Diabetic Complications Consortium

Application Title: Role of NADPH Oxidase 5 (Nox5) in Diabetic Neuropathy

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1. Project Accomplishments:

The overall objectives of this proposal were to: 1) generate and characterize a transgenic (Tg) mouse model expressing human Nox5 in Schwann cells (SCs; $Tg^{SC\text{-}Nox5}$) and 2) determine the therapeutic efficacy of Nox5 inhibition on peripheral neuropathy (PN). The rationale for this proposal is that understanding the role of Nox5, as a major source of reactive oxygen species (ROS) that contributes to PN progression, will allow us to identify targeted antioxidant therapies that will restore oxidative balance and nerve function.

Support from the Diacomp Pilot and Feasibility grant has allowed us to genetically manipulate human Nox5 in a new clinically relevant mouse model for therapeutic development purposes for PN. For aim 1, we first generated the SC-specific Mpz-rtTA mouse line in conjunction with the Tg core. These mice were then crossed with the Nox5 mice to generate the inducible $Tg^{SC\text{-}Nox5}$ mouse model selectively expressing human Nox5 in SCs. During the course of the grant, we confirmed Nox5 gene expression specifically in myelinated peripheral nerves. In order to examine the effect of Nox5 expression on metabolic health and nerve function, metabolic and neuropathy phenotyping of the $Tg^{SC\text{-}Nox5}$ male and female mice were assessed following high fat diet (HFD) feeding at 16 and 24 week of age. We are showing the 24 wk time point due to space, but will publish both the 16 and 24 week.

In initial studies for aim 2, we sought to evaluate the effect of global Nox4 deletion, another major Nox isoform in the peripheral nervous system, on the onset and progression of PN and compare the extent of nerve injury in this mouse model to that observed in $Tg^{SC\text{-}Nox5}$ mice. We found that Nox4 knockout (KO) mice placed on a HFD had less fat mass and plasma insulin levels, but developed similar levels of impaired glucose tolerance compared to HFD mice. Although thermal sensitivity was improved in KO mice at early disease stages, this effect was abolished after longer periods of high-fat feeding. Additionally, Nox4 deletion had no beneficial effects on large fiber neuropathy. Finally, we examined whether Nox4 deletion triggered a compensatory induction of other Nox isoforms that may influence nerve function. Interestingly, we found that Nox2 protein expression is increased in KO mice. These results suggest that the increase in Nox2 expression may mediate HFD-induced nerve dysfunction in the absence of Nox4. Ongoing studies in our laboratory will determine whether Nox5 expression will affect Nox4 and Nox2 levels in $Tg^{SC\text{-}Nox5}$, as well as Nox inhibition efficacy on PN progression.

2. Specific Aims:

Specific Aim 1. Generate and characterize a Tg mouse model expressing human Nox5 in SCs.

Results:

Generation of the SC-specific Mpz-rtTA mouse line: We selected the *Mpz* promoter because it is essential for physiological peripheral nerve myelination and its protein expression is restricted to SCs. To that end, CRISPR/Cas9 technology was used to insert rtTA coding sequence before the MPZ termination codon of the mouse *Mpz* gene. CRISPR reagents and a synthetic DNA donor with arms of homology were co-microinjected in fertilized eggs. In the fertilized mouse egg, the DNA donor carrying P2A-rtTA was introduced through repair of the chromosome break by homology directed repair. Because P2A is a self-cleaving peptide, one rtTA molecule was produced for every molecule of Mpz produced (**Figure 1**). Following pronuclear microinjection, six potential founders were positive by the rtTA specific primers and subjected to analysis with primers outside of the arms of homology that spanned the rtTA transgene. One of the six G0 founders showed the 1033 bp PCR amplicon. The amplicon was gel purified and subjected to TOPO TA cloning and Sanger sequencing to confirm correct integration of rtTA into the *Mpz* gene. The male G0 founder was mated to C57BL/6J mice (Jackson Laboratory Stock number 000664) for germline transmission of the targeted *Mpz* gene. In the first litter of 11 G1 pups, four animals were positive by PCR for rtTA. Sequencing of DNA isolated from obligate heterozygote G1 pups showed that they inherited the correctly targeted Mpz rtTA knockin. These animals were used for further colony expansion. Mice were housed in an AAALAC accredited facility in accordance with the National Research Council's guide for the care and use of laboratory animals.

