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Mitochondrial dysfunction in distal axons contribute to HIV sensory neuropathy

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Abstract

Objective—Accumulation of mitochondrial DNA (mtDNA) damage has been associated with aging and abnormal oxidative metabolism. We hypothesized that in human immunodeficiency virus associated sensory neuropathy (HIV-SN), damaged mtDNA accumulates in distal nerve segments and that a spatial pattern of mitochondrial dysfunction contribute to the distal degeneration of sensory nerve fibers.

Methods—We measured levels of common deletion mutations in mtDNA and expression levels of mitochondrial respiratory chain complexes of matched proximal and distal nerve specimens from patients with and without HIV-SN. In mitochondria isolated from peripheral nerves of simian immunodeficiency virus (SIV) infected macaques, a model of HIV-SN, we measured mitochondrial function and generation of reactive oxygen species.

Results—We identified increased levels of mtDNA common deletion mutation in post-mortem sural nerves of patients with HIV-SN as compared to uninfected patients or HIV patients without sensory neuropathy. Furthermore, we found that common deletion mutation in mtDNA was more prevalent in distal sural nerves compared to dorsal root ganglia. In a primate model of HIV-SN, freshly isolated mitochondria from sural nerves of macaques infected with a neurovirulent strain of SIV showed impaired mitochondrial function compared to mitochondria from proximal nerve segments.

Interpretation—Our findings suggest that mtDNA damage accumulates in distal mitochondria of long axons, especially in patients with HIV-SN, and that this may lead to reduced mitochondrial function in distal nerves relative to proximal segments. Although our findings are based on HIV-SN, if confirmed in other neuropathies, these observations could explain the length-dependent nature of most axonal peripheral neuropathies.

Keywords

HIV; peripheral neuropathy; mitochondria

Human immunodeficiency virus (HIV)-associated sensory neuropathy (HIV-SN) is one of the most frequent complications of HIV infection with prevalence rates between 10–35% in HIV infected individuals^{1–3}. Risk factors for the development of HIV-SN are typically those that are associated with advanced disease course and include age, previous use of certain dideoxynucleoside agents, prior abuse of opiates, low CD4 cell nadir, and increased plasma HIV RNA levels^{4–6}. HIV-SN can be further distinguished into a subtype associated with HIV infection per se and an antiretroviral toxic neuropathy associated with the use of antiretroviral agents. These two forms are clinically indistinguishable and dominated by neuropathic symptoms such as hyperalgesia and allodynia accompanied by mild to severe sensory deficits^{1, 3}. Like many other forms of polyneuropathies, symptoms in HIV-SN develop in a strict length dependent fashion starting in the feet progressing proximally⁷. Pathologically, HIV-SN is characterized by distal axonal degeneration of small myelinated and unmyelinated nerve fibers⁸. Development of HIV-SN is predicted by a common European mitochondrial haplogroup⁹. In this study, relationship between mitochondrial haplogroup T and HIV-SN was strongest among subjects randomized to dideoxynucleoside agents and this relationship was independent of age, protease inhibitor use, baseline CD4 or viral load.

In most eukaryotic cells, including neurons, mitochondria are essential in managing oxidative stress and providing energy by generating adenosine triphosphate (ATP) through oxidative phosphorylation. In neurons, ATP is required for axonal transport and maintenance of ionic gradients for generation of action potentials and synaptic activity¹⁰. Mitochondria have their own genetic material (mtDNA) and are assembled in the neuronal cell body and subsequently transported down the axon. Reactive oxygen species (ROS), which are generated at low levels during normal function of mitochondrial respiratory chain, can cause mtDNA mutations. Accumulation of mtDNA mutations in turn results in further increases in ROS and subsequently in additional increase in respiration-inactivating mtDNA mutations. This vicious cycle is believed to be a critical factor for the exponential increase in oxidative damage and subsequent loss of cellular functions during aging and in diseases associated with mitochondrial dysfunction^{11–15}.

Based on the fact that axonal mitochondria are assembled in neuronal cell body and transported down the length of axons, we hypothesized that mitochondria in distal axons are more likely to have mtDNA mutations and functional impairment and that this may play a role in pathogenesis of HIV-SN. Thus, we measured levels of mtDNA mutations in human nerve samples and explored the functional characteristics of freshly isolated neuronal mitochondria in simian immunodeficiency virus (SIV) infected macaques, an animal model of HIV-SN.

