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High dietary fat consumption impairs axonal mitochondrial function *in vivo*

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Abstract

Peripheral neuropathy (PN) is the most common complication of prediabetes and diabetes. PN causes severe morbidity for type 2 diabetes (T2D) and prediabetes patients, including limb pain followed by numbness resulting from peripheral nerve damage. PN in T2D and prediabetes is associated with dyslipidemia and elevated circulating lipids; however, the molecular mechanisms underlying PN development in prediabetes and T2D are unknown. Peripheral nerve sensory neurons rely on axonal mitochondria to provide energy for nerve impulse conduction under homeostatic conditions. Models of dyslipidemia *in vitro* demonstrate mitochondrial dysfunction in sensory neurons exposed to elevated levels of exogenous fatty acids. Herein, we evaluated the effect of dyslipidemia on mitochondrial function and dynamics in sensory axons of the saphenous nerve of a male high-fat diet (HFD)-fed murine model of prediabetes to identify mitochondrial alterations that correlate with PN pathogenesis *in vivo*. We found that the HFD decreased mitochondrial membrane potential (MMP) in axonal mitochondria and reduced the ability of sensory neurons to conduct at physiological frequencies. Unlike mitochondria in control axons, which dissipated their MMP in response to increased impulse frequency (from 1 to 50 Hz), HFD mitochondria dissipated less MMP in response to axonal energy demand, suggesting a lack of reserve capacity. The HFD also decreased sensory axonal Ca^{2+} levels and increased mitochondrial lengthening and expression of PGC1α, a master regulator of mitochondrial biogenesis. Together, these results suggest that mitochondrial dysfunction underlies an imbalance of axonal energy and Ca^{2+} levels and impairs impulse conduction within the saphenous nerve in prediabetic PN.

Significance Statement

Diabetes and prediabetes are leading causes of peripheral neuropathy (PN) worldwide. PN has no cure, but development in diabetes and prediabetes is associated with dyslipidemia, including

elevated levels of saturated fatty acids. Saturated fatty acids impair mitochondrial dynamics and function in cultured neurons, indicating a role for mitochondrial dysfunction in PN progression; however, the effect of elevated circulating fatty acids on the peripheral nervous system *in vivo* is unknown. In this study, Sajic et al. identify early pathogenic events in sensory nerve axons of mice with high-fat diet-induced PN, including alterations in mitochondrial function, axonal conduction, and intra-axonal calcium, that provide important insight into potential PN mechanisms associated with prediabetes and dyslipidemia *in vivo*.

Introduction

Diabetes and prediabetes incidence is rising at alarming rates. In 2019, ~463 million people globally had diabetes, with a projected increase to ~700 million by 2045 (Saeedi et al., 2019). Peripheral neuropathy (PN), the most prevalent prediabetic and diabetic complication, affects ~50% of type 2 diabetes (T2D) patients and 33% of prediabetic patients in the United States (Feldman et al., 2019). Characterized by nerve damage, PN manifests as pain or sensory loss starting in the feet and then the hands in a “stocking and glove” pattern. At end-stages, complete loss of sensation in peripheral tissues results in morbidity, including gait problems, foot ulcerations, and lower limb amputation. Early attempts to prevent PN in prediabetes and T2D patients focused on glycemic control alone, which was only marginally successful (Callaghan et al., 2012). As the increased prevalence of prediabetes and T2D is associated with obesity and high-fat “Western” diets, saturated fatty acids (SFAs) and dyslipidemia likely play important roles in PN progression (Eid et al., 2019). Indeed, recent clinical studies have correlated PN progression with the metabolic syndrome and dyslipidemia in both prediabetes and T2D, independent of glycemic control (Callaghan et al., 2016b; Callaghan et al., 2016a; Callaghan et al., 2018; Callaghan et al., 2020).

Although overwhelming preclinical and clinical evidence indicate that a SFA-rich diet correlates with PN development (Savelieff et al., 2020), molecular mechanisms remain poorly understood. Dorsal root ganglion (DRG) neurons, the primary sensory neurons affected by PN, have axons up to a meter long in humans and thus require long-distance axonal transport of functional mitochondria to satisfy distal ATP demands and maintain adequate Ca^{2+} buffering (Sajic, 2014). Elevated long-chain SFA levels impair mitochondrial metabolism (Schonfeld and Wojtczak, 2016), and we demonstrated that exogenous long-chain SFAs reduce mitochondrial membrane potential (MMP), bioenergetics capacity, ATP production, and motility of axonal mitochondria in cultured DRG neurons, while physiological glucose had no effect (Rumora et al.,

2018; Rumora et al., 2019a). Since PN is characterized by distal-to-proximal axonal degeneration, we hypothesized that SFA-induced impairment in mitochondrial function and transport *in vivo* are an important cause of PN progression.

Mitochondrial function has been investigated *in vitro* (Feldman et al., 2017), and in *in vivo* models where myelination affects mitochondrial function and dynamics (Zhang et al., 2010; Ohno et al., 2011; Sajic et al., 2013). *In vitro*, MMP and mitochondrial motility are constantly influenced by growth cues from neurite outgrowth in culture (Morris and Hollenbeck, 1993; Chada and Hollenbeck, 2004), but these cues are missing *in vivo* in healthy adult animals. Cultured or *ex vivo* preparations also lack a perfused vasculature to ensure physiological nutrient and oxygenation levels (Sajic, 2014). To overcome these constraints and validate our *in vitro* findings (Rumora et al., 2018; Rumora et al., 2019a), we examined mitochondria *in vivo* within the homeostatically-regulated environment of adult, electrically active saphenous nerve axons in a high-fat diet (HFD)-induced prediabetes mouse model. The HFD chow contains 45% lard, mimicking a “Western” SFA-rich diet (O'Brien et al., 2014). HFD-fed mice recapitulate salient features of prediabetes, including impaired glucose tolerance, obesity, and neuropathy, and they develop electrophysiological and histological characteristics of human PN, *e.g*., reduced sensory and motor nerve conduction velocities and decreased intraepidermal nerve fiber density (IENFD) (O’Brien et al., 2014), making it a useful model for evaluating PN mechanisms.

We report that mitochondrial transport in saphenous nerve axons of prediabetic HFD mice is intact *in vivo.* However, axonal mitochondria suffer from significantly reduced MMP, which does not dissipate in response to physiological increases in axonal energy demand in HFD mice versus control mice. These changes also associate with significant decreases in intra-axonal calcium levels ($[Ca^{2+}]i$) and lower expression of PGC1α, a master regulator mitochondrial biogenesis, in prediabetic versus control mice. These results suggest that partial MMP loss and

reduced [Ca^{2+}]i play important roles in regulating sensory neuron axonal impulse conduction in peripheral nerves of prediabetic HFD mice with PN.

Materials and methods

Animals

All experiments were carried out under the United Kingdom (UK) 1986 Animals (Scientific Procedures) Act, the UK 2006 Animal Welfare Act, and the European Directive 2010/63/EU. All experiments were approved by the institutional ethics committee and followed the ARRIVE guidelines. Mouse strains used in this study included positive and negative littermates of genetically modified Tn-XXL mice (Mank et al., 2008) and B6.CG-Tg(Thy1-CFP/COX8A)S2Lich/J mice (The Jackson Laboratories, catalog # 007967). All mice were housed in individually ventilated cages with a maximum of 5 mice per cage that were provided with bedding, nesting material, playing tunnels, and wood blocks. Mice had access to food, as specified in the experimental design, and water *ad libitum*, and were kept on a 12h light/12h dark cycle. At study termination, plasma was collected and submitted to the Cincinnati Mouse Metabolic Phenotyping Center for a complex lipid panel analysis to measure circulating phospholipids, cholesterol, triglycerides, and non-esterified fatty acids. Both male and female mice were also evaluated for sex differences related to food consumption, weight gain, hyperglycemia, glucose tolerance, and tactile hypersensitivity prior to assessment of *in vivo* mitochondrial events. Food consumption was evaluated by measuring the gram amount of food consumed per mouse per day for both male and female mice.

Fasting glucose

Blood glucose measurements were performed after overnight fasting, using a commercially available Accu-Check Mobile blood glucose monitor (Roche), from a single, minimally invasive tail puncture.

Intraperitoneal glucose tolerance test

The intraperitoneal (i.p.) glucose tolerance test (ipGTT) was performed according to the DiaComp protocol (https://www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=11). Briefly, after the fasting period, mice were weighed, and a baseline blood glucose measurement was obtained, as described above. An intraperitoneal injection of glucose (1 mg/g) (Sigma-Aldrich, UK) was administered, and further blood glucose measurement were acquired 15, 30, 60, and 120 min after the i.p. injection.

Tactile hypersensitivity

Tactile responses were evaluated as described before (Obrosova et al., 2007). In short, mice were transferred into a clear Plexiglas box with mesh floor, and allowed to acclimatize. Following the acclimatization period, a series of flexible von Frey filaments (NC12775-99, North Coast, USA) were applied to the surface of the hind paws with adequate force to bend the filament for 6 seconds. Paws were stimulated alternately and stimulations were repeated 6 times. Short paw withdrawal or paw flinching was classified as a positive response. Stimulation was stopped when > 50% of the 6 stimuli were positive, otherwise, the filament with the next greater force was applied. Tactile response thresholds were calculated with the formula $[(w_1 \times r_1) + (w_2 \times r_2) + (w_x \times r_x)/r_{total}]$, with w corresponding to filament weight and r corresponding to number of positive responses for that filament.

