-gauge recording electrodes were placed in the interosseous muscle to determine motor NCV (MNCV) in the tibial, sciatic, and ulnar nerves of the right pelvic and thoracic limbs. The sciatic nerve was stimulated at the stifle and at the level of the femoral head, and the tibial nerve was stimulated at the tarsus and stifle. The ulnar nerve was stimulated at the carpus and the elbow. Monopolar 26-gauge stimulating electrodes were placed subcutaneously to stimulate each nerve. Stimulation duration was 100 microseconds, filter settings were 20 Hz and 2 kHz, gain was 2 mV/cm, and analysis time was 20 milliseconds. For sensory NCV (SNCV), recording electrodes were placed subcutaneously lateral to the radial nerve at the level of the elbow. Subcutaneous monopolar stimulating electrodes were placed in the skin over the dorsum of the paw. A 100-microsecond stimulus duration, band pass of 20 Hz to 2 kHz, gain of 2 μV/cm, and analysis times of 10 milliseconds were used. Motor NCV was determined by dividing the distance between the stimulating electrodes by the difference in onset latency between the 2 recorded evoked potentials. Sensory NCV was determined by dividing the distance between stimulating and recording electrodes by the latency to the first peak. Amplitude was measured from peak to trough of the evoked response. Electromyography was recorded using a 25-mm, 26-gauge concentric needle electrode inserted into the muscle. Amplifier filters were 5 Hz to 5 kHz; responses were recorded at a sensitivity of 100 μV/cm and a sweep speed of 10 milliseconds per centimeter. Niemann-Pick disease type C–affected and control cats (10 per group) were compared at 8 weeks of age. Six NPC disease–affected and 15 control cats were compared at 24 weeks of age.

**Postmortem Examination**

Cats were killed using an overdose of barbiturates in accordance with the American Veterinary Medical Association guidelines. All NPC disease cats were killed when they were no longer able to maintain sternal recumbency without assistance, which occurs at a mean age of 20.5 ± 4.8 weeks (17). Control cats were killed between 20 and 29 weeks of age for histologic comparison. Immediately before death, each cat was given 0.5 mL of heparin (1,000 units/mL) intravenously. After death, the cats were perfused with 500 mL of 0.9% cold saline, and samples of brain, liver, spleen, lung, and peripheral nerves (radial, ulnar, sciatic, tibial, and peroneal nerves) were collected for histologic and biochemical analyses.

**FIGURE 1.** Nerve conduction velocities (NCVs) of peripheral nerves. (A) In radial and sciatic nerves, at 8 weeks of age, there were significant differences in NCV between normal and Niemann-Pick disease type C–affected cats (*p < 0.05). (B) There were significant differences in the tibial, radial, and sciatic nerves at 24 weeks of age (*p < 0.05). Means and SD are presented for normal and affected cats in each age range.

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Light and Electron Microscopy

Nerve specimens as necropsy samples were collected immediately after death and saline perfusion. Specimens were either immersion fixed in 10% neutral buffered formalin or placed on a tongue depressor, wrapped in a saline-dampened gauze sponge, placed into a watertight container, and kept chilled during shipping. On receipt, fixed nerves were transferred to 2.5% glutaraldehyde, postfixed in 1% aqueous OsO₄, and processed to araldite resin blocks. One-micron-thick sections were cut and stained with toluidine blue for light microscopic examination. Thin sections (60–90 nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate for electron microscopic examination.

Teased fibers from the ulnar nerve were processed as above except that the araldite resin used for overnight infiltration lacked hardener. After infiltration, excess araldite resin

![Figure 2](http://jnen.oxfordjournals.org/)

**FIGURE 2.** Pathologic changes affecting myelin in peripheral nerve biopsies from Niemann-Pick disease type C (NPC)–affected versus unaffected cats. **(A–D)** Compared with a control ulnar nerve (**A**), there was variability in thickness of the myelin sheath in affected cats (**B, C**). Area enclosed in (**C**) is expanded in (**D**) and shows inappropriately thin myelin sheaths (white arrows) and Schwann cell cytoplasm containing myelin debris and vacuoles (black arrows). Scale bars = (**A–C**) 50 μm; (**D**) 25 μm.
was wiped off, and the nerve was subsequently teased in Epon 812 resin without hardener before coverslipping and examination by light microscopy.