Materials and Methods

Patient samples

Matched frozen tissue from proximal (dorsal root ganglia / sciatic nerve) and distal (sural nerves) peripheral nerves from 13 patients with HIV infection without HIV-SN, 11 patients with HIV-SN, and 11 HIV negative controls were used for this study. De-identified samples were obtained from the National NeuroAIDS Tissue Consortium (NNTC), supplied by 3 centers in the U.S (funded by N01MH32002). Only samples collected within 24 hours *post mortem* were included in the study. Neuropathy was diagnosed according to established

clinical and pathological criteria. The clinical criteria used for the diagnosis of neuropathy included two out of three examination findings of reduced pinprick and vibration sensation distally in the feet and reduced ankle deep tendon reflexes. Pathological evaluations were done by a board-certified pathologist and diagnosis of neuropathy was given to patients with reduced axonal density in their sural nerves at the ankle level. Antemortem clinical evaluations were done within 6 months of death and pathological diagnosis of neuropathy was carried out in all of the autopsied sural nerve samples. Patients with other potential causes of neuropathy such as chronic alcohol use or diabetes were excluded from the study. CD4 T-cell count and plasma virus load were available for all cases. The median age of the patients was 45.5 years (range 37–78 years) and did not differ among the 3 groups. Table 1 lists details of the clinical information on patients.

PCR detection of the common mtDNA⁴⁹⁷⁷ deletion mutation

Standard quantitative real-time-PCR technique was used to determine the relative levels of mtDNA⁴⁹⁷⁷ deletion mutation as described previously¹⁴. DNA was isolated from fresh-frozen lumbar DRG and distal sural nerves using a proteinase-K digestion and DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in a total volume of 100µl, of which five was used for subsequent PCR. The break point of the 4977 base pair (bp) common deletion was amplified as a 350bp fragment with the primers CCCCTCTAGAGCCCACTGTA (forward) and GAGTGCTATAGGCGCTTGTC (reverse), while the reference region (hypervariable region 2, HVR2) was amplified as a 400bp fragment with the primers CTCTACCCTATTAACCACT (forward) GTTAAAAGTGCATACCGCCA (reverse) in a DNA Engine Opticon 2 (MJ Research) using the SYBR Green method (QuantiTect SYBR Green PCR Kit, Qiagen). The cycle parameters were 95C (denaturing) x 30 seconds, 56 C (annealing) x 45 seconds, 72 C (extension) x 1 minute, 40 cycles. The relative amount of the mtDNA⁴⁹⁷⁷ deletion mutation product was normalized to the internal control, HVR2, using the $2^{-\Delta\text{ct}}$ method. Each sample was measured in duplicate and data are shown as mean values $\pm$ SEM.

Distribution of Mitochondria

Distribution of mitochondria was determined in sural nerve biopsy specimens from eight patients and four SIV infected and two uninfected macaques. Human sural nerves were biopsied for diagnostic purposes; of the eight patients five had normal sural nerves on final diagnosis and three had mild axonal neuropathy. The biopsy specimens were fixed in 2.5% glutaraldehyde in Sorensen's buffer, post-fixed in 1% OsO₄ and embedded in epoxy resin. For electron microscopy, 70–80 nm sections were cut on to 200 mesh copper grids, double stained with uranyl acetate and lead citrate and examined with a Hitachi H600 electron microscope. Fiber cross sections were randomly selected and micrographs were taken at low magnification (x5000–12000). The number of mitochondria was determined for the four compartments (myelinated axons, unmyelinated axons, Schwann cells and other cells) in 20–30 fields per specimen. For statistical analysis, the number of mitochondria per compartment was averaged for each specimen.

Westernblotting

Frozen nerve samples were lysed in buffer (Tris 50mM, 0.1% SDS, 150mM NaCl, 0.5% Sodium Deoxycholate, 1% NP40) in the presence of protease inhibitors. Protein concentration was determined by BCA protein assay (Pierce, Rockford IL) and 10 µg of protein was subjected to electrophoresis on 15% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. After washing in 0.1% Tween-PBS, blots were blocked in 5% milk powder for 1 hour. The following antibodies were used: anti-COX-I (1:2000; Invitrogen, CA), anti-beta-tubulin (1:2000; Promega, WI), anti-mitochondrial protein (recognizes a 65kDa mitochondrial membrane protein, 1:500; Millipore, MA), and

anti-porine (Invitrogen, 1:1000). Membranes were incubated overnight at 4°C and horseradish-peroxidase conjugated anti-goat and anti-mouse antibodies (1:1000; Invitrogen, CA) were applied.