Experimental design

Mitochondrial dynamics experiments: Cyan fluorescent protein (CFP) transgenic mice (B6.Cg-Tg(Thy1-CFP/COX8A)S2Lich/J, The Jackson Laboratories, catalog # 007967) with constitutive expression in 40-60% of mitochondria where used for imaging mitochondria *in vivo*. Male littermates were randomly assigned to either a control chow diet (Lab Diet 5001 Cat#: 0001319, 13% kcal fat) or HFD (Research Diets Cat#: D12451, 45% kcal fat) according to the following regimens: (i) 4-week-old mice fed control or HFD for 16 weeks (4+16 group, aged 20 weeks at experiment termination), (ii) 4-week-old mice fed control or HFD for 36 weeks (4+36 group, aged 40 weeks at experiment termination), or (iii) 24-week-old mice fed control or HFD for 16 weeks (24+16, aged 40 weeks at experiment termination). Dietary composition, fatty acid makeup, and energy density of the control diet and HFD chows are detailed in Figure 1-1. Animals were weighed weekly and examined for signs of pain or discomfort.

Calcium imaging experiments: Tn-XXL transgenic mice, which express a calcium-binding troponin C-based biosensor with two fluorescent probes, citrine and CFP, were used for imaging calcium (Mank et al., 2008). Two experiments were performed. In the first, 9-week-old male littermates were randomly assigned to either a control diet or HFD for 5 weeks (9+5 group, aged 14 weeks at experiment termination). In the second, 24-week-old male littermates were randomly assigned to either a control diet or HFD for 16 weeks (24+16 group, aged 40 weeks at experiment termination).

Surgical procedure, *in vivo* imaging, and electrophysiology

Animals were terminally anesthetised (urethane; i.p., 1.25 g/kg) (Sigma-Aldrich), the leg was immobilised using a mold made from dental polymer (Examix NDS Monophase Purple, GC

America) (Misgeld et al., 2007). The left saphenous nerve was exposed at three locations, at the mid-thigh for confocal imaging, at the groin for the stimulating electrode, and at the ankle for the recording electrode. The left saphenous nerve exposed at mid-thigh was desheathed, as described previously (Sajic et al., 2013), and stained *in situ* with tetramethylrhodamine methyl ester [TMRM (Invitrogen/Thermo Fisher Scientific, UK); 0.5 µM in artificial cerebrospinal fluid (aCSF) (Harvard Apparatus, Holliston, MA, USA) for 30 min], and the surrounding structures with fluorescein isothiocyanate-conjugated *Griffonia* isolectin B4 (1:20 in sterile saline for 10 min) (Sigma-Aldrich), in addition to the constitutive CFP labeling. While the dyes incubated at the mid-thigh, the saphenous nerve was exposed in the groin to create a “well” with the exposed part of the nerve at its bottom. Teflon tape, approximately 2 cm × 0.5 cm, was placed under the nerve to electrically isolate it from the surrounding muscle. A pair of stimulating platinum wire electrodes was placed under the saphenous nerve, resting on the Teflon tape. The well was then filled with mineral oil to prevent tissue drying. Next, the saphenous nerve was exposed at the ankle to attach the active recording electrode, which was placed directly next to the exposed distal part of the saphenous nerve. A reference needle electrode was inserted into the fifth toe, and a ground electrode was placed in the abdominal muscle. At this stage, the animal was transferred to the confocal microscope (LSM Pascal 710, Zeiss, Germany). Fresh saline was applied to the exposed saphenous nerve at mid-thigh and covered with a glass coverslip sealed with petroleum jelly. Confocal time-lapse and single images were taken using either a Zeiss Apochromat 10x objective (NA 0.3; overview only) or a Zeiss Apochromat Plan 63x oil objective (NA 1.4). The pinhole size was set to 1 AU and time-lapse sequences were recorded at 1 frame/s for 100 s. Successive dual-colour CFP / TMRM (405 nm / 543 nm) images were taken to distinguish axonal mitochondria from mitochondria found in other cells. However, time-lapses were based on the TMRM signal alone because of its superior contrast and because repeated CFP imaging causes a prohibitive amount of bleaching. Time-lapse images of mitochondria were taken simultaneously with electrophysiological stimulation and recording. First, a single

supramaximal stimulus was delivered at frequencies of 1 Hz and 50 Hz. Sensory compound action potentials (sCAPs) were averaged (n = 30) and stored (Sigma 60, Nicolette Technologies). Next, to examine the refractory period of transmission (RPT), two successive supramaximal stimuli were delivered while sequentially increasing the delay between the first and second stimulus in 0.1 ms increments, up to 2.5 ms. sCAPs were averaged (n = 30) and stored (Sigma 60, Nicolette Technologies). The amplitudes were measured using Matlab.

In vivo calcium imaging

Tn-XXL mice were prepared for imaging as described above. However, no dyes were applied to the nerve and images were acquired using a Zeiss Apochromat Plan 63x oil objective (NA 1.4). First, axons of interest were identified by exciting the citrine fluorophore of Tn-XXL directly via a 514 nm laser. Next, two 512 x 512 pixel images were taken for every axon: (i) a 'ratiometric' two-channel image with an excitation of 405 nm and detection ranges of 460 nm – 510 nm (for CFP) and 520 nm – 570 nm (for citrine), and (ii) an 'anatomical' image with 514 nm excitation and a broad detection range of 515 nm – 650 nm. Laser settings were constant across all animals. The initial calibration was performed in naïve Tn-XXL animals, which was removed from the microscope stage after visible axons were imaged. Then ionomycin (100 µM in aCSF) was applied to the nerve for 10 min, a new coverslip was placed over the leg, and imaging was repeated.

Tissue collection

After sCAPs and/or images were acquired, mice were perfused transcardially with paraformaldehyde (PFA, 4% in 0.4 M phosphate buffer, pH 7.4) (VWR Chemicals, UK). Sciatic nerves and spinal cord lumbar segments (L3, L4, and L5) were dissected and post-fixed for 2 h

(PFA, 4% in 0.4 M phosphate buffer), whereas glabrous and hairy skin were post-fixed overnight. After post-fixation, tissues were transferred to 0.1% sodium azide (VWR Chemicals) in phosphate buffered saline (PBS). A block of hairy skin from the medial side of the foot dorsum, innervated by saphenous nerve, and a block of glabrous skin from the foot plantar innervated by the sciatic nerve were dissected. Skin blocks and spinal cord tissue were separately embedded into paraffin wax. Eight micron sections were cut using a microtome (Leitz 1512, Germany), transferred into a water bath (Medlite Tissue Flotation Bath, TFB- 45, Medizintechnik, Germany) set at a temperature of 40 °C, mounted onto slides (Superfrost, Thermo Scientific, UK), and left to air-dry overnight.

Immunohistochemistry

To assess PGC1α expression in DRGs or skin intraepidermal nerve fibre density, sections were incubated with anti-PGC1α (Abcam, ab54481, RRID:AB_881987) and anti-β-III-tubulin (Abcam, ab78078, RRID:AB_2256751) antibodies, respectively. First, the slides were deparaffinised by incubating in xylene (VWR Chemicals) for 5 min, xylene/100% ethanol 1:1 for 3 min, and then 3 min each in serial ethanol dilutions: 100%, 90%, 70%, 50% (VWR Chemicals). Slides were rinsed in distilled water for 3 min followed by cold acetone (VWR Chemicals) for 5 min. After deparaffinization, the slides were washed twice in PBS for 5 min. Antigen retrieval was performed by heating slides to boiling in 10% citrate solution (pH 6; Dako, UK) and then transferring to an oven at 40 °C for 40 minutes. The slides were then rinsed in distilled water, followed by two washes in PBS-T (PBS with 0.2% Triton X-100, Sigma-Aldrich). Slides were then blocked in 5% rabbit serum in PBS-T (Vector Laboratories Inc., UK) for 1 h in a humidified chamber at room temperature. The serum was removed with a Pasteur pipette, and the primary antibodies, polyclonal rabbit anti-PGC1α (1:600) and monoclonal mouse anti-neuronal β-III tubulin (1:1,000) diluted in 2% blocking solution were applied overnight at 4 °C. The next day,

