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# Loss and damage in large-diameter sensory neurons in the db/db diabetic mouse

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## Abstract

Diabetic peripheral neuropathy (DPN) is the most common complication of diabetes. Half of DPN patients experience sensory deficits including loss of sensation and pain. Loss of sensation increases the risk of unnoticed foot injuries which combined with poor circulation and healing lead to amputation. Type 2 diabetes accounts for 50% of foot amputation highlighting the significant impact sensory loss can have on patients' quality of life. However, the cellular basis underlying sensory loss in DPN remains unclear. We characterized diabetes-induced neuronal loss and damage in dorsal root ganglia (DRG) in the db/db mouse model of type 2 diabetes. Morphometric characterization was carried out on two neuronal populations in lumbar DRGs of 32-week diabetic (db/db) mice. These are the N200-positive neurons, a marker for low and high-threshold mechanosensitive sensory and proprioceptive neurons, and peripherin (PRPH)-positive neurons, a marker for pain sensing neurons. In diabetic mice, N200-positive neurons were reduced by 30%. Furthermore, diabetes increased the percentage of N200-positive neurons with cytoplasmic vacuoles, a sign of damage and stress, by 2.44 fold. In addition, the average number of vacuoles was 1.6 fold higher in diabetic mice. Therapies aimed at reducing this loss could help patients better protect their limbs from injuries and thus reduce amputations.

## Keywords

Dorsal root ganglia, diabetic peripheral neuropathy, sensory neurons, N200-positive neurons, cytoplasmic vacuoles

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## Introduction

Diabetic peripheral neuropathy (DPN) is a common complication of type 2 diabetes affecting approximately 50% of patients. DPN is characterized by progressive damage to peripheral sensory nerves particularly those innervating distal limbs (feet and hands).[1] DPN symptoms include increased pain sensitivity (e.g. allodynia, hyperalgesia) and sensory deficits (e.g. numbness, sensory loss), with the latter increasing the risk of unnoticed foot injuries that with poor healing may lead to amputations.[2] Globally, type 2 diabetes accounts for nearly 50% of foot amputations.[3]

Pain and touch are detected by primary sensory neurons in the dorsal root ganglion (DRG). DRG neurons are classified by their morphological, molecular, and functional properties. Large-diameter neurons expressing the Neurofilament 200 (N200) protein are mostly involved in touch and proprioception while pain sensing neurons (nociceptors) are small or medium in diameter and express the cytoskeletal protein peripherin (PRPH).[4] In mice, 40% of DRG neurons express N200, 55% express PRPH, and 5% co-express both markers.[5]

The db/db mouse (Lepr^db/db) is a widely used model of type 2 diabetes for studying DPN.[6] Mice exhibits peripheral neuropathy similar to human DPN, including sensory deficits, axonal damage, and altered skin innervation. The aim of this study is to examine diabetes-induced neuronal loss in lumbar DRGs which innervate hind paws. We hypothesize

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that diabetes leads to a loss of N200-positive neurons, which are involved in the detection of innocuous stimuli and typically give rise to myelinated fibers.

## Materials and methods

*Animals:* Eight adult male db/db mice (BKS.Cg-+Leprdb/+Leprdb/OlaHsd), and eight lean littermates (heterozygous and wildtype) male mice were purchased from (Inotiv, The Netherlands). Max of four animals were kept in a single box with food and water provided *ad libitum*. Diabetic mice were examined twice a week for signs of ill health. At 32-week of age, mice were sacrificed in accordance with Schedule 1 of the Animal (Scientific procedure) Act 1986. The whole spinal column was dissected out and immersed in ice cold phosphate buffered saline (PBS).

*DRG isolation:* Spinal columns were halved, and L4–L5 DRG were identified using spinal ribs as reference points, then extracted and placed in ice-cold PBS. After trimming the peripheral and central nerves to about 3–4 mm, each DRG was embedded in optimal cutting temperature (OCT) medium (Leica Biosystems, UK) and frozen on dry ice. Blocks were stored at −70°C. DRGs were sectioned at 15 µm, each slide contained two sections ~225 µm apart.