Animals and mitochondria isolation

Samples from five uninfected and seven juvenile macaques (*Macaca nemestrina* and *fascicularis*) that were infected with simian immunodeficiency virus (SIV) were used in this study. Two of the seven infected macaques were treated with a four-drug combination, referred to hereafter as HAART, beginning on day 12 postinfection and continuing until necropsy (range, days 161 to 175). These animals were administered 205 mg/kg body weight saquinavir, 10 mg/kg of L-870812, an integrase inhibitor, and 270 mg/kg atazanavir orally twice/day. In addition, 30 mg/kg of tenofovir was administered once daily intramuscularly¹⁶. Pathological analysis of SIV-infected macaques shows a predominantly small fiber neuropathy^{17, 18}. All experiments were carried out in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals and local institutional review committee regulations. SIV-infected macaques were inoculated intravenously simultaneously with the neurovirulent clone SIV/17E-Fr and the immunosuppressive swarm SIV/DeltaB670 as previously described^{19, 20}. After euthanasia with intravenously administered pentobarbital, sural and sciatic nerve samples were removed and isolated with MitoProfile® Benchtop Mitochondria Isolation Kit (Mitosciences, OR) according to the manufacturer's instructions. Briefly, tissue homogenates were washed, centrifuged at 1,000g for 10 minutes at 4°C and the supernatant was centrifuged again at 12,000 g for 15 minutes at 4°C. Protein concentration was determined by BCA protein assay (Pierce, Rockford IL) and mitochondria were immediately processed for functional assays. All mitochondrial experiments were run within 4 h following tissue harvest.

Mitochondrial Swelling Assay

Isolated mitochondria (~0.2 mg) were added to 0.2 ml of buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, 50 μM Ca²⁺ and 1 μM rotenone) and swelling was measured by decrease in absorbance at 540 nm using a 96-well plate reader (Molecular Devices, CA).

Mitochondrial H₂O₂ Production

Isolated Mitochondria (~0.2 mg) were added to 0.5 ml buffer containing 100 mM potassium phosphate (pH 8.0), 5 mM succinate, 25 μM luminol and 0.7 IU of horseradish peroxidase. Chemiluminescence was monitored continuously for 20 min and H₂O₂ production was determined by area under the curve.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was determined by use of TMRM fluorescence intensity (excitation/emission = 550/575 nm). Isolated mitochondria (0.1 mg) were added to 100 μl of buffer (70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, and 1 μM rotenone) containing TMRM (0.4–1 μM), and membrane potential was assessed by quenching of the fluorescent signal.

Protein oxidation assay

Oxidative modification of proteins by oxidation reactions results in generation of protein carbonyl derivatives. Carbonylated proteins were measured using Oxyblot™ oxidized protein detection kit (Oncor, Gaithersburg, MD). This kit, using western blot analysis, immunochemically detects carbonyl groups that have been derivatized with 2,4-

dinitrophenylhydrazine. Briefly, 10µg of protein from freshly isolated macaques' nerves (n=8) were derivatized with 2,4-dinitrophenylhydrazine, separated by electrophoresis on 15% SDS-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. After washing and blocking, membranes were developed with specific primary and secondary antibodies, according to the manufacturer's instructions. Negative controls included non-derivatized protein samples and omission of primary or secondary antibodies.

Protein nitrosylation assay

Excess oxidative reactions result in formation of peroxynitrite, which lead to stable nitrotyrosine products in proteins²¹. Nitrotyrosine levels in primate sciatic and sural nerves were measured using Bioxytech[®] nitrotyrosine enzyme immunoassay kit. Briefly, the fresh-frozen nerves (n=8) were homogenized with T-PER Tissue Protein Extraction Reagents (Thermo Scientific, Rockford, IL). Protein concentration was determined using Pierce BCA Protein Assay Kit (ThermoScientific, Rockford, IL). Nitrotyrosine level was measured using the BIOXYTECH Nitrotyrosine ELISA kit (OxisResearch, Portland, USA) according to the protocol provided by the manufacturer. Lysed tissue samples were centrifuged at 10,000g x 5min to pellet the tissue debris. 40 µg of total protein in 100 µl lysis buffer were used for each detection. All samples were done in triplicates.