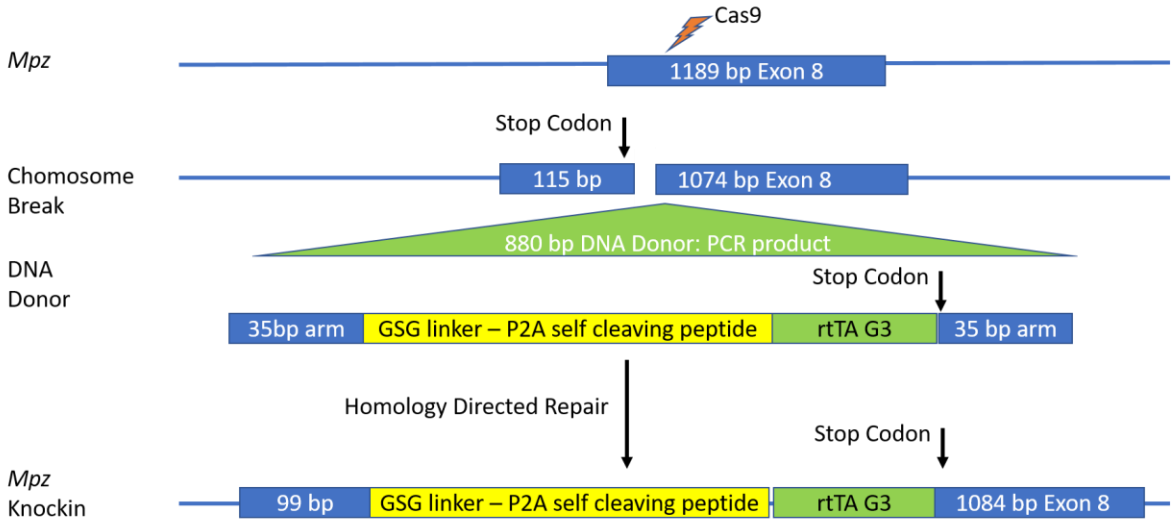

Figure 1. Mpz-rtTA mouse generation.

Generation and validation of the humanized Tg$^{SC\text{-}Nox5}$ mouse that expresses Nox5 in SCs: Nox5 mice were crossed with Mpz-rtTA mice to generate Tg$^{SC\text{-}Nox5}$ mice. Nox5 gene expression in myelinated peripheral nerves was then confirmed by RT-qPCR, which showed significant Nox5 expression in the sciatic nerves of Tg mice, whereas Nox5 levels were undetectable in nerves from WT mice (**Figure 2A**). Nox5 expression was also confirmed by genotyping of genomic DNA extracted from ear punches (**Figure 2B**).