**Morphometry**

For quantitative assessment, high-quality nerve specimens that were adequately fixed and free of artifacts were chosen. Using point counting techniques (22) and a grid with a magnified distance of 0.08 mm between intersection points, fascicular areas, defined as the number of points falling on the endoneurium of nerve fascicles, were determined. The total number of myelinated fibers, the number of fibers with inappropriately thin myelin sheaths relative to their axon diameter and those with myelin splitting and ballooning, and the number of probable regenerative clusters (defined as 2 or more closely apposed myelinated fibers) were assessed in each nerve specimen and normalized to fascicular area (23). Only those fibers with clear faintly staining space evident between separated myelin with an asymmetric profile are considered to have splitting and ballooning, whereas profiles containing paranodal regions or Schmidt-Lanterman clefts were not counted (24, 25). In addition, computer-assisted analyses of axonal size-frequency distributions of myelinated fibers, nerve fiber diameter, axonal diameter, myelin thickness, and G ratio (structural

![Electron micrographs of the ulnar nerve from a Niemann-Pick disease type C (NPC) affected cat confirmed the light microscopic findings. (A, B) There were myelin debris and vacuoles in Schwann cell cytoplasm with normal-appearing axons (asterisks) and Schwann cell nuclei (B). (C, D) There were inappropriately thin myelin sheaths (C) and large vacuoles containing lipid substrates (D). Scale bar in (D) = 0.87 μm for (A) and (B); 0.95 μm for (C) and (D).](http://jnen.oxfordjournals.org/DownloadedFrom/3.jpg)
index of axonal myelination defined as axonal diameter/fiber diameter based on measurements by perimeter) were determined using SPOT Advanced Imaging software and modifications of Adobe Photoshop (26).

Statistical Analysis

Differences between nerve conduction velocity and morphometric parameters of affected and control cats were tested using 2-tailed unpaired t-tests after determining that variances were not significantly different. When variances were unequal, an unpaired t-test with Welch correction was used.

RESULTS

Clinical Examinations

All NPC cats developed progressive intention tremors and cerebellar ataxia, eventually resulting in their inability to walk; however, none developed appendicular muscle flaccidity, hyporeflexia, or evidence of diminished sensation. All affected cats were killed when they were no longer able to maintain sternal recumbency without support at a mean age of 22.4 ± 3.7 weeks. Unaffected cats developed no neurologic deficits and were killed to serve as age-matched controls.

Peripheral Nerve Electrodiagnostic Testing

Significantly diminished sciatic MNCV was found in NPC disease cats at both 8 and 24 weeks of age. Tibial MNCV was significantly slower in affected cats at 24 weeks of age (Fig. 1). Radial SNCV was also significantly slower in affected cats compared with that in control cats at both 8 and 24 weeks of age. In control cats, the tibial, ulnar, and radial nerves showed increasing NCV over time (8–24 weeks) and reached statistical significance (p < 0.05). Individual nerves in affected cats at both ages all showed increasing NCV over time, but only the radial and sciatic nerves reached statistical significance (p < 0.05). No significant differences in amplitudes of motor or sensory evoked potentials were found between affected and control cats at either age (data not shown). No electromyographic abnormalities were identified in any cats evaluated.

Light and Electron Microscopy

At the light microscopic level, similar pathologic changes were identified in all nerves of affected cats; representative changes are illustrated for the ulnar nerve (Fig. 2). Compared with control ulnar nerve (Fig. 2A), the density of myelinated fibers was subjectively appropriate, but in NPC-affected nerves, the thickness of myelin sheaths varied considerably among nerve fibers, with many showing inappropriately thin myelin sheaths (Fig. 2B–D). Scattered nerve fibers also contained membranous debris and lipid substrates (Fig. 2D). Axonal degeneration was not found. Electron microscopic changes were consistent with those observed at the light level (Fig. 3). Demyelinated nerve fibers were evident with membranous debris, myelin figures, and vacuoles containing lipid substrates within the Schwann cell cytoplasm (Fig. 3A, B). Schwann cell nuclei and axons appeared normal. Specifically, axons showed no evidence of neurofilament abnormalities and no axoplasmic dense bodies. We also performed a survey of high-quality plastic thick sections of all nerves for evidence of darkly staining axoplasm, which was not identified. Nerve fibers with inappropriately thin myelin sheaths (Fig. 3C) and fibers with appropriate myelin sheaths but containing large vacuoles and myelin debris in the Schwann cell cytoplasm (Fig. 3D) were observed. Variability in thickness of the myelin sheath and expanded paranodal areas containing lipid substrates and debris were also noted in teased fibers from the ulnar nerve of affected cats compared with those in unaffected controls (Fig. 4).