*Immunohistochemistry:* Slides were air-dried for 30 min, washed for 5 min in PBS then fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. After washing with PBS, slides were permeabilized with 0.1% Triton-X100, blocked with a buffer containing 2% bovine serum albumin, 0.1% Tween-20, and 2% fish skin gelatin for 2 h at room temperature (Sigma-Aldrich, UK). Primary antibodies (rabbit NF200, 1:2000; Sigma-Aldrich, UK) and (mouse PRPH, 1:2000; Santa Cruz, UK) were applied in the blocking buffer overnight at 4°C. Secondary antibodies (goat anti-rabbit Alexa-Fluor 488 and goat anti-mouse Alexa-Fluor 594, 1:2000, invitrogen) were added in blocking buffer for 2 h. Slides were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Thermo Fisher, UK).

*Image analysis:* Fluorescent images were captured using a Leica DMi8 microscope and analyzed with Fiji-ImageJ (v1.54f). The "Analyze Particles" plugin was used to quantify immunopositive neurons, excluding artifacts via size thresholds. Neuronal diameters were calculated as Diameter $(d) = 2\sqrt{\frac{area}{\pi}}$.

## Results

### Loss of N200-positive profiles in lumbar DRG of diabetic mice

To investigate possible neuronal loss, DRG sections from diabetic and lean littermates were immunolabeled with Neurofilament 200 (N200), a marker of large-diameter touch neurons, and peripherin (PRPH), a marker for small-diameter nociceptive neurons. A total of 4042 neuronal profiles from lean mice and 2975 from diabetic mice were sampled from eight lean and eight db/db mice.

The percentage of N200-positive profiles decreased in diabetic mice, Figure 1. The percentage decreased from 45.6% ± 11.1% to 30.3% ± 8.10% in L4 and 39.0% ± 9.42% to 27.7% ± 5.53% in L5, Figure 1(a). The combined decrease for both L4 and L5, 41.2% ± 8.6%–28.8% ± 4.8% is equivalent to the loss of 30% of N200-positive profiles. As a result of the decrease in the N200-positive neurons, the nominal percentage of PRPH-positive profiles in lumbar DRG increased, Figure 1(c).

We then examined the size of the N200-positive profiles to assess if the neuronal loss affects a particular soma size, Figure 1(d). The size distribution of N200-positive profiles in L4 (30.4 ± 7.9 µm vs 28.6 ± 8.2 µm) and L5 (29.05 ± 8.1 µm vs 28.7 ± 8.1 µm) are not significantly different. As expected, there is no difference in the distribution or means of PRPH-positive profiles.

### Increase in the percentage of N200-positive neurons with cytoplasmic vacuoles in diabetic lumbar DRG

We then examined N200-positive profiles for histological signs of damage. We detected cytoplasmic vacuolation in N200-positive profiles in lean mice and db/db, with the extent of vacuolation worsening by diabetes, Figure 2(a). The percentage of N200-positive profiles with 1 or more cytoplasmic vacuole increased from 7.8% ± 3.5% to 19.1% ± 3.2% in diabetic lumbar L4 and L5 DRG, equivalent to a 2.44-fold increase, Figure 2(b). Furthermore, diabetes significantly increased the number of cytoplasmic vacuoles in N200-positive profiles, Figure 2(c). The mean number of vacuoles increased from 3.1 ± 1.0 to 5.2 ± 1.4, a 1.6-fold increase. The mean diameter of vacuolated N200-positive profiles of 37.4 ± 5.5 µm versus 37.2 ± 5.7 µm were not different, Figure 2(c).

## Discussion

This study aimed to examine diabetes-induced sensory neuron loss in DRG that may underlie the numbness and loss of sensation reported in human DPN patients.[10] We found a significant loss of N200-positive sensory neurons accompanied by an increase in signs of damage and stress in the remaining N200-positive neurons.

We chose the db/db mouse model as it is a well-characterized model of type 2 diabetes with sensory deficits that closely mimic those in human patients.[6] We examined DRGs from mice of at least 32 weeks of age, a time point where diabetes induced sensory changes are well established.[7] This time point corresponds to <40 year old aged diabetic human patients.[8] Because of the stock and glove nature of DPN