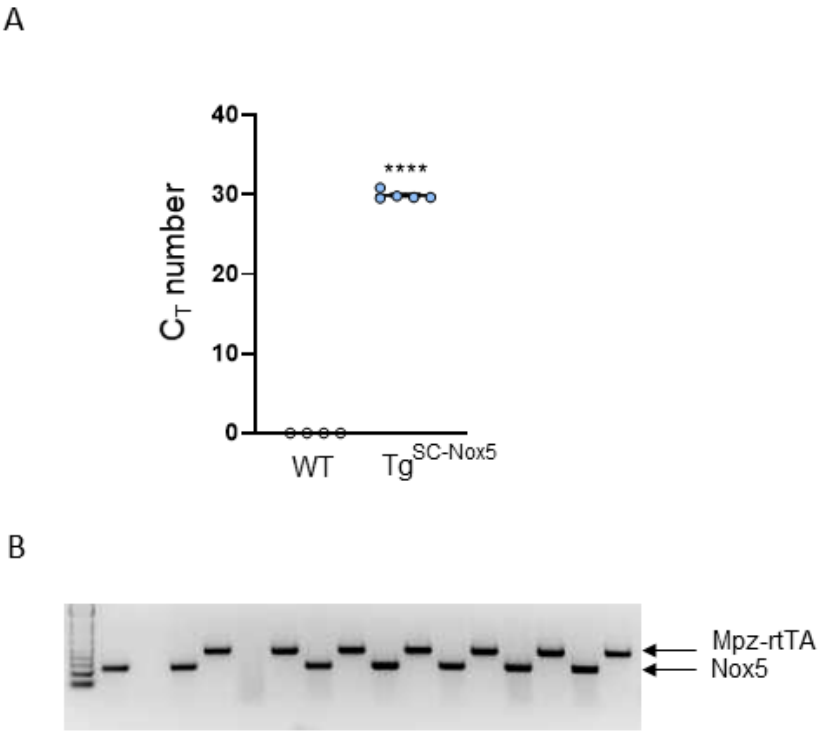

Figure 2. Nox5 expression validation by RT-qPCR (A) and conventional genotyping (B). Data are mean±s.e.m. Unpaired *t*-test, ****P<0.0001 vs WT.

The effect of Nox5 expression on the metabolic phenotype: Following expression validation, we determined the effect of Nox5 expression on metabolic parameters in male (**Figure 3A, B**) and female (**Figure 3C, D**) $Tg^{SC-Nox5}$ at 24 wk of age. Similar to HFD-fed mice, HFD-$Tg^{SC-Nox5}$ male mice develop a prediabetic phenotype with increased body weight (**Figure 3A**) and impaired glucose tolerance (**Figure 3B**). While we did not observe a statistically significant increase in body weight following HFD in female mice (**Figure 3C**), HFD-$Tg^{SC-Nox5}$ female mice displayed a significant increase in body weight relative to control groups. Interestingly, female Tg mice did not exhibit impaired glucose tolerance after HFD at 24 wk of age (**Figure 3D**).