Statistical analysis

For statistical analysis Kruskal-Wallis test with Dunn's post-test was used to compare group data. $P < 0.05$ was considered statistically significant.

Results

In sensory nerves most mitochondria are located within the axoplasm

It is assumed that mitochondria are not randomly distributed in tissue, but concentrated in cells and organs that typically have a high demand for ATP. Since it is unknown to what extent mitochondria are localized in axons and/or Schwann cells within sensory nerve fibers, we determined the distribution of mitochondria in cross-sections of human and primate sural nerve specimens by electron microscopy. Consistent with the idea that in peripheral nerve fibers, axons are the largest consumer of mitochondria-derived ATP, we found that in humans and primates, the vast majority of mitochondria (~84% in humans and ~93% in primates) were located within unmyelinated and myelinated axons, whereas Schwann cells and other cells harbored only a small percentage of all nerve fiber mitochondria (Fig. 1a-e). This distribution did not differ in normal and pathological human nerve biopsies. Likewise, in primates, there was no difference in the mitochondria per cell distribution in control and SIV infected animals.

The mtDNA⁴⁹⁷⁷ deletion mutation is higher in distal nerve segments in HIV-SN

Previous studies have shown that somatic mutations, such as the common mtDNA⁴⁹⁷⁷ deletion mutation are a marker of mitochondrial damage and accumulate in brain and other tissues with advanced age^{11, 12, 22–24}. Due to the fact that mitochondria are primarily assembled in the neuronal cell body and subsequently transported along the long axons we hypothesized that mitochondria in the distal sural nerves are considerably older than those in dorsal root ganglia and may harbor increased levels of the mtDNA⁴⁹⁷⁷ deletion mutation. Furthermore, either the HIV infection or the treatments used to treat patients may contribute to an increase in mtDNA⁴⁹⁷⁷ deletion mutation distally. As predicted, sural nerve specimens of patients with HIV-SN had significantly higher levels of the mtDNA⁴⁹⁷⁷ deletion mutation than age matched controls or HIV+ individuals without neuropathy. In contrast, lumbar DRGs of control, HIV+ and HIV-SN samples showed comparable levels of mtDNA⁴⁹⁷⁷

deletion (Fig. 2a,b). Patients were also grouped according to age, last CD4 cell count, CD4 nadir, and highest and last plasma viral load. From these characteristics a CD4 cell count <100cells/ μ l was associated with significantly elevated levels of the mtDNA⁴⁹⁷⁷ deletion mutation in sural nerve samples (Fig. 2c).

Sural nerve samples from patients with HIV-SN show decreased expression of mitochondrial proteins and catalytic subunit of complex IV (COX-I)

We next asked if the distal accumulation of the common deletion mutation in HIV-SN is also associated with reduced levels of mitochondrial-encoded subunits of the mitochondrial respiratory chain complexes. Samples from controls (n=6), HIV+ individuals without neuropathy (n=6) and those with HIV-SN (n=6) were immunoblotted and developed with an antibody against COX-I (Fig. 3). Afterwards the membranes were stripped and re-developed with antibodies against human mitochondria (MT, recognizes a 65kDA membrane bound mitochondrial glycoprotein) and beta-III-tubulin (β -III-tub). Densitometric analysis of the ratios between COX-I, MT and beta-III-tubulin COX-I showed a decrease in COX-I and in mitochondrial protein levels in HIV-SN in comparison to controls and neuropathy-free HIV + samples. Some membranes were developed with an antibody against porine, instead of MT and similar results were obtained (not shown). These data suggest that in HIV-SN, an increase in mitochondrial DNA damage in distal nerve segments is associated with defects in the mitochondrial respiratory chain complex and subsequent loss of (mostly) axonal mitochondria.

The same approach was used to determine the expression of mitochondrial proteins / subunits in peripheral nerve tissue of SIV infected macaques (n=7) and control animals (n=5). The SIV infected macaque model shares many clinical features of human HIV infection and replicate pathogenic mechanisms of HIV infection including small fiber neuropathy and damage to peripheral nervous system somatosensory ganglia^{17, 18, 20, 25}. As shown in Fig. 4, sural nerve samples from SIV infected macaques showed reduced expression of mitochondrial proteins and COX-I as compared to sural nerve samples from uninfected animals.