the slides were washed twice in PBS and once in distilled water, each for 5 min. The goat anti-rabbit AlexaFluor 546 (Invitrogen Thermo Fisher Scientific, catalog # A11010, RRID: 2534077) or goat anti-mouse AlexaFluor 647 (Invitrogen Thermo Fisher Scientific, catalog # 32728, RRID: AB_2633277), were added to their respective primary for 1 h in the dark. Next, the slides were washed three times in PBS for 5 min and then rinsed with distilled water. The slides were air dried in the dark. Coverslips were mounted using 4′,6-diamidino-2-phenylindole (DAPI) fluoromount (Vectashield, Vector Laboratories, UK) and sealed using nail varnish. Additionally, in processing tissue for DRGs, a 70% methanol (VWR Chemicals) pre-treatment step was carried out after deparaffinization. Also, two rounds of incubation in 1% sodium borohydrite (Sigma-Merck) for 5 min each were carried out after antigen retrieval, followed by incubation in blocking solution with 20% rabbit serum. All sections were immunolabeled in the same session. For image acquisition, fluorescence emission was recorded through a 10×/0.3, Zeiss EC Plan Neofluar 10x/0.3 Ph2 or Plan-Apochromat 63×/1.4 Oil Ph3 (Zeiss) objective lens. All images were acquired with the following settings: beam-splitter HFT 488/543; filter BP 505–570, argon laser irradiation at 488 nm and filter LP 585 helium neon laser irradiation at 543 nm. First, tile scans were carried out using Zeiss EC Plan Neofluar 10x/0.3 Ph2 magnification using blue (DAPI, 405 nm) and red (AlexaFluor546, 565 nm) channels. For images of β-III-tubulin labeled hairy and glabrous skin sections, Z-stack images from five, randomly chosen, non-overlapping positions were acquired using Zeiss EC Plan Neofluar 40x/0.75 Ph2 (water) objective (Zeiss, Germany) and ZEN black acquisition software (Zeiss). If the image did not appear to be uniformly labeled across the field of view, the next random area was chosen for image acquisition. Each Z-stack contained ten images, each at 1 µm thickness. For images of PGC1α-labeled DRGs, Z-stacks were taken in the same way, while using the Zeiss Plan-Apochromat 65x/1.40 (oil) Ph3 objective. Care was taken not to saturate pixel intensity during image acquisition. Tissue dissection, embedding, sectioning, immunolabeling, and image acquisition and image analysis were performed in a blinded fashion.

Western blot analysis

VDAC1/2, MFN2, and PGC1α protein expression level was evaluated in the L1-L5 lumbar DRG or sural nerves from a separate cohort of mice fed either standard diet (Research Diets, D12450B) or 45% HFD (Research Diets, D12451) from 4-36 weeks of age. Western blotting was conducted per our previous study (Vincent et al., 2010). Briefly, L1-L5 DRG or sural nerve from each mouse were pulverized with a pestle, resuspended in 50 μl of RIPA lysis buffer (Sigma-Aldrich) with protease inhibitors (Roche), and evaluated for protein concentration. A total of 10 μg of protein/lane were separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Antibodies against PGC1α (Abcam, catalog # ab188102), MFN2 (Protein Tech, catalog # 12186-1-AP, RRID: AB_2266320), and VDAC1/2 (Protein Tech, catalog # 10866-1-AP, RRID: RRID:AB_2257153) were used to detect proteins. Membranes were then washed and probed for loading controls tubulin (Abcam, catalog # ab6160, RRID: AB_305328) and YWHAZ (ProteinTech, catalog #1 4881-1-AP, RRID: AB_2218248).

***In vivo* confocal image analyses**

Mitochondrial movement was analyzed as described previously (Sajic et al., 2013) using the *Difference Tracker* plugin for ImageJ Fiji (SciJava) (Andrews et al., 2010). Mitochondrial size was evaluated in single images from a minimum of 5 axons from 5 or more non-overlapping regions totaling at least 25 axon regions analyzed per mouse. Each single image showed 50-100 mitochondria, which were analyzed by the Analyse Particle and Measure plugins for ImageJ, using the blue (CFP) channel, as described previously (Sajic et al., 2013). For the analysis of mitochondrial membrane potential, TMRM intensity was measured separately for every mitochondrion relative to its local background. First, the foreground TMRM pixels were

identified from overlap with CFP pixels to identify axonal mitochondria. The median intensity of all foreground TMRM pixels of the particle was measured in ImageJ. Next, a local background selection was created containing all pixels within a 3-pixel radius around the mitochondrion of interest which were not part of, or immediately adjacent to, another mitochondrion, and the median intensity of these particles was calculated. The final intensity of a particle was defined as the median foreground intensity divided by the median background intensity. For analyzing $[Ca^{2+}]i$, image analysis was performed using Matlab (MathWorks). First, all images were filtered using a 3x3 pixel median filter to reduce noise. Individual axons were extracted by manually selecting a rough axon outline. Then, all pixels of interest were selected by thresholding the anatomical (citrine-only) image. For each pixel of interest, the ratio of citrine to CFP in the ratiometric image was calculated. For the images of immunolabeled skin and DRG sections, Z-stacks were collapsed using Zen Black imaging software, which were opened in ImageJ. For the β-III tubulin labeled images, dermis and epidermis in each image were carefully manually selected and the area measured using the *Measure* plugin for ImageJ. The images were then carefully thresholded to ensure that the resulting image faithfully represented the original image. The number of labeled pixels was determined using the *Measure* plugin for ImageJ and expressed per normalised area unit. For the analysis of PGC1α labeling in DRGs, and β-III tubulin in the skin, three cells in each field of view, with a minimum three fields of view per animal, were randomly chosen from the collapsed Z-stacks. The cell area was carefully outlined and measured using ImageJ. Images were carefully thresholded to ensure that the resulting image faithfully reproduced the original image, and areas of PGC1α and β-III tubulin labeling were measured again, and expressed as % of the cell or skin area, respectively.

Data analysis

Prior to all statistical analyses, the data were tested for normality of distribution using D'Agostino and Pearson normality test with Prism v5 (GraphPad Software). Differences between groups in weight, food consumption, ipGTT, hyperglycemia, tactile hypersensitivity, and all parameters of mitochondrial dynamics and size, and amplitudes of second response in RPT, were calculated using two-way ANOVA with Bonferroni's multiple comparison tests. Two-tailed Student's t-test was used for analyzing differences in terminal blood glucose, MMP, and loss of amplitude after 1 h conduction at 50 Hz in HFD versus control mice. Mann-Whitney test was applied to analyze differences in $[Ca^{2+}]i$ in HFD 9+5 mice and PGC1α and β-III tubulin coverage. Finally, Kruskal-Wallis test was used to analyze differences between groups in $[Ca^{2+}]i$ in mice fed HFD for 16 weeks starting from 24 weeks of age. An ordinary one-way ANOVA was used to evaluate complex lipid panel data. A two-tailed unpaired t-test was used to assess VDAC1/2, MFN2, and PGC1α protein expression level in DRG and sural nerves. Parametric data are presented as mean ± standard error of the mean, whereas non-parametric data, or data that did not follow a normal distribution, are presented as median ± interquartile range. A p-value < 0.05 was considered statistically significant.

Results

HFD mice exhibit weight gain and impaired glucose tolerance

We examined the effect of HFD on mitochondrial trafficking and function *in vivo* in three experimental groups of mice fed a HFD (45% calories from fat), compared to their respective controls. The first and second groups consisted of "young" mice fed a short- (16 weeks) or long-term (36 weeks) diet, starting at 4 weeks of age, *i.e.*, HFD 4+16 and HFD 4+36 groups, respectively (Figure 1, top). The third group consisted of "older" mice fed short-term (16 weeks) HFD starting from 24 weeks of age (HFD 24+16 group). Corresponding control littermates received control diet (13% calories from fat) for the same duration as their HFD counterparts.

HFD mice gained significantly more weight than their control counterparts (Figures 1A-1C). Furthermore, mice from the HFD 4+36 and 24+16 groups developed pronounced hyperglycemia compared to control mice (Figures 1B' and 1C'), whereas glucose tolerance was impaired across all HFD groups (Figures 1A"-1C"). Both HFD and control mice consumed the same amount of food per day, suggesting that the changes to body weight and hyperglycemia are related to diet composition rather than differences in food intake (Figure 1-2). We next assessed the effect of HFD on circulating lipids. Compared to control mice, HFD 4+36 mice had elevated plasma cholesterol and phospholipids levels, but no differences in triglycerides and non-esterified fatty acid (NEFA) levels (Figures 1D-1G). Finally, we evaluated the PN phenotype by quantifying IENFD, which was reduced in glabrous skin of HFD versus control mice (Figure 1-3). Our combined findings show that HFD mice recapitulate metabolic dysfunction in prediabetic patients.

An additional cohort of HFD female mice developed hyperglycemia and gained a similar amount of weight to HFD male mice (Figure 1-2), although HFD females had a higher percent increase in body weight versus HFD males starting at 22 weeks. Male and female mice displayed similar tactile hypersensitivity at 20 and 40 weeks, when *in vivo* mitochondrial dynamics were measured, although development was slightly delayed in females (Figure 1-2). Therefore, *in vivo* mitochondrial analyses were only performed in male mice.