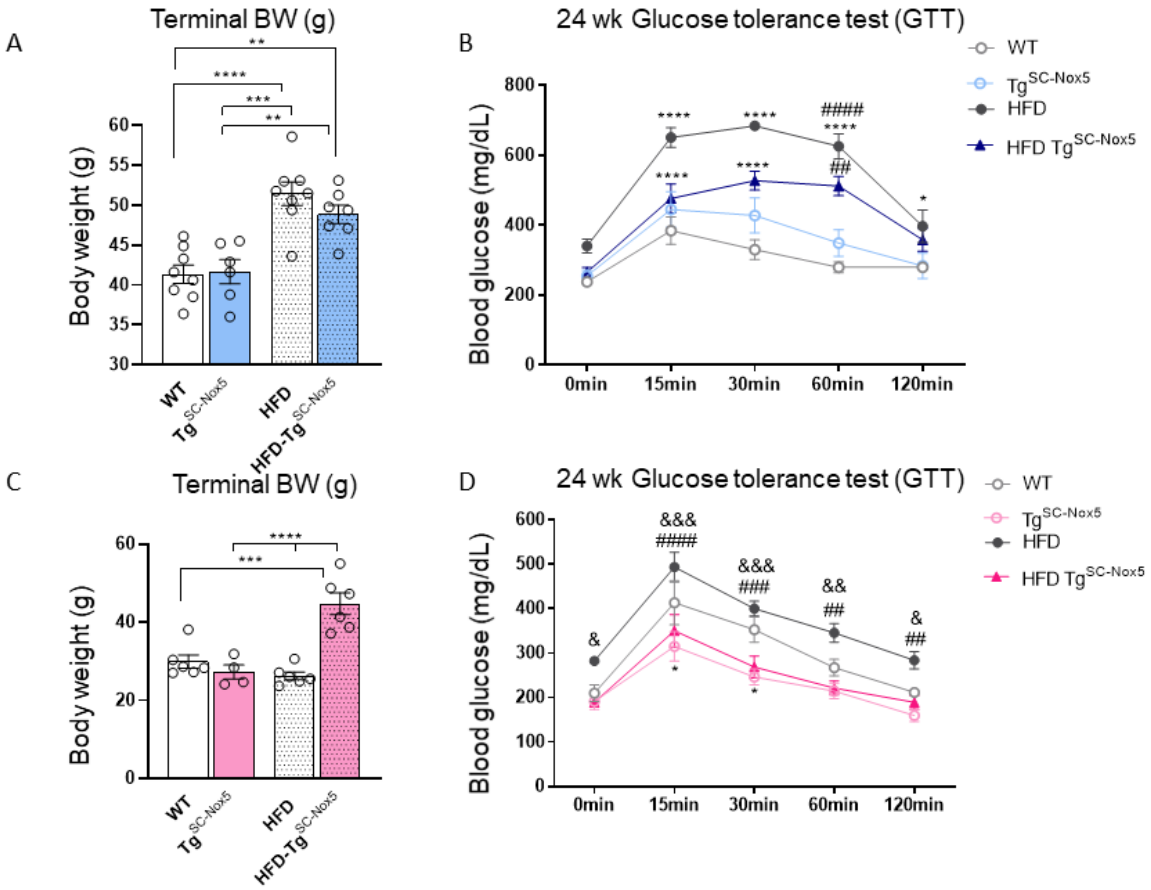

Figure 3. The effect of Nox5 expression on the metabolic phenotype. Data are mean±s.e.m. One-way ANOVA with Tukey's multiple comparisons test, **P*<0.05, ***P*<0.01, ****P*<0.001, or *****P*<0.0001 vs WT; $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 or $^{\#\#\#\#}$ *P*<0.0001 vs Tg$^{SC\text{-}Nox5}$; $^{\delta}P$<0.05, $^{\delta\delta}P$<0.01, $^{\delta\delta\delta}P$<0.001 vs HFD-Tg$^{SC\text{-}Nox5}$.

The effect of Nox5 expression on nerve function: We next examined the effects of Nox5 expression on large fiber neuropathy (nerve conduction velocity; NCV) and sensory neuropathy (nociceptive behavior testing of paw withdrawal latencies in response to a painful thermal stimulus) at 24 wk of age. As expected, male (**Figure 4A**) and female (**Figure 4D**) mice on HFD showed significantly increased hind paw latency relative to WT mice on standard diet, indicating sensory neuropathy. However, Nox5 expression in SCs had no clear effect on thermal sensitivity. At 24 wk, both male (**Figure 4B, C**) and female (**Figure 4E, F**) HFD mice developed delayed sensory NCVs (SNCV) and motor NCVs (MNCVs) relative to WT mice. Interestingly, expression of Nox5 in male and female Tg$^{SC\text{-}Nox5}$ mice, even without HFD, was sufficient to significantly reduce NCVs compared to WT HFD mice.