Mitochondria from sural nerves of SIV infected macaques show functional defects and increased reactive oxygen species production

Based on our observations that i) sural nerve specimens in HIV-SN showed increased mitochondrial DNA damage and loss of mitochondrial proteins and ii) that this loss of mitochondrial proteins could also be detected in SIV sural nerve samples, we further explored mitochondrial function of isolated mitochondria from peripheral nerve tissue of SIV infected macaques and uninfected controls in various functional assays.

A common mechanism by which oxidative stress mediates mitochondrial damage is the mitochondrial permeability transition (mPT). MPT is caused by a sudden, irreversible increase of membrane permeability by opening pores in the inner mitochondrial membrane that results in free diffusion of solutes and subsequent loss of the proton gradient²⁶. The morphological swelling of isolated mitochondria induced by mPT can be indirectly assessed by a decrease in light scattering in the presence of Ca^{2+} . As shown in Fig. 5, freshly isolated mitochondria from sural nerves of SIV infected macaques showed a rapid decrease in light scattering as compared to mitochondria from proximal (sciatic) nerve segments of the same animals or mitochondria from uninfected controls; this indicates a higher susceptibility to mPT. Of note, the decrease was most prominent in mitochondria isolated from animals that received HAART treatment, suggesting additive mitochondrial toxicity of antiretroviral drugs.

Likewise spontaneous generation of ROS, as determined by the mitochondrial production of H_2O_2 , was increased in freshly isolated mitochondria from sural nerves of SIV infected macaques in comparison to mitochondria from sciatic nerve segments or from uninfected control animals (Fig. 5c,d). The mitochondrial membrane potential, as measured by fluorescence of TMRM (tetramethyl rhodamine methyl ester), showed a progressive loss of mitochondrial potential in “sural” mitochondria from SIV infected macaques suggestive for mPT (Fig. 5e), whereas mitochondria from control animals maintained the membrane potential and could be depolarized by addition of FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone), a typical mitochondrial uncoupler. In contrast, mitochondria from SIV infected macaques could not be further depolarized by FCCP, indicating a relative loss of membrane potential. Measurement of mitochondrial H_2O_2 production and mitochondrial membrane potential was restricted to mitochondria of controls and SIV infected animals only, because mitochondria from HAART treated animals were not available in sufficient quantities.

Sural nerves from SIV infected macaques show increased oxidative modification of proteins

We hypothesized that the spatial accumulation of dysfunctional mitochondria in distal sural nerve segments of SIV infected macaques results in increased oxidative stress. To assess oxidative stress in peripheral nerve tissue we determined levels of protein oxidation in sural and sciatic nerve specimens of SIV infected and uninfected macaques. Protein samples from sural nerves were separated by SDS-Page and carbonyl groups from proteins that were modified by oxidation were detected after derivatization with 2,4-dinitrophenylhydrazine. As shown in Fig. 6, sural nerve samples (Fig 6a,c) but not sciatic nerve samples (Fig 6b,d) had increased carbonyl groups indicating increased protein modification by oxidative stress. In addition, sural nerve samples (Fig 6e) but not sciatic nerves (Fig 6f) had increased nitrotyrosine modification supporting the hypothesis that sural nerves of SIV animals had increased levels of oxidative stress.

Discussion

Our data demonstrate that mtDNA deletion mutations are higher in distal nerve segments in HIV-SN and are associated with impaired mitochondrial function and loss of mitochondrial respiratory chain complexes. Somatic mtDNA mutations, such as the common mtDNA⁴⁹⁷⁷ deletion, are considered markers of mtDNA damage in general that are known to accumulate in post-mitotic tissues during normal aging^{11, 12, 14}. Increased levels of mtDNA mutations can also be found in some brain regions of patients with Parkinson's²⁷ and Huntington's disease²⁸. These observations have led to the hypothesis that in neurodegenerative disorders, somatic mtDNA mutations accumulate with local variability that lead to a decrease in cellular energetic capacity and subsequent death of neurons in specific brain regions such as the substantia nigra. As an extension to this hypothesis, we studied spatial differences in mtDNA damage in proximal and distal parts of peripheral sensory nerves in HIV-SN and found that mtDNA deletion mutations are higher in distal nerve segments in patients affected in HIV-SN. Our analysis of the mitochondrial distribution showed that in cross-sections of human sural nerves around 84% of mitochondria are localized in axons rather than in Schwann cells, endothelial cells, fibroblasts or macrophages. Thus we conclude that increased levels of the common mtDNA⁴⁹⁷⁷ deletion are more likely to reflect changes in intra-axonal mitochondria rather than mitochondria in other cells (i.e. Schwann cells).