HFD mice do not exhibit impaired mitochondrial transport in saphenous nerve axons *in vivo*

Terminal mitochondrial transport was examined in adult, electrically active, myelinated saphenous nerve axons *in vivo* (Figure 1, top). In this transgenic mouse, about 40 to 60% of axonal mitochondria constitutively express CFP (CFP^+, Figures 2A and 2B) (Sajic et al., 2013). Nerves were also stained *in situ* with TMRM, a cationic dye that accumulates in mitochondria in

proportion to their MMP, staining them red. CFP^+, unpolarized mitochondria (do not stain with TMRM) will appear cyan, whereas CFP^+, polarized mitochondria (stain with TMRM) will appear magenta, pink, or white. Unpolarized mitochondria that do not express CFP (CFP^-) will not be visible, whereas polarized, CFP^- mitochondria will be red. At the experimental endpoint, we used time-lapse confocal microscopy to track CFP (axonal mitochondria; non-axonal mitochondria do not express CFP) and TMRM (polarized) labeled motile mitochondria (Movies 1 and 2).

Sham-stimulated axons, that are presumed to be electrically silent, had a significantly greater number of motile mitochondria in both anterograde and retrograde directions in HFD 4+16 mice than corresponding controls (Figure 2C), while other parameters of mitochondrial motility and size were similar. We then analyzed mitochondrial transport in mice fed long-term HFD (4+36 mice, Figure 2D), or in older mice fed the short-term HFD (24+16 mice, Figure 2E, Movies 1 and 2). In addition, in order to mimic physiological axonal activity in live animals, we electrically stimulated saphenous nerve axons at either low (1 Hz) or high (50 Hz) frequency during imaging. Electrical stimulation places an energy demand on axons and, thus, mitochondria mobilize in response. However, regardless of the impulse load on the axons, we found no difference in the number of motile mitochondria between the groups (Figures 2D and 2E). Detailed analysis showed that HFD 4+36 mice (Figure 2D) exhibited a significant increase in the *size* of anterogradely transported mitochondria in nerves conducting at 50 Hz, and in the *average speed* of retrogradely transported mitochondria in nerves conducting at 1 Hz, in comparison with controls. Also, in HFD 24+16 mice (Figure 2E), the *average speed* of anterograde mitochondrial transport was significantly higher than in control nerves, at both 1 and 50 Hz, and the anterograde *maximal speed* of mitochondrial transport was significantly higher versus controls at only 1 Hz. Overall, however, our results indicate that, in contrast to our expectations from *in vitro* data (Rumora et al., 2018; Rumora et al., 2019a), prediabetic mice do

not exhibit impaired mitochondrial transport along saphenous nerve axons *in vivo*. Although there was a small increase in the number, size, and speed of mitochondria in some HFD groups, it was not sufficiently pronounced to affect overall axonal mitochondrial transport.

HFD mice exhibit increased stationary mitochondrial size in saphenous nerve axons *in vivo*

In healthy axons, the majority of axonal mitochondria remain stationary regardless of the level of impulse activity. However, stationary mitochondria in electrically stimulated axons are shorter than mitochondria in electrically silent or low frequency conducting axons. This finding has been interpreted as activity-induced mitochondrial fission intended to generate smaller more easily transportable mitochondria (Sajic et al., 2013). To examine the effect of HFD on stationary, polarized mitochondria *in vivo*, we next measured their size by confocal microscopy (Figure 3). There were no differences in the length of stationary mitochondria in young mice fed a short 16-week HFD (4+16) versus control diet (Figures 3A and 3D). However, in mice fed HFD for 36 weeks, stationary mitochondria in axons conducting at 1 Hz were significantly *longer* compared to control mice (Figures 3B and 3E). Further, the average size of stationary mitochondria was significantly longer in older mice fed HFD (24+16) versus controls at both frequencies (Figures 3C and 3F). To illustrate the change in the size distribution of stationary mitochondria, we stratified the average size data (Figures 3E' and 3F') and presented them as a proportion of all stationary axonal mitochondria (Figures 3B' and 3C'). A trend towards a greater proportion of larger (*i.e*., longer than 5 μm) versus shorter stationary mitochondria was found in mice fed HFD versus control diet in the 4+36 and 24+16 week groups.

HFD fed mice exhibit increased PGC1α expression in DRGs *in vivo*

The transcriptional coactivator PGC1α is a master regulator of mitochondrial biogenesis, fatty acid oxidation (Scarpulla, 2011), and intracellular calcium levels (Chen et al., 2010). Thus, we next sought to determine whether PGC1α levels are altered in peripheral nerves of HFD mice. We used confocal fluorescence microscopy to quantify PGC1α protein expression in spinal cord tissue sections from the lumbar L3 and L4 segments by immunohistochemistry (IHC). The HFD 4+36 mice displayed elevated PGC1α immunoreactivity compared to controls in both L3 (Figures 4A-4C) and L4 DRGs (Figures 4D-4F). PGC1α protein level in lumbar DRG was also significantly increased in HFD versus control mice in a separate cohort fed respective diets from 4-36 weeks (Figure 4G). PGC1α protein level did not differ in HFD versus control sural nerves (Figure 4H). Although PGC1α can trigger MFN2-mediated mitochondrial fusion, there was no difference in DRG MFN2 protein levels in HFD and control mice (Figure 4-1). VDAC1/2, an important regulator of mitochondrial function, was significantly decreased in HFD sural nerves, but unchanged in HFD DRG (Figure 4-1). Therefore, increased PGC1α expression occurred concomitantly with increased mitochondrial length (Figure 3), and reduced mitochondrial MMP (Figure 5) and $[Ca^{2+}]i$ in HFD saphenous nerve axons (Figure 7).

HFD mice exhibit reduced MMP in saphenous nerve axons *in vivo*

MMP is a key requirement for mitochondrial ATP synthesis (Zorova et al., 2018). We showed previously that SFAs decrease MMP in primary DRG neurons *in vitro* (Rumora et al., 2018; Rumora et al., 2019a). Therefore, we next examined whether HFD impaired MMP *in vivo* in axons under conditions of both low and high energy demand. We quantified MMP *in vivo* by acquiring confocal images of TMRM-labeled CFP^+ axons and calculating the ratio of TMRM intensity in CFP^+ mitochondria versus TMRM intensity in the adjacent surrounding axoplasm. To monitor MMP within electrically active axons, we imaged saphenous nerve axons while simultaneously stimulating them. In sham-stimulated (*i.e.*, basal energy demand) HFD 4+16 and

1 Hz-stimulated (*i.e.*, low energy demand) HFD 4+36 and 24+16 saphenous nerve axons, mitochondria stained more weakly with TMRM than in control mice, indicating lower MMP in HFD mitochondria (Figures 5A, 5A', 5B, and 5C). We next increased axonal energy demand by stimulating the saphenous nerves at 50 Hz, which markedly decreased MMP in control mitochondria (Figures 5B' and 5C'). This is consistent with the basic tenet of mitochondrial function whereby MMP (the proton gradient) is dissipated through complex V to generate ATP. In contrast, in mice fed HFD, conducting at 50 Hz did not change MMP. Overall, this suggests that the lower MMP in HFD mice may suffice to meet basal energy demands for axon survival, but not for meeting the energy demands for the full range of physiological functions, including high frequency impulse activity.

HFD mice exhibit impaired axonal conduction in saphenous nerve axons *in vivo*

Impulse conduction requires increased ATP production (van Hameren et al., 2019); however, we found herein that HFD mitochondria fail to dissipate MMP in response to electrical stimulation (Figure 5), suggesting that axons from HFD mice may not be able to conduct at the physiological frequencies required for normal functioning, *e.g.*, sensory perception during walking (Prochazka and Gorassini, 1998). Therefore, we examined whether HFD saphenous nerve axons could sustain conduction at physiological frequency *in vivo* by measuring sCAPs, *i.e.*, simultaneous action potentials from several axons. Low frequency (1 Hz) stimulation generated synchronous sCAPs from HFD mice saphenous axons that were comparable to those in control mice (Figure 6-1). Only in some mice on long-term HFD (4+36 weeks) were sCAPs at 1Hz more dispersed, indicating slowing of conduction velocity in some axons (Figure 6-1). These findings suggest that even though MMP was significantly lower in HFD versus control axons, HFD mitochondria still generate sufficient ATP to conduct impulses at 1 Hz. Importantly, however, such low level of impulse activity is atypical for non-resting sensory

axons, which require sustained impulse conduction between 50 to 150 Hz for moderate physical activity such as walking.

Thus, we repeated sCAP recordings during 50 Hz electrical stimulation. In control mice, sCAPs maintained the same shape and amplitude for at least 60 minutes (Figures 6A and 6D); however, sCAP amplitudes steadily declined in both 4+36 (Figures 6B and 6D) and 24+16 (Figures 6C and 6D) HFD mice. The reduced sCAP amplitudes were sometimes accompanied by the appearance of additional, late peaks (Figure 6B) and an overall latency increase (Figure 6C). The reduced amplitudes during sustained impulse activity were more pronounced in animals fed HFD for a longer period. After 16 weeks of HFD diet, the average sCAP amplitude dropped to 80 ± 19% [mean ± standard deviation] of the initial amplitude (Figure 6C'), significantly less than in controls ($p = 0.03$, unpaired Student's t-test). After 36 weeks on HFD, the sCAP amplitudes dropped to as little as only 27% in some animals, with a trending average drop of 71 ± 34% of the initial amplitude (Figure 6B') ($p = 0.10$, unpaired Student's t-test).