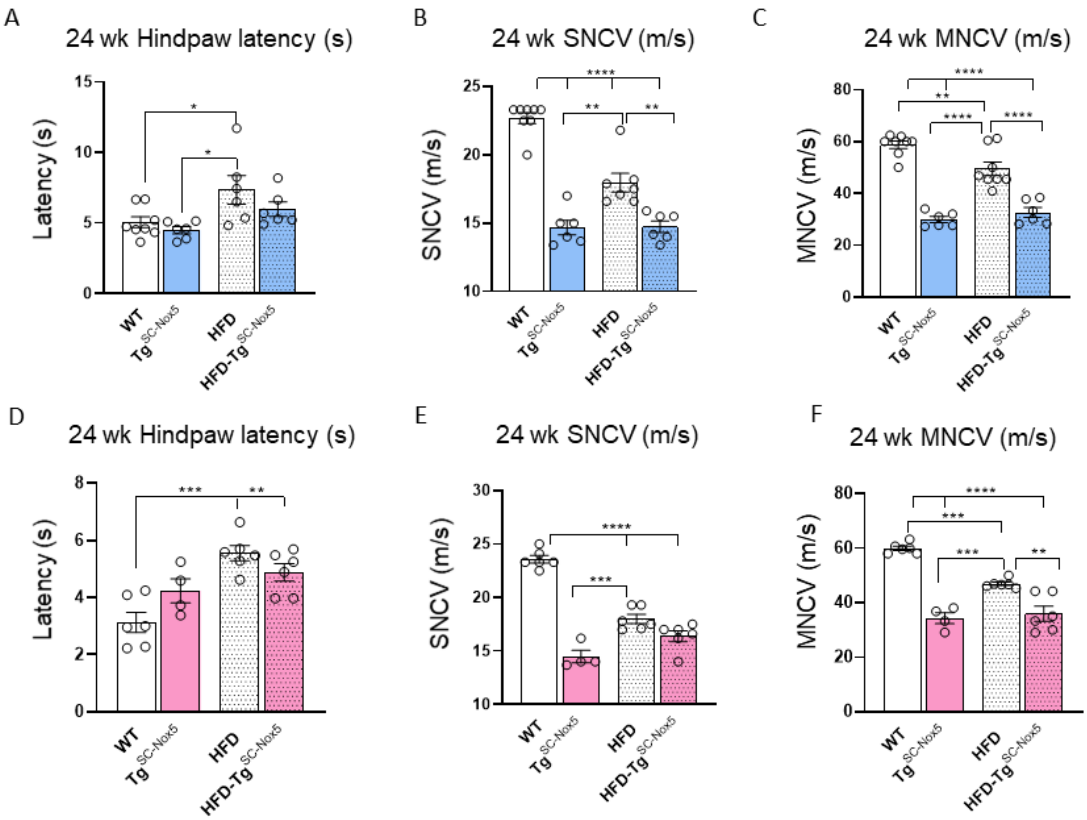

Figure 4. The effect of Nox5 expression on nerve function. Data are mean±s.e.m. One-way ANOVA with Tukey's multiple comparisons test, **P*<0.05, ***P*<0.01, ****P*<0.001, or *****P*<0.0001.

Conclusions and additional studies: These data confirm the superior effect of Nox5 in SCs on accelerated myelinated nerve fiber dysfunction even in the absence of metabolic insults. We are currently assessing morphological changes of sciatic nerves following Nox5 expression in the absence or presence of HFD, specifically analyzing collapsed myelinated fibers and overall axon numbers. Our findings are in agreement with published findings implicating Nox5 expression in accelerated oxidative damage in complication-prone tissues.

Specific Aim 2. Determine the therapeutic efficacy of Nox5 inhibition on PN.

Due to the COVID-19 pandemic and lockdown, we were unable to perform daily injections using GKT, the Nox inhibitor, in $Tg^{Sc\text{-}Nox5}$ mice. We opted instead to study the effect of global Nox4 deletion, another major Nox isoform in the peripheral nervous system, on the onset and progression of PN and compare the extent of nerve injury in this mouse model to that observed in $Tg^{Sc\text{-}Nox5}$ mice.

Results:
The effect of Nox4 deletion on the metabolic phenotype: The study included four groups of male mice that were placed on a HFD for 24 weeks to induce a prediabetic phenotype and consisted of the following groups: WT mice on standard chow, Nox4 knockout (KO) mice on

standard chow, HFD, and KO-HFD mice. We first evaluated the effect of Nox4 deletion on metabolic parameters. KO and KO-HFD mice gained weight at a slower pace compared to WT mice, throughout the duration of the study (**Figure 5A**). Consequently, body composition was evaluated 3 weeks after high-fat feeding (**Figure 5B, C**) and showed that KO mice had significantly reduced fat mass compared to WT controls (**Figure 5B**), with no differences in lean mass across all experimental groups (**Figure 5C**). At study termination, both HFD and KO-HFD mice were significantly heavier than their respective controls on standard diet (**Figure 5D**). Both HFD and KO-HFD mice had impaired terminal glucose tolerance relative to their respective controls (**Figure 5E**). Interestingly, while plasma insulin levels were significantly increased in HFD compared to control mice, KO mice displayed reduced basal insulin levels compared to WT mice, which were increased following high-fat feeding, but not as much as those observed in HFD mice (**Figure 5F**).