The human samples that we used for this study were from HAART-treated patients with end-stage HIV disease, thus HIV-SN in those cases is likely to be caused by both the use of antiretroviral agents and HIV infection. Examination of the specific role of antiretroviral

toxicity versus HIV infection would be desirable but is impossible to achieve due to the fact that DRG and sural nerve samples obtained at post-mortem from untreated late stage HIV patients are not readily available in the era of HAART. Nucleoside reverse transcriptase inhibitors (NRTIs), the backbone of HAART, are well known to cause mitochondrial injury and subsequent neuropathy 1· 29. Previous morphological studies on nerve biopsy specimens from patients treated with the dideoxycytidine (ddC) have noted ultrastructural abnormalities and loss of mitochondria in myelinated and unmyelinated axons of sural nerves 30. NRTIs inhibit mitochondrial DNA polymerase- γ 31, which is a key enzyme for replication and repair of the mitochondrial genome. Further, ddC, stavudine (d4T), and other NRTIs such as didanosine (ddI) are known to cause toxicity to primary neurons and neuronal cell lines in vitro by mitochondrial dysfunction 32· 33. Recently, Zhu and colleagues demonstrated that in an animal model of HIV-1, the feline immunodeficiency virus (FIV) infection, ddI induced axonal injury and decreased mitochondrial COX-I expression in cultured feline DRG neurons and in DRG of FIV infected animals 34. In accordance with these findings we observed loss of mitochondrial proteins and a decrease in expression of COX-I in sural nerves of patients affected with HIV-SN. Furthermore, we found that in macaques infected with SIV, mitochondrial-swelling assay was most abnormal in tenofovir-treated animals supporting the observation that NRTIs contribute to development of distal axonal degeneration by impairing mitochondrial function. Taken together, these data suggest that mitochondrial toxicity contributes to the pathogenesis of HIV-SN.

Clinical and pathological studies indicate that HIV-SN is a typical length-dependent “dying back” axonopathy, in which axonal degeneration starts in the distal end and continues in a centripetal manner. This pattern of involvement is not unique to HIV-SN and is common to most length-dependent peripheral neuropathies, including diabetic and most toxic neuropathies. This distal-to-proximal progression of axonal degeneration is responsible for what we clinically refer to as “stocking and glove” distribution of sensory and motor involvement. A long-standing hypothesis to explain length-dependency of most peripheral neuropathies has been the assumption that distal axons are akin to last field of an irrigation system getting the least “nutrients” from the cell body 35. What is unknown is the identity of “nutrients” from the cell body. Based on our observations we suggest that differences in health of mitochondria in proximal versus distal axons are responsible for the distal axonal degeneration seen in most peripheral neuropathies.

Can these observations also explain why most peripheral neuropathies, including HIV-SN, affect the unmyelinated small sensory fibers first? Although we don't have a direct proof, we can speculate that differences in mitochondrial numbers in relation to the energy needs of an axon in myelinated versus unmyelinated fibers may underlie this selective vulnerability. We hypothesized that unmyelinated axons have lower numbers of mitochondria per axonal volume and thus at a disadvantage to meet local energy demands compared to myelinated axons. To our surprise, when we calculated number of mitochondria per axonal volume in human and primate sural nerves, the unmyelinated axons had almost twice the number of mitochondria compared to myelinated axons. This relative high density of mitochondria in unmyelinated axons has been observed in the optic nerve between unmyelinated and myelinated segments of the retinal ganglion neuron axons 36. A review of the literature, however, suggests that despite a higher density of mitochondria, unmyelinated axons are at an “energy” disadvantage due to huge inefficiencies in the way they conduct action potentials 37–39. When there is a local energy deficit (i.e. ATP depletion), Na^+/K^+ ATPase failure can lead to reverse flow of calcium through the $\text{Na}^+-\text{Ca}^{2+}$ exchanger and result in axonal degeneration 40. This mechanism has been proposed to underlie axonal degeneration seen in demyelinating lesions in multiple sclerosis (reviewed by Waxman 41).