Morphometry

Axonal size-frequency analysis was performed on the ulnar nerves of cats affected with NPC disease and compared with those of unaffected cats (Fig. 5). There was a shift in axon size-frequency distribution from large myelinated fibers more than 5 μm to small myelinated fibers 5 μm or less in NPC disease–affected compared with controls. A statistically significant decrease in fiber diameter (p = 0.03), axonal diameter (p = 0.04), and myelin thickness (p = 0.02) was also noted, with a nonsignificant trend toward an increased myelinated fiber density (Table).

DISCUSSION

The brains of NPC disease patients show widespread neuronal and glial cytoplasmic vacuolization, neuronal loss most severely affecting Purkinje cells, neuroaxonal dystrophy, cerebrocortical meganeurite formation, ectopic dendritogenesis, and gliosis (1, 3, 7, 27). Cerebral white matter is generally normal, although demyelination with perivascular accumulation of macrophages has been reported (28). The histologic abnormalities contribute to the phenotype of ataxia, impaired mental development, seizures, dysphagia, dysarthria, and dementia. However, unlike the CNS, the peripheral nervous system shows more subtle clinical and morphologic changes. Diminished tendon reflexes and superficial sensation have been described in a small number of patients, and a demyelinating motor and sensory polynuropathy has been identified through electrodiagnostic testing and nerve biopsy (10–12). Although

FIGURE 4. Teased fiber preparations from the ulnar nerve of a control cat (A) and a Niemann-Pick disease type C (NPC) disease–affected cat (B, C) showed variability in nerve fiber thickness (B) and paranodal swellings containing myelin debris (C). Scale bar = 50 μm.

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Demyelinating polyneuropathy in cats with Niemann-Pick type C disease

FIGURE 5. Histogram showing the axonal size-frequency distribution in the ulnar nerves of Niemann-Pick disease type C (NPC)-affected cats compared with those in unaffected cats. There was a significant shift to smaller caliber fibers ($p = 0.03$) in NPC-affected versus unaffected cats.

The incidence of clinical polyneuropathy in NPC disease seems low (4), the incidence of subclinical neuropathy is not known.

The feline NPC disease model has clinical, neuropathologic, and biochemical CNS abnormalities similar to those in juvenile-onset patients, including Purkinje cell death, ectopic dendritogenesis, neuroaxonal dystrophy, and central myelin deficits (7, 15, 17). No signs of peripheral nervous system involvement are present on clinical examination, but electrodagnostic studies show clear evidence of slow nerve conduction velocity in both motor and sensory nerves in the absence of changes in evoked potential amplitude and in the absence of electromyographic abnormalities. These changes are consistent with a demyelinating polyneuropathy that was confirmed by histopathologic analysis. Our findings in the feline model are similar to those described in the mouse model (29). The lipid inclusions within Schwann cells evident by light microscopy, the many myelin figures observed by electron microscopy, and the paranodal swellings corresponding to sites of inclusion accumulations observed with teased nerve fibers are similar among the species. There was no evidence of segmental demyelination or axonal degeneration. Furthermore, histograms showed a reduced percentage of large myelinated fibers in the NPC cats compared with those of unaffected cats and an increased population of small fibers (Fig. 5). Morphometric analysis demonstrated statistically significant differences in cluster density, fiber diameter, axonal diameter, and myelin thickness between normal and NPC-affected cats (Table). The increased population of small-caliber nerve fibers may reflect the statistically significant increase in cluster density in addition to the decreased myelin thickness. Similarly, a reduced percentage of large myelinated fibers with an increased population of small-caliber fibers was also noted in the mouse model (29). However, unlike the mouse model, distended axons containing peripherally located massive accumulations of small membrane-bound electron-dense bodies were not observed in the cat model. Indeed, decreased axonal diameters were found in NPC cats. Such electron-dense bodies in the mouse model are identical to those found in dystrophic axons in the CNS (29). Although axonal spheroids packed with electron-dense material have been described in the CNS of affected cats (13), they are absent in peripheral nerves. In 1 human patient examined, electron-dense bodies were identified within some peripheral nervous system axonal spheroids but also not to the extent described in the murine model (11). The absence of axonal electron dense bodies in peripheral nerves of the NPC cat may reflect a species variation or, alternatively, an age-related change that could develop if the cats were allowed to reach a more advanced age; however, the latter is less likely because affected mice were examined at 60 days of age.