To explore the extent of PN in these mice, we next measured their axonal RPT (Smith, 1980), a sensitive method for detecting early PN (Braune, 1999). RPT is the period after one action potential has propagated that the axon is refractory to the propagation of a second action potential. We quantified RPT in HFD and control axons by delivering a first stimulus and increasing the delay a second stimulus was applied in steps of 0.1 ms up to a 2.5 ms delay (Figures 6E and 6F). We quantified the percentage of axons that conducted the second stimulus at each stimulus interval (Figures 6G and 6H). In control nerves, some axons were still able to conduct the second stimulus after a delay of only 0.3 ms (Figure 6E, green arrow), approximately 50% still conducted after a 0.8 ms delay, and 80% after a 1.2 ms delay (Figure 6H). In contrast, in HFD 24+16 mice, no axons conducted after a 0.3 ms delay, only approximately 10% conducted after a 0.8 ms delay, and about 50% conducted after a 1.2 ms (Figure 6H). These results confirm that 16 weeks on HFD is sufficient to detectably impair

conduction. Interestingly, after a longer 36-week HFD duration, RPT tended to revert towards the control pattern (Figure 6G). Nonetheless, these mice showed greater deterioration in their ability to conduct at 50 Hz compared to mice on a shorter diet duration (Figure 6B' and 6C'). Overall, our data show that the loss of MMP coincides with the loss of axonal conduction in HFD mice.

HFD mice exhibit reduced intra-axonal [Ca^{2+}] levels in saphenous nerve axons *in vivo*

Since HFD causes axonal mitochondrial deficits, and mitochondria are important regulators of intra-axonal [Ca^{2+}] ([Ca^{2+}]i) (Rizzuto et al., 2012), we next examined whether HFD alters [Ca^{2+}]i in saphenous nerve axons *in vivo*. We used the transgenic Tn-XXL mouse, which expresses a calcium-binding troponin C-based biosensor with two fluorescent probes, citrine and CFP (Mank et al., 2008). Calcium binding to the sensor results in fluorescence resonance energy transfer (FRET); thus, [Ca^{2+}]i can be quantified by taking the ratio of citrine to CFP fluorescence intensity from axons imaged by confocal microscopy (Figures 7A-7C). We first validated our system by incubating saphenous nerve axons *in vivo* in Tn-XXL mice in artificial CSF followed by adding ionomycin, an ionophore that raises [Ca^{2+}]i. As anticipated, the citrine to CFP fluorescence intensity ratio increased (Figure 7D).

In preliminary experiments, we examined the consequences of a relatively short duration of HFD (5 weeks) on [Ca^{2+}]i, employing 13-week old Tn-XXL mice (13+5 group). This regimen only caused a moderate body weight increase (22.23 ± 1.13 g in control versus 26.13 ± 0.83 g in HFD), yet, surprisingly, it significantly reduced [Ca^{2+}]i ($p = 0.0005$, Mann-Whitney test, $n = 4$ animals per group, Figure 7C). We examined [Ca^{2+}]i in HFD 24+16 mice, given that this cohort displayed significantly impaired axonal conduction (Figure 6). This HFD regimen in Tn-XXL mice induced progressive metabolic dysfunction, including accelerated weight gain, hyperglycemia, and glucose intolerance (Figure 7-1), similar to the effects seen in CFP mice (Figure 1).

529 Interestingly, unmyelinated axons (C-fibers, distinguishable by caliber < 1.5 μm), which are
530 typically affected early in the course of PN and manifest as pain, showed significantly greater
531 $[Ca^{2+}]i$ than larger diameter myelinated axons (4-6 μm) in control mice (Figure 7E). In 24+16
532 HFD Tn-XXL mice, we found a small, but very significant, decrease in $[Ca^{2+}]i$ in both myelinated
533 and unmyelinated saphenous nerve axons when compared to Tn-XXL controls (Figure 7F, $p <$
534 0.0001, unpaired t-test, n = 9 animals per group). Taken as a whole, our results show that a 16-
535 week HFD diet caused an unexpected decease in $[Ca^{2+}]i$ *in vivo* in both myelinated and
536 unmyelinated saphenous nerve axons, compared to control mice on control diet.

537

538 **Discussion**

539 PN in prediabetes and T2D exhibits distal-to-proximal nerve damage and axonal dysfunction;
540 however, underlying molecular mechanisms are undefined. Our clinical studies indicate that
541 dyslipidemia, through the metabolic syndrome, contributes to PN, even in normoglycemic
542 individuals (Callaghan et al., 2020). We also reported that long-chain SFAs impair mitochondrial
543 MMP, bioenergetics capacity, ATP production, and motility in cultured DRG neurons (Rumora et
544 al., 2018; Rumora et al., 2019a). Herein, we postulated that similar mechanisms contribute to
545 sensory neuron degeneration *in vivo* during PN. Using a HFD prediabetic mouse model that
546 develops PN (O'Brien et al., 2014) and accumulates ectopic nerve fat (O'Brien et al., 2020), we
547 characterized mitochondrial function within the saphenous nerve, a sensory nerve of bundled
548 axons that relies on mitochondrial axonal trafficking to maintain energy homeostasis (Sajic et
549 al., 2013). Surprisingly, HFD only marginally affected mitochondrial transport; however, it
550 significantly increased stationary mitochondrial size, lowered MMP, and impaired saphenous
551 nerve conduction at physiological frequencies. This suggests that lower MMP in HFD
552 mitochondria may produce insufficient ATP to sustain physiological conduction. Additionally,
553 HFD decreased $[Ca^{2+}]i$ and increased DRG PGC1α expression.

554 We first evaluated whether HFD-feeding has a sex-specific effect on PN. All male and
555 female HFD mice developed metabolic dysfunction and tactile hypersensitivity. Although HFD
556 female mice had delayed onset of tactile hypersensitivity, both sexes exhibited similar tactile
557 responses at 20 and 40 weeks when *in vivo* experiments were conducted. Therefore, the
558 molecular changes underlying PN may not be sex-dependent so we used HFD male mice
559 throughout the study. Male HFD 4+36 mice had elevated plasma cholesterol and phospholipids
560 associated with PN, similar to previous studies (Tesfaye et al., 2005). Alterations in cholesterol
561 level (Madamanchi and Runge, 2007) and phospholipid composition (Lu and Claypool, 2015)
562 are reported to compromise mitochondrial function in dyslipidemia models, so we evaluated the
563 effect of HFD on mitochondrial events *in vivo*.

564 We anticipated HFD-induced mitochondrial transport deficiencies *in vivo* in saphenous nerve
565 based on our *in vitro* data (Rumora et al., 2018); however, no significant differences were
566 observed. Indeed, short-term HFD increased motile mitochondria numbers in young mice, but
567 not in old mice. The response to short-term HFD in young mice may represent a compensatory
568 mechanism to counteract partial MMP loss and impaired energy production (Baqri et al., 2009),
569 which may fail in old animals. In young mice, healthy polarized mitochondria are rapidly
570 transported into areas of localized, experimentally-induced saphenous nerve axon damage to
571 avert local bioenergetics collapse (Sajic et al., 2018). The discrepancy between our *in vitro* and
572 *in vivo* results perhaps reflects myelination state, which is absent *in vitro* but present around
573 many saphenous axons *in vivo* (Ohno et al., 2011).

574 We found that HFD significantly increased the average mitochondrial size *in vivo*, indicating
575 a shift towards mitochondrial biogenesis. Similar to PGC1α-overexpressing cardiac myocytes
576 (Russell et al., 2004), we observed increased PGC1α protein levels in HFD DRG that correlated
577 with increases in mitochondrial size. Since PGC1α stimulates fatty acid oxidation (Scarpulla,
578 2011; Cheng et al., 2018), higher PGC1α levels in HFD DRGs may be a compensatory

579 mechanism to boost mitochondrial biogenesis and offset MMP loss and energy deficits (Zhang
580 et al., 2019). PGC1α can also induce mitochondrial fusion by activating MFN2 (Zorzano, 2009).
581 However, as seen in skeletal muscle (Jheng et al., 2012) and hepatic tissue (Lionetti et al.,
582 2014), we found no change in DRG and sural MFN2 protein levels, suggesting that
583 mitochondrial fusion is not responsible for the HFD-induced increases in axonal mitochondrial
584 size in sensory nerves. PGC1α also regulates mitochondrial metabolism and apoptosis through
585 VDAC1/2 (Shoshan-Barmatz et al., 2010; Gill et al., 2019). Indeed, we found a significant
586 decrease in VDAC1/2 protein expression in HFD sensory nerves. Therefore, increased DRG
587 PGC1α expression may not be sufficient to offset mitochondrial energy deficits in the sensory
588 nerve axons resulting in a loss of VDAC1/2 expression indicative of mitochondrial dysfunction.