Compared to other cell types in the body, long peripheral nerves in humans provide a unique challenge to the biogenesis and maintenance of mitochondria. Although mitochondria are transported via fast axonal transport, movement of mitochondria is often bi-directional and the majority of mitochondria are stationary, often at nodes of Ranvier^{42–45}. This results in an “effective” rate of axonal transport of mitochondria closer to the rate of slow axonal transport. If we assume that mitochondrial biogenesis occurs only in the DRG neuronal body, then intra-axonal mitochondria in nerves in human feet are 2–3 years “older” than the mitochondria in proximal segments of the same axon. The evidence for this hypothesis is indirect at this point. Our data demonstrate higher levels of mtDNA mutations in distal axons but does not provide direct data that these are in fact “older” mitochondria compared to their counterparts in proximal axons. Furthermore, future studies may show that mitochondrial biogenesis may occur within the axon during transport of mitochondria. One hypothesis to explain our data in view of such findings may be that mtDNA replication and mitochondrial biogenesis maybe inefficient and prone to mistakes and thus result in higher levels of mtDNA mutations in distal axons. Nevertheless, as aging is associated with mtDNA mutations, we can assume that distal axonal mitochondria are more likely to be dysfunctional with reduced capability to handle oxidative stress or meet the energy demands of the distal axonal terminal. In support of this hypothesis, we can look at the observation that axonal regeneration is less efficient in taller people. In a model of human nerve regeneration after capsaicin-induced denervation of the intraepidermal nerve fibers, height was an independent risk factor predicting the rate of reinnervation⁴⁶. Of note, peripheral neuropathies, including HIV-SN, are more common among taller and older people^{47–50}. This epidemiological observation supports the hypothesis that long axons are more vulnerable to degeneration from a variety of causes, likely because mitochondria in distal axons in older people have increased mtDNA modifications that hamper their ability to handle oxidative stress and local energy demands.

By examining only the common deletion mutation, we probably underestimated the total mtDNA damage seen in distal nerves. There are likely to be other mutations or modifications in mtDNA that are outside the common deletion region and still result in mitochondrial dysfunction. Future studies aimed at evaluating mtDNA damage and mitochondrial heteroplasmy with more sophisticated techniques can extend these observations. If our findings are confirmed in other peripheral neuropathies, it would help establish the central hypothesis that dysfunctional mitochondria in distal axons is a common mechanism to explain length-dependency of peripheral neuropathies. These findings will also provide a new direction for development of therapies aimed at maintaining mitochondrial health and function as a way to prevent and treat peripheral neuropathies and, perhaps, other neurodegenerative diseases.

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Figure 1. In sensory nerves most mitochondria are located within the axoplasm

The distribution of mitochondria was determined in cross-sections of human and primate sural nerve specimens by EM. (a, b) Representative electron micrograph of myelinated (a, bar = 5 μ m) and unmyelinated (b, bar = 1 μ m) human sensory nerve fibers with mitochondria (arrows). (c) Electron micrograph of mitochondria in unmyelinated axons at high magnification (bar = 0.5 μ m). (d, e) Quantification of mitochondria in different cell compartments in normal (black bars) and pathologic (grey bars) human (d) and primate (e) sural nerve cross sections reveals that most mitochondria are located within the axoplasm.

Figure 2. The mtDNA⁴⁹⁷⁷ deletion mutation accumulates in distal nerve segments in patients with HIV-SN and is associated with CD4+ T cell count < 100/μl

The mtDNA⁴⁹⁷⁷ deletion mutation was measured in human autopsy samples from controls, patients with HIV and patients with HIV-SN and normalized to a fragment of the mitochondrial control (HVR2). **(a)** qRT-PCR analysis of mtDNA⁴⁹⁷⁷ deletion in sural nerve and dorsal root ganglia samples of controls, HIV patients and patients with HIV-SN. The mtDNA⁴⁹⁷⁷ deletion is detectable in most nerve samples and is amplified as 350pb product. In two samples, a second product was detected (*), indicating an additional mtDNA deletion. Those samples were excluded from further analysis. **(b)** Quantification of mtDNA⁴⁹⁷⁷ deletion mutation in DRG and sural nerve samples from controls (n = 11), HIV patients (13) and patients with HIV-SN (n=12). **(c)** Quantification of mtDNA⁴⁹⁷⁷ deletion mutation in sural nerve samples from HIV patients with CD4+ T cell count < 100/μl (n=21) and patients with CD4+ T cell count > 100/μl (n=24). All graphs show means ± SEM. (p<0.05, Kruskal-Wallis-Test with Dunn's post hoc test).