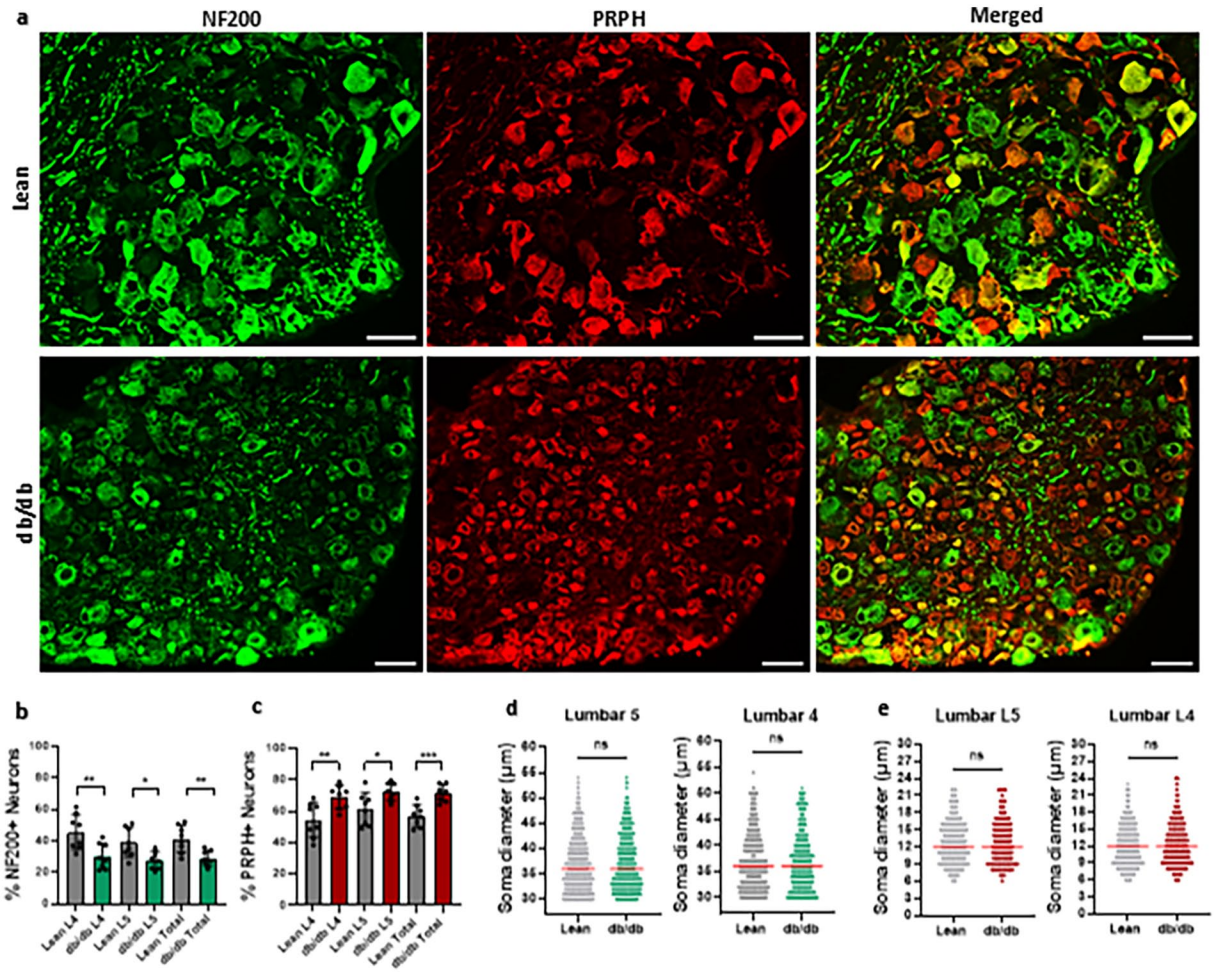


**Figure 1.** Loss in N200-positive profiles in lumbar DRG of db/db mice. (a) Representative images of lumbar DRG labeled with anti-N200 (green) and anti-PRPH (red) antibodies. (b) The percentage of N200-positive profiles in db/db mice is reduced by 30% compared to lean mice; $p = 0.0031$. (c) The percentage of PRPH-positive profiles has increased in db/db mice compared to lean mice. (d) Size distribution of N200-positive profiles is not different between lean and db/db mice ($n = 6$ mice). (e) Size distribution of PRPH-positive neurons is not different between lean and db/db mice ($n = 8$ mice). Results are mean ± SD. Scale bars = 20 µm.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

symptoms, we focused our investigation on L4 and L5 lumbar DRGs because they have long axons that innervate the paws.