589 *In vitro*, SFAs decreased MMP in DRG neurons (Rumora et al., 2018; Rumora et al., 2019a;
590 Rumora et al., 2019b), and HFD exerted a similar effect *in vivo*, even after short-term HFD.
591 Similarly, SFAs decreased MMP in C2C12 mouse muscle cells (Jheng et al., 2012) and primary
592 mouse cardiomyocytes (Joseph et al., 2016). The partial MMP loss we measured in HFD mouse
593 saphenous mitochondria didn't completely abrogate axonal function. This suggests that
594 elevated SFAs in the HFD reduce MMP by partially uncoupling the proton gradient from ATP
595 production in the saphenous nerve. Partial uncoupling of the proton gradient allows more
596 electron carriers ($NADH/FADH_2$) to enter the electron transport chain, thereby increasing fatty
597 acid β-oxidation and ATP production to sustain axonal function (Skulachev, 1998; Hinder et al.,
598 2019). However, increased energy demand may result in a complete loss of MMP and energy
599 production within HFD saphenous nerves. Therefore, at low energy demand, the HFD
600 saphenous nerve conducted; however, increasing demand to 50 Hz simulation, levels
601 commensurate with daily activities, *e.g.*, walking (Prochazka and Gorassini, 1998), prompted
602 limited conduction, suggesting inability to supply additional ATP for impulse conduction. This
603 progressive conduction slowing/blocking likewise precedes overt PN in diabetics (Dyck et al.,

604 2005). Moreover, further MMP reductions may completely depolarize axonal mitochondria,
605 triggering degradation, and chronic ATP shortages might increase intra-axonal Na^+ levels,
606 causing axonal degeneration (Stys et al., 1992). Conversely, healthy saphenous axons from
607 control mice readily conducted at 50 Hz, as expected since MMP drives ATP synthesis (Nicholls
608 and Ferguson, 2013). Indeed, impulse conduction in healthy saphenous nerve coincides with
609 increased ATP production (van Hameren et al., 2019). Our study is the first, to our knowledge,
610 to quantify MMP changes in adult mammalian axons during impulse conduction *in vivo*. The
611 partial dissipation of MMP may be an important step to increase ATP synthesis during impulse
612 conduction.

613 Saphenous nerve MMP and axonal conduction were impaired even though distal axonal
614 terminals were structurally intact in the hairy skin they innervate on the medial foot dorsum,
615 supporting human studies demonstrating conduction defects before PN onset (Dyck et al.,
616 2005). HFD animals developed PN in glabrous skin on the plantar foot innervated by the sciatic
617 nerve, evidenced by lower IENFD compared to controls. This result is supported by studies
618 evaluating effects of 45-60% HFD in prediabetic mice on glabrous skin, which exhibit IENFD
619 loss as a PN marker (Vincent et al., 2009; Rumora et al., 2019b; O'Brien et al., 2020). The
620 differential impact of HFD on IENFD in glabrous versus hairy skin may reflect effects of
621 increased body weight on the plantar foot in HFD mice (Collongues et al., 2018).

622 Importantly, we found lower saphenous axonal $[Ca^{2+}]i$ in HFD mice versus controls before
623 overt PN. SFAs increase $[Ca^{2+}]i$ at the expense of endoplasmic reticulum Ca^{2+} stores, prompting
624 excessive intra-mitochondrial Ca^{2+} in T2D models (Ly et al., 2017). Palmitate raises $[Ca^{2+}]i$ in
625 mouse (Remizov et al., 2003) and human (Gwiazda et al., 2009) primary β-cells, rat INS-1
626 insulinoma cells (Schnell et al., 2007), and mouse podocytes (Xu et al., 2015), and elevated
627 $[Ca^{2+}]i$ is a unifying axonal degeneration mechanism across multiple neuronal injury types
628 (LoPachin and Lehning, 1997), including diabetic PN (Fernyhough and Calcutt, 2010). DRGs

from streptozotocin-induced type 1 diabetes and genetic leptin signaling-deficient *db*/*db* T2D mice exhibit elevated [Ca^{2+}]i (Kostyuk et al., 1999); however, shorter diabetes durations induced no discernible effects (Kostyuk et al., 2001), suggesting calcium dyshomeostasis occurs early. Thus, lower [Ca^{2+}]i may be an earlier pathological consequence of metabolic disturbance and higher [Ca^{2+}]i likely occurs during later irreversible stages of PN.

Our study had several limitations. First, although we did not find sex differences in the development of metabolic dysfunction or tactile hypersensitivity, there may be sex dimorphism in mitochondrial activity *in vivo* in response to the HFD. Future studies will assess the effect of HFD on MMP, [Ca^{2+}]i, and PGC1α expression in the saphenous nerve from female and male mice. Second, we identified an early decrease in axonal [Ca^{2+}]i before overt PN in HFD mice, suggesting that decreased axonal [Ca^{2+}]i is an early pathological response to metabolic dysfunction and that axonal [Ca^{2+}]i may increase in later-stage PN pathogenesis. Although we did not test this hypothesis herein, future studies will focus on calcium flux in saphenous nerve axons. Although low number of biological replicates is a study limitation, we observed replicable and significant results. Future studies will focus on increasing the number of mice for sCAP analyses and axonal [Ca^{2+}]i assessment, to reduce variance between animals.

Our findings lend new insight into early pathological events in saphenous nerve axons underlying PN development during HFD-induced prediabetes. MMP reduction was a key pathogenic event and may consequently reduce the ability of mitochondria to increase ATP production to meet energy demands, which is relevant to daily activities, like sensory perception during walking. Impairment of mitochondrial function also associated with several compensatory mechanisms, including increased mitochondrial size and transport and elevated PGC1α expression. Importantly, these mechanisms were accompanied by reduced [Ca^{2+}]i, which may have profound consequences for axonal signaling, maintenance, and regeneration. We suggest that diminished MMP in HFD mitochondria creates unbalanced axonal energy supply and

654 demand concomitant with decreased axonal [Ca^{2+}]i, thereby contributing to PN progression in
655 prediabetes.

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840 **Figures legends**

841 **Figure 1. HFD mice exhibit weight gain and impaired glucose tolerance typical of**

842 **prediabetes**

843 (Top) Scheme of the three experimental groups: (i) mice fed HFD from 4 weeks of age for 16
844 weeks (4+16 weeks), (ii) mice fed HFD from 4 weeks of age for 36 weeks (4+36 weeks), and
845 (iii) mice fed HFD from 24 weeks of age for 16 weeks (24+16 weeks). The control diet and HFD
846 fatty acid composition is provided in Figure 1-1 (Extended data). Respective control mice
847 received control chow. At the endpoint, mitochondrial transport and morphology, and axonal
848 $[Ca^{2+}]i$, were measured *in vivo* along with electrophysiological measurements. (A-B) HFD 4+16
849 and 4+36 mice gain more weight than their respective controls and the percentage weight
850 increase is significant starting from 15 and 19 weeks of HFD, respectively, and onwards ($p <$
851 0.001, 2-way ANOVA, n = 5-28 animals per time point). (C) HFD 24+16 mice gain more weight
852 than their respective controls and the percentage weight increase is significant starting from 3
853 weeks of HFD and onwards ($p < 0.001$, 2-way ANOVA, n = 3-10 animals per time point). (A')
854 Terminal fasting blood glucose (FBG) at the experiment endpoint in HFD 4+16 mice was not
855 significantly different to the controls (n = 5 control, n = 8 HFD). (B') Terminal FBG in HFD 4+36
856 mice was significantly higher than the controls (**$p < 0.01$, unpaired 2-tailed Student's t-test, n =
857 10 control, n = 7 HFD). (C') Terminal FBG in HFD 24+36 mice was significantly higher than the
858 controls (*$p < 0.05$, unpaired 2-tailed Student's t-test, n = 8 control, n = 7 HFD). The impact of
859 HFD on food consumption, FBG, body weight, and tactile response in male and female mice is
860 shown in Figure 1-2 (Extended data). (A'') Intraperitoneal glucose tolerance test (ipGTT) in HFD
861 4+16 mice was significantly different to controls at all time points (*$p < 0.05$, **$p < 0.01$, ***$p <$
862 0.001, Bonferroni post-hoc test, n = 4-5 per group). The overall effect from the HFD diet was
863 extremely significant ($p < 0.0001$, 2-way ANOVA). (B'') ipGTT in HFD 4+36 mice was

significantly different to controls at 60 and 120 min (***p < 0.001, Bonferroni post-hoc test, n = 4-7 per group). The overall effect from the HFD diet was extremely significant (p < 0.0001, 2-way ANOVA). (C'') ipGTT in HFD 24+16 mice was significantly different to controls at 30, 60, and 120 min (***p < 0.001, Bonferroni post-hoc test, n = 10 per group). The overall effect from the HFD diet was extremely significant (p < 0.0001, 2-way ANOVA). (D) Plasma cholesterol levels were significantly increased in HFD versus control 4+36 mice (****p < 0.0001, 1-way ANOVA, n = 3 per group). (E) Plasma phospholipid levels were significantly increased in HFD versus control 4+36 mice (****p < 0.0001, 1-way ANOVA, n = 3 per group). (F) Non-esterified fatty acid (NEFA) levels did not differ in HFD versus control 4+36 mice (1-way ANOVA, n = 3 per group). (G) Triglyceride levels did not differ in HFD versus control 4+36 mice (1-way ANOVA, n = 3 per group). Quantitation of hairy skin and glabrous skin IENFD is shown in Figure 1-3 (Extended data).