Figure 3. Sural nerves from patients with HIV-SN show reduced expression of mitochondrial proteins and COX-I

Sural nerve samples from controls (n=6), patients with HIV (n=6) and with HIV-SN (n=6) were immunoblotted and developed with an antibody against COX-I, human mitochondria (MT, recognizes a 65kDA membrane bound mitochondrial glycoprotein) and beta-III-tubulin (β -III-tub). **(a)** Representative westernblots of sural nerve samples from controls, patients with HIV and patients with HIV-SN. **(b)** Densitometric analysis of the ratios between MT and beta-III-tubulin (normalized to controls = 1) shows a decrease in mitochondrial protein levels in HIV and HIV-SN. **(c)** Ratios of COX-I and MT are reduced in HIV-SN indicating a relative decrease of COX-I in HIV-SN. **(d)** Ratios of COX-I and beta-III-tubulin are also reduced in HIV-SN. All graphs show means $\pm$ SEM. ($p < 0.05$, Kruskal-Wallis-Test with Dunn's post hoc test). (a.u. refers to arbitrary units based on density measurements and normalized to controls)

Figure 4. Sural nerves from SIV infected macaques show reduced expression of mitochondrial proteins and COX-I

Sural nerve samples from controls (n=8) and SIV infected macaques (n=5) were separated, blotted and developed with antibodies against COX-I, human mitochondria (MT) and beta-III-tubulin (β -III-tub). (a) Representative westernblots of sural nerve samples from controls, and SIV infected macaques. (b) Densitometric analysis of the ratios between MT and beta-III-tubulin (normalized to controls = 1) shows a decrease in mitochondrial protein levels in SIV infected macaques. (c) Ratios of COX-I and MT are also reduced in SIV indicating a relative decrease of COX-I in mitochondria. (d) Ratios of COX-I and beta-III-tubulin were not statistically different between SIV infected macaques and controls. All graphs show means $\pm$ SEM. ($p < 0.05$, Kruskal-Wallis-Test with Dunn's post hoc test).

Figure 5. Mitochondria from distal nerves of SIV infected and HAART treated macaques show increased susceptibility to mitochondrial permeability transition and show increased production of H₂O₂

(a,b) Swelling of isolated mitochondria from sural (a) and sciatic (b) nerve samples measured as a decrease in light scattering. Mitochondria from sural nerves of SIV infected (squares) and HAART treated (triangles) macaques show rapid swelling, compared to mitochondria from controls (circles) or from sciatic nerve samples. (c,d) Representative recordings of H₂O₂ production from sural and sciatic mitochondria of SIV infected macaques and controls. Mitochondria from sural (c) but not sciatic nerves (d) of SIV infected macaques (grey) show increased H₂O₂ production compared to controls (black). (e) Representative recording of membrane potential of isolated mitochondria from SIV infected macaques (dashed line) shows a steady decrease in membrane potential. FCCP causes immediate depolarization in control mitochondria, whereas mitochondria from SIV infected macaques cannot be further depolarized.

Figure 6. Sural nerves from SIV infected macaques show increased oxidative modification of proteins

Protein samples from sural and sciatic nerve specimens from SIV infected and uninfected macaques were separated by SDS-Page and carbonyl groups from proteins that were modified by oxidation were detected after derivatization with 2,4-dinitrophenylhydrazine. Sural nerve samples (a) but not sciatic nerve samples (b) show increased carbonyl groups as indicators for protein modification by oxidative stress. Lower panel = Blots were developed with antibody for GAPDH as loading control. (c,d) Densitometric analysis of modified proteins normalized to controls show an increase of proteins modified by oxidation in sural nerve samples of SIV infected macaques. Increased nitrotyrosine formation in proteins from excess peroxynitrate was measured using Bioxytech® nitrotyrosine enzyme immunoassay kit. Sural nerve samples (e) but not sciatic nerve samples (f) show increased nitrotyrosine levels (n=4 for each group) (means $\pm$ SEM, $p < 0.05$, Mann-Whitney-U-test).

Table 1

Patient data

	<u>Control</u>	<u>HIV</u>	<u>HIV-SN</u>
Number of patients	11	13	11
Age (years)	45 (38–78)	47 (36–55)	43 (34–52)
Ratio (male/female)	7/4	6/7	7/4
CD4 T cell count (cells/ μ l)	-	91 (2–333)	60 (7–259)
Virus load	-	91,700 (307–750,000)	47,631 (108–750,000)