A significant and similar neuronal loss was previously reported in L5 DRG of 32 week old db/db mice.[9] However, the subset of neurons lost was not characterized. Our results show that the loss occurs in the N200-positive population of neurons, Figure 1. The percentage of PRPH-positive neurons nominally increased as a result. On one hand, this fits with an 18% reduction of the conduction velocity of the fastest-conducting (i.e. heavily myelinated typically N200-positive) fibers with little changes in slow-conducting (i.e. unmyelinated typically PRPH-positive) fibers.[10] However, our findings contradict those of a study where neuronal loss was not observed.[11] On the other hand, the absence of small diameter neuron loss confirms it as a key difference between type 1 and type 2 mouse models.[12]

Furthermore, the remaining N200-positive neurons showed a much higher incidence of cytoplasmic vacuolation, a sign of stress and damage that may lead to functional changes and/or proceed neuronal death, Figure 2. We did not find similar changes in PRPH-positive neurons therefore it is unlikely that neuronal loss and vacuolation underlie increased pain sensitivity in DPN patients. Interestingly, vacuolation was observed in lean mice indicating that the process is initiated by age rather than diabetes. However, diabetes significantly exacerbates vacuolation, increasing the number of affected neurons by 2.44 fold and number of vacuoles per neuron by 1.6 fold, Figure 2(b) and (c). In N200-positive neurons in db/db mice, neither the neuronal loss nor vacuolation were linked to a particular neuronal soma size, Figures 1(d) and 2(d).

Vacuoles in diabetic DRG neurons were reported in induced and genetic type 1 models.[12,13] However, vacuoles in