SEM, standard error of the mean.

Figure 2. HFD mice do not exhibit impaired mitochondrial transport in saphenous nerve axons *in vivo*

(A) The saphenous nerve (black arrow), blood vessels, and the surrounding connective and fat tissue are exposed in the left thigh after skin incision. Scale bar = 1 cm. (B) Time-lapse high magnification confocal image of saphenous nerve axons in CFP transgenic mouse with CFP^+ axonal mitochondria (blue). Isolectin-IB4 (green) labels a proportion of unmyelinated C-fibers and tetramethylrhodamine methyl ester (TMRM) labels polarized mitochondria (CFP^+, $TMRM^+$ mitochondria are magenta, pink, or while; CFP^-, $TMRM^+$ mitochondria are red). Scale bar = 50 µm. (C) Spider-web diagram showing characteristics of polarized, motile mitochondria in sham-stimulated saphenous nerve axons of HFD 4+16 mice. There were significantly more motile anterograde and retrograde mitochondria in HFD than in control axons (*p < 0.05, 2-way

ANOVA, data represent mean of 37-66 axons per group for each category analyzed, n = 2 mice per group). Videos of mitochondrial trafficking in control diet and HFD mice are shown in Movies 1 and 2 respectively. (D-E) Spider-web diagrams showing characteristics of polarized, motile mitochondria in stimulated saphenous nerve axons of 4+36 (D) or 24+16 (E) mice. There were no overall differences in axonal mitochondrial transport. However, HFD 4+36 mice exhibited larger anterogradely transported mitochondria in nerves conducting at 50 Hz and higher average retrograde speed at 1 Hz (*$p < 0.05$, 2-way ANOVA, data represent mean of 77-97 total axons per group, n = 3 mice per group). HFD 24+16 mice exhibited larger average speed of anterograde mitochondrial transport at both 1 and 50 Hz and higher anterograde maximum speed of mitochondrial transport at only 1 Hz (**$p < 0.01$, 2-way ANOVA, data represent mean of 77-97 total axons per group, n = 3 mice per group).

Figure 3. HFD mice exhibit increased stationary mitochondrial size in saphenous nerve axons *in vivo*

(A-C and A'-C') High magnification confocal image of saphenous nerve axons in CFP transgenic mouse with CFP^+ axonal mitochondria (blue) stimulated at 1 Hz. TMRM labels polarized mitochondria; CFP^+, $TMRM^+$ mitochondria are magenta, pink, or white; CFP^-, $TMRM^+$ mitochondria are red. Scale bar = 10 μm. (A-C) In saphenous nerve from control 4+16 (A), 4+36 (B), and 24+16 (C) mice. (A'-C') In saphenous nerve axons from HFD 4+16 (A'), 4+36 (B') and 24+16 (C') mice. Several axonal mitochondria were longer in older HFD (white arrows) than in control mice. (D) HFD 4+16 mice had similar average stationary mitochondrial size at the experiment endpoint in their saphenous nerve axons compared to controls (mean ± SEM, n = 2 mice per group, n = 2996 control mitochondria, n = 9987 HFD mitochondria). (E-F) HFD 4+36 and 24+16 mice had increased average stationary mitochondrial size at the experiment endpoint in their saphenous nerve axons stimulated at 1 Hz and for HFD 24+16 mice also at 50

914 Hz versus controls (*p < 0.05, ***p < 0.001, 2-way ANOVA, mean ± SEM, n = 4-5 mice per
915 group). (E'-F') Distribution of stationary mitochondrial size at the experiment endpoint in
916 saphenous nerve axons shows a shift towards larger mitochondria in 4+36 and 24+16 HFD
917 versus control mice.

918 CI, confidence interval; SEM, standard error or the mean.

919

920 **Figure 4. HFD fed mice exhibit increased PGC1α expression in lumbar DRG *in vivo***

921 (A-B) Representative, flattened Z-stack confocal image of a L3 section from control and HFD
922 4+36 mice, labeled by IHC with anti-PGC1α antibody (red) and counterstained with DAPI.
923 Punctate PGC1α labeling is noticeable in several DRGs (white arrows), particularly in HFD
924 mice. Scale bar = 10 μm. (C) Quantification of percent area labeled with anti-PGC1α antibody in
925 L3 tissue shows significant increase in PGC1α expression in HFD versus control mice (***p <
926 0.001, Mann-Whitney test median [25%-75% percentile]: HFD 3.96 [3.2-6.5] versus control 1.75
927 [0.9-2.5], n = 5 mice per group). (D-E) Representative, flattened Z-stack confocal image of a L4
928 section from control or HFD 4+36 mice, labeled by IHC with anti-PGC1α antibody (red) and
929 counterstained with DAPI. Punctate PGC1α staining is noticeable in several DRGs. Scale bar =
930 10 μm. (F) Quantification of percent area labeled with anti-PGC1α antibody in L4 tissue shows
931 significant increase in PGC1α expression in HFD versus control mice (*p < 0.05, Mann-Whitney
932 test, median [25%-75% percentile]: control 2.7 [1.6-5.3] versus HFD 4.5 [2.2-7.3], n = 5 mice per
933 group). (G) Western blot analysis of right and left L1-L5 lumbar DRG shows a significant
934 increase in PGC1α protein level in HFD versus control mice at 36 weeks of age (*p < 0.05, t-
935 tailed unpaired t-test, n = 8-9 mice per group). (H) Western blot analysis of sural nerves shows
936 no change in PGC1α protein level in HFD versus control mice at 36 weeks of age (t-tailed

unpaired t-test, n = 9-10 mice per group). Western blot analysis of MFN2 and VDAC1/2 protein level in DRG and sural sensory nerve is shown in Figure 4-1 (Extended data).

CTRL, control; DRG, dorsal root ganglion; HFD, high-fat diet; IQR, interquartile range.

Figure 5. HFD mice exhibit reduced MMP in saphenous nerve axons *in vivo*

(A-C) High magnification confocal image of saphenous nerve axons in CFP transgenic mouse with CFP^+ axonal mitochondria (blue, top panel) stimulated at 1 Hz. TMRM labels polarized mitochondria (red, middle panel). Bottom panel is merged; CFP^+, $TMRM^+$ mitochondria are magenta, pink, or white; CFP^-, $TMRM^+$ mitochondria are red. In control mice, all axonal mitochondria were polarized and were either magenta, pink, or white on merged images (bottom; red mitochondria are either axonal, non-CFP expressing or non-axonal). In contrast, in all HFD groups, some axonal mitochondria were depolarized and appear blue on merged images (bottom, white arrows). (A') The ratio of TMRM intensity in CFP^+ mitochondria versus the adjacent axoplasm indicates MMP and is significantly lower in HFD 4+16 mice versus controls (***p < 0.001, unpaired Student's t-test, mean ± SEM, n = 4-5 mice per group). (B') The ratio of TMRM intensity in CFP^+ mitochondria versus the adjacent axoplasm indicates MMP and is significantly lower in HFD 4+36 mice versus controls in 1 Hz-stimulated axons. Increasing the stimulation frequency to 50 Hz lowers MMP in control mitochondria but has no effect in HFD mitochondria (*p < 0.05, **p < 0.01, ***p < 0.001, 2-way ANOVA, mean ± SEM, n = 4-5 mice per group). (C') The ratio of TMRM intensity in CFP^+ mitochondria versus the adjacent axoplasm indicates MMP and is significantly lower in HFD 24+16 mice versus controls in axons stimulated at 1 Hz. Increasing the stimulation frequency to 50 Hz lowers MMP in control mitochondria but has no effect in HFD mitochondria (*p < 0.05, **p < 0.01, ***p < 0.001, 2-way ANOVA, mean ± SEM, n = 4-5 mice per group).