The cause of the demyelinating polyneuropathy associated with NPC disease is not well understood. The light and electron microscopic findings in the NPC mouse were postulated to indicate defective myelin turnover, and a defect in the utilization of cholesterol was proposed as the cause (29). In

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**TABLE.** Morphometric Analysis of the Ulnar Nerve in Cats Affected With Niemann-Pick Disease Compared With That in Control Cats

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 6)</th>
<th>Affected (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF density</td>
<td>10,826 ± 1,140 (8,721–11,980)</td>
<td>13,007 ± 4,876 (7,211–22,200)</td>
<td>Ns</td>
</tr>
<tr>
<td>Split MF density</td>
<td>40 ± 31 (5–80)</td>
<td>78 ± 61 (13–79)</td>
<td>Ns</td>
</tr>
<tr>
<td>% Split MF</td>
<td>0.4 ± 0.3 (0.0–0.8)</td>
<td>0.6 ± 0.4 (0.1–1.2)</td>
<td>Ns</td>
</tr>
<tr>
<td>Thin MF density</td>
<td>871 ± 291 (297–1,360)</td>
<td>1,162 ± 721 (467–2,564)</td>
<td>Ns</td>
</tr>
<tr>
<td>% Thin MF</td>
<td>7.8 ± 3.1 (3.4–11.7)</td>
<td>8.4 ± 2.6 (6.2–12.4)</td>
<td>Ns</td>
</tr>
<tr>
<td>Cluster density</td>
<td>1 ± 1 (0–2)</td>
<td>27 ± 27 (0–70)</td>
<td>0.03</td>
</tr>
<tr>
<td>No. myelinated sprouts/cluster</td>
<td>1 ± 1 (0–3)</td>
<td>2 ± 1 (0–3)</td>
<td>Ns</td>
</tr>
<tr>
<td>Fiber diameter, μm</td>
<td>8.82 ± 1.53 (7.27–11.73)</td>
<td>6.91 ± 0.82 (6.20–8.76)</td>
<td>0.03</td>
</tr>
<tr>
<td>Axonal diameter, μm</td>
<td>5.40 ± 0.86 (4.34–6.90)</td>
<td>4.38 ± 0.60 (3.08–5.58)</td>
<td>0.04</td>
</tr>
<tr>
<td>G ratio</td>
<td>0.61 ± 0.02 (0.59–0.65)</td>
<td>0.63 ± 0.03 (0.59–0.68)</td>
<td>Ns</td>
</tr>
<tr>
<td>Myelin thickness, μm</td>
<td>1.73 ± 0.35 (1.42–2.42)</td>
<td>1.26 ± 0.17 (1.04–1.59)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (range) and were analyzed by a 2-tailed unpaired t-test. When variances were unequal, a t-test with Welch correction was used.

MF, myelinated fiber; split MF, fibers with split myelin sheaths including ballooned myelin sheaths; thin MF, fibers with myelin sheaths that are thin in comparison with fibers with similar axonal diameters; G ratio, structural index of axonal myelination defined as axonal diameter/fiber diameter based on measurements by perimeter; ns, not significant.

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support of this hypothesis, trauma to the sciatic nerve in NPC mice resulted in delayed myelination compared with that in normal mice, and reutilization of cholesterol, as assayed by labeling with radioactive acetate, was reduced (30). The cause for defective myelination in the CNS in NPC is also poorly understood. In the CNS, expression of myelin basic protein in oligodendrocytes is decreased in the NPC1 mouse model compared with that in normal mice (31, 32). No increase in oligodendrocyte apoptosis and no defect in differentiation of oligodendrocyte progenitor cells into premyelinating oligodendrocytes was seen in affected mice. The mechanisms of the defect in myelination was proposed to be associated with an observed increase in the polysialylated-neuronal cell adhesion molecule, a negative regulator of myelination, and/or a defect in differentiation of premyelinating oligodendrocytes into myelinating oligodendrocytes (32). The latter hypothesis is supported by the findings of increased numbers of premyelinating oligodendrocytes in the cerebral cortex of NPC mice and a decrease in myelin gene regulatory factor, a transcription factor that promotes formation of myelin proteins and is necessary for the formation of myelinating oligodendrocytes (33).

In conclusion, we provide evidence for a subclinical motor and sensory polyneuropathy in the feline NPC disease model that is similar to what has been described in some human patients and in the murine model. Because experimental therapies are evaluated for NPC disease, it will be necessary to identify additional surrogates markers of disease progression and severity that can be quantified and evaluated over time to provide evidence of therapeutic effect. It is also necessary to identify subclinical disease, which may manifest with new clinical signs when the more dominant CNS dysfunction is ameliorated.

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