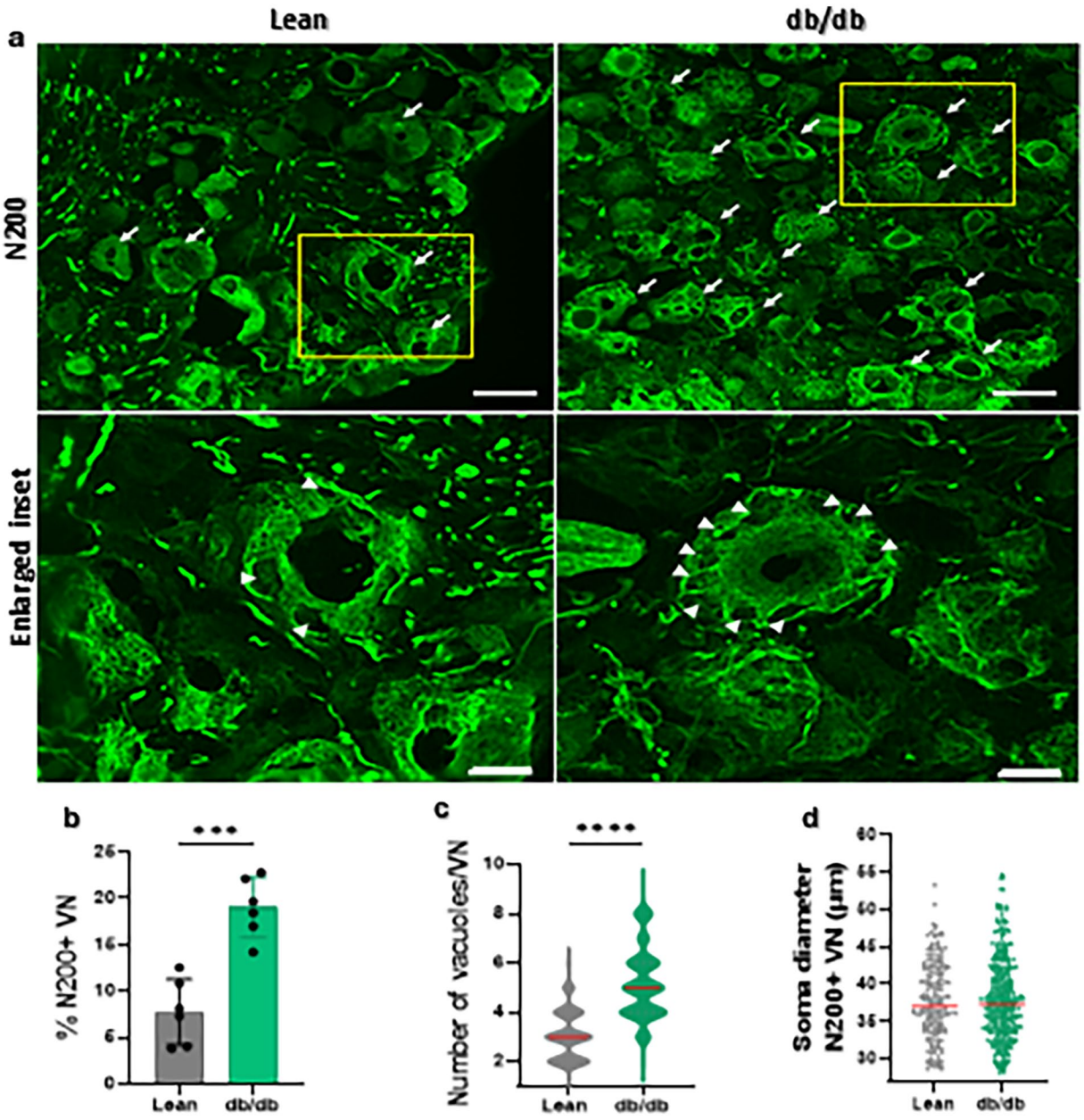


**Figure 2.** Cytoplasmic vacuoles in N200-positive profiles in lumbar DRG of db/db mice. (a) N200-positive (green) neurons with cytoplasmic vacuoles (arrows) in lean and db/db mice. Lower panels, enlarged view of a neuron with multiple vacuoles (arrow heads). (b) Percentage of N200-positive profiles with vaccoules is 2.44 fold higher in db/db compared to lean mice ($p < 0.0001$). (c) The number of vaccoules in N200-positive neurons increased in db/db mice. (d) The distribution of soma diameter of vacuolated N200-positive neurons is not different between lean and db/db mice. Scale bars = 20 μm.

type 1 models differed in two ways. Firstly, the percentage increase in the number of vacuolated neurons (13.2%–17.7%, 1.34-fold[12]) was much smaller than what we observed (7.8%–19.1%, 2.44-fold). Secondly, vacuoles in the type 1 model were more numerous and of much smaller size.[13]

Diabetes-induced death and damage of sensory neurons are expected to result in the presence of dead neurons and a reduction in the overall size of DRG. Evidence of both has been reported in samples from human DPN patients. The volume of L5 DRG in patients with severe DPN symptoms was 21% smaller (decreasing from 170 $mm^3$ to 134 $mm^3$) than in those with mild or moderate DPN.[14]

Furthermore, the number of Nageotte nodules, representing dead neurons surrounded by non-neuronal cells, increased threefold in DPN patients (rising from 8% to 25%).[15] Notably, this threefold increase in 'dead' neurons in human DRGs is comparable to the 2.44-fold increase in vacuolated neurons observed in mouse DRGs. Additionally, the presence of

Nageotte nodules in non-diabetic human DRGs, similar to the vacuolation observed in lean mouse DRGs, supports our suggestion that diabetes significantly exacerbates aging-induced degeneration. We have not examined the peripheral nerve (e.g. the sural nerve) in db/db mice; therefore, it remains to be determined whether they exhibit the observed increase in the cross-sectional area of sensory nerves in human samples.[16]

N200-positive neurons give rise to heavily myelinated fibers with high conduction velocities. Loss and damage to this population are expected to impact the conduction velocity of sensory nerves (e.g. the sural nerve). Indeed, a reduction in conduction velocity has been observed in the db/db mouse model[10] and in human patients.[17]

In summary, our findings demonstrate a significant loss of N200-positive DRG neurons in diabetic mice. This loss could, at least in part, be attributed to an exacerbation of aging-induced damage. We propose that the loss and damage of N200-positive neurons serve as a cellular basis for foot numbness in patients, a hallmark of DPN. Reversing or slowing the changes in N200-positive neurons may help patients better protect their feet, potentially reducing the incidence of amputations. Furthermore, our results reinforce the suitability of the db/db mouse as a model of type 2 diabetes.

## Author contributions

R M Filfilan carried out all experimental work, prepared all figures and contributed to writing the manuscript. MA Nassar contributed to tissue extraction and writing the manuscript.

## Declaration of conflicting interests

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