961 IQR, interquartile range; SEM, standard error of the mean.

962

963 **Figure 6. HFD mice exhibit impaired axonal conduction in saphenous nerve axons *in vivo***

964 (A) Waterfall graph of sCAPs from a control mouse, which remain relatively unchanged over 1
965 hour of sustained impulse activity at 50 Hz. The earliest records are at the front of this plot and
966 all other waterfall plots. (B) Waterfall graph of sCAPs from a 4+36 HFD mouse, which
967 progressively change over 1 hour of sustained impulse activity at 50 Hz. The records show a
968 loss of amplitude over time and the labile appearance of axons conducting at longer latency.
969 (B') Plot of change in sCAP amplitude after 1 hour of conducting at 50 Hz, expressed as a
970 percentage of the starting amplitude. There is no mean loss of amplitude in controls, but a mean
971 reduction of 29% in HFD 4+36 mice ($p = 0.05$, unpaired Student's t-test, n = 3 control, n = 5
972 HFD). Example traces of conduction at 1 Hz in HFD and control 4+36 mice are shown in Figure
973 6-1 (Extended data). (C) Waterfall graph of sCAPs from a 24+16 HFD mouse, which
974 progressively change over 1 hour of sustained impulse activity at 50 Hz. The records show a
975 loss of amplitude, indicative of conduction block or partial depolarization, and increase in
976 latency, indicating conduction slowing. (C') Plot of change in sCAP amplitude after 1 hour of
977 conducting at 50 Hz, expressed as a percentage of the starting amplitude. There is no mean
978 loss of amplitude in controls, but a mean reduction of 20% in HFD 24+16 mice ($p = 0.03$,
979 unpaired Student's t-test, n = 3 control, n = 6 HFD). (D) Loss of sCAP amplitude over time in a
980 control (green), 4+36 HFD (dark red), and 24+16 HFD (red) mouse, showing dramatic loss in %
981 of axons conducting 50 Hz in HFD mice. (E) Waterfall graph of RPTs in a control mouse,
982 revealing that a proportion of axons can still conduct a second stimulus delivered 0.3 ms after
983 the first (green arrow); the proportion of axons that conduct the second stimulus increases as
984 the interval increases. (F) Waterfall graph of RPTs in a HFD 24+16 mouse, revealing, in
985 contrast to controls (E), axons require at least a 0.8 ms interval before they can conduct the

second stimulus (red arrow). (G) Graph comparing RPT recovery in control (green) and HFD (red) 4+36 mice, expressed as the recovery in amplitude of the second as a proportion of the first sCAP, is similar between the control and HFD 4+36 mice. (H) Graph comparing RPT recovery in control (green) and HFD (red) 24+16 mice, expressed as the recovery in amplitude of the second as a proportion of the first sCAP, is significantly prolonged in HFD versus control mice (**p < 0.01, 2-way ANOVA, n = 3 control, n = 6 HFD). This shows impaired ability of HFD axons to conduct closely spaced impulses.

Figure 7. HFD mice exhibit reduced intra-axonal [Ca^{2+}] levels in saphenous nerve axons *in vivo*

(A) Confocal image of an exposed saphenous nerve *in vivo* in a Tn-XXL mouse. Scale bar = 400 µm. (B-C) High magnification confocal image in CFP, citrine, and citrine FRET channels with a merge of CFP and citrine FRET, of saphenous nerve axons bathed *in vivo* with artificial CSF (ACSF) (B) or ionomycin (100 µM) for 20 minutes (C). Scale bar = 50 µm. (D) Citrine to CFP intensity ratio increases after incubating control Tn-XXL mouse saphenous nerve axons in ionomycin (100 µM) for 20 minutes (n = 4 mice per group, n = 257-318 axons per group). (E) Pilot data of HFD 9+5 mice exhibit reduced [Ca^{2+}]i in myelinated axons compared to controls (*p < 0.05, Mann-Whitney test, mean ± SEM, n = 4 control mice with n = 318 axons, n = 4 HFD mice with n = 148 axons). (F) HFD 24+16 mice exhibit reduced [Ca^{2+}]i in both myelinated and unmyelinated fibers compared to controls (**p < 0.01, ***p < 0.001, Kruskal-Wallis test, mean ± SEM, n = 9 control mice with n = 70 axons, n = 10 HFD mice with n = 60 axons). Weight gain and impaired glucose tolerance in Tn-XXL (calcium reporter) mice is shown in Figure 7-1 (Extended data).

SEM, standard error of the mean.

Extended data files:

Figure 1-1. Fatty acid composition of the control diet and 45% high-fat diets.

The protein, carbohydrate, and fat content in the control diet (Lab Diets, catalog # 5001) and 45% HFD (Research Diets, catalog # D12451) is provided along with the percentage of saturated, monounsaturated, and polyunsaturated fatty acids in each diet.

Figure 1-2. Male and female mice exhibit similar metabolic and neuropathy phenotypes.

(A) All male and female HFD and control (CTRL) mice consumed around 4 g of food per day. (B) Fasting blood glucose was significantly increased to approximately 8 mmol/L in HFD versus control mice, regardless of sex. (C) Male and female HFD mice gained weight throughout the study. Female HFD mice gained a significantly higher percent weight between 22-26 weeks of age compared to male HFD mice. (D-E) A von Frey test was used to measure tactile sensitivity of HFD and control mice to calibrated filaments. Male HFD mice exhibited tactile hypersensitivity between 24-28 weeks of age while female HFD mice developed tactile hypersensitivity at 32 weeks of age. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 2-way ANOVA with Bonferroni's multiple comparison. CTRL, control; HFD, high-fat diet.

Figure 1-3. HFD mice do not exhibit IENFD loss in the hairy skin innervated by the saphenous nerve but do show IENFD loss in the glabrous skin innervated by sciatic nerve.

(A-B) Left panel: Confocal images of hairy skin labeled with anti-β-III-tubulin (red) to visualize saphenous nerve axonal terminals (white arrows). Right panel: Quantification of epidermal skin layer area labeled with anti-β-III-tubulin showed no differences in IENFD in HFD 24+16 (A) or 4+36 (B) versus control mice. (C-D) Left panel: Confocal images of glabrous skin labeled with anti-β-III-tubulin (red) to visualize saphenous nerve axonal terminals. Right panel: Quantification of the epidermal skin layer area labeled with anti-β-III-tubulin showed a significant reduction in IENFD in HFD 24+16 (C) or 4+36 (D) versus control mice, $p < 0.0001$, Mann-Whitney test.

IENFD, intraepidermal nerve fiber density, GSK, glabrous skin; HSK, hairy skin.

Movie 1. Time-lapse video of axonal mitochondrial trafficking in a control mouse *in vivo* in saphenous nerve conducting at 1 Hz. High magnification, time-lapse confocal image of saphenous nerve axons in a control 24+16 CFP transgenic mouse with CFP+ axonal mitochondria (blue) stimulated at 1 Hz. TMRM labels polarized mitochondria, CFP+, TMRM+, which are magenta, pink, or white (blue arrow). CFP-, TMRM+ Schwann cell mitochondria are red (white arrow). Relative to the imaging site, axons were stimulated at 1 Hz proximally (left of the image) and conducted compound action potentials were recorded distally (right of the image). Many motile mitochondria move in either direction. A node of Ranvier is present in the upper part of the image (yellow arrowhead). Scale bar = 10 μm.

Movie 2. Time-lapse video of axonal mitochondrial trafficking in a HFD mouse in vivo in saphenous nerve conducting at 50 Hz. High magnification, time-lapse confocal image of saphenous nerve axons in a control 24+16 CFP transgenic mouse with CFP+ axonal mitochondria (blue) stimulated at 50 Hz. TMRM labels polarized mitochondria, CFP+, TMRM+, which are magenta, pink, or white (blue arrow). CFP-, TMRM+ Schwann cell mitochondria are

red (white arrow). Relative to the imaging site, axons were stimulated at 50 Hz proximally (left of the image) and conducted compound action potentials were recorded distally (right of the image). Many motile mitochondria move in either direction. A node of Ranvier is present in the upper part of the image (yellow arrowhead). Scale bar = 10 μm.

Figure 4-1. MFN2 and VDAC1/2 expression in HFD versus control mice in DRG and sural nerves *in vivo*

(A-B) Western blot analysis of L1-L5 lumbar DRG (A) and sural nerves (B) shows no change in MFN2 protein level in HFD versus control mice at 36 weeks of age (2-tailed unpaired t-test, n = 9-10 mice per group). (C) Western blot analysis of right and left L1-L5 lumbar DRG shows no change in VDAC1/2 protein level in HFD versus control mice at 36 weeks of age (2-tailed unpaired t-test, n = 7-8 mice per group). (D) Western blot analysis of sural nerves shows a significant decrease in VDAC1/2 protein level in HFD versus control mice at 36 weeks of age (*p < 0.05, 2-tailed unpaired t-test, n = 9 mice per group).

Figure 6-1. Example traces of conduction at 1 Hz in HFD and control 4+36 mice.

(A) Typical size and shape of sensory compound action potential (sCAP) recorded from saphenous nerve in control mice. (B) Most HFD 4+36 mice exhibited similar sCAP size, shape, and latency as control mice. (C) Some HFD 4+36 mice exhibited delayed and dispersed sCAP, with smaller amplitude compared with control mice.

Figure 7-1. Tn-XXL (calcium reporter) mice exhibit pronounced weight gain and impaired glucose tolerance typical of prediabetes.

(A) Tn-XXL 24+16 HFD mice gain more weight than their respective controls and the percentage weight increase is significant starting from 3 weeks of HFD and onwards (*p <

0.001, 2-way ANOVA, n = 9 for control group, n=10 for HFD group). (B) Terminal fasting blood glucose in Tn-XXL 24+16 HFD mice was significantly different to the controls (*p = 0.03, Mann-Whitney test, n = 9 control group, n = 10 HFD group). (C) Intraperitoneal glucose tolerance test in Tn-XXL 24+16 HFD mice was significantly different to controls at 30, 60, and 120 min (***p < 0.01, ****p < 0.0001, Bonferroni post-hoc test, n = 9 control group, n = 10 HFD group). The overall effect from the diet was significant (p < 0.001, 2-way ANOVA).