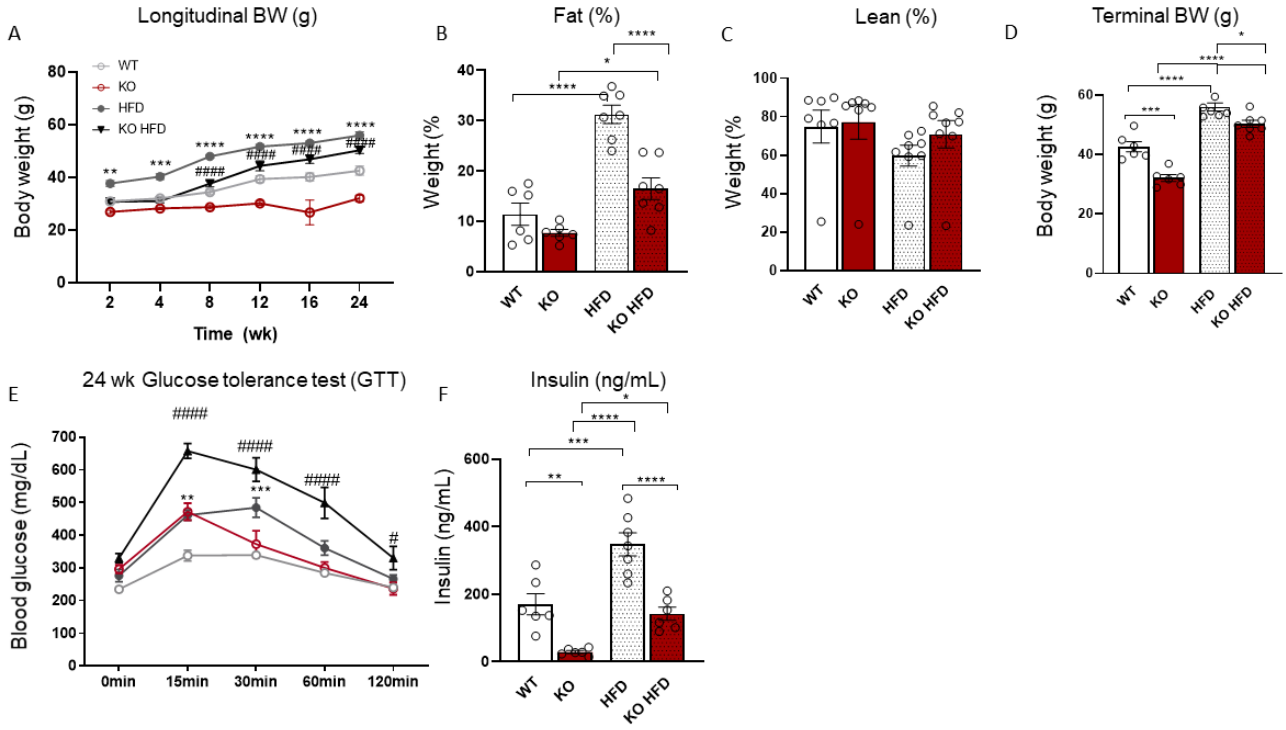

Figure 5. The effect of Nox4 deletion on the metabolic phenotype. Data are mean±s.e.m. One-way ANOVA with Tukey's multiple comparisons test, **P*<0.05, ***P*<0.01, ****P*<0.001, or *****P*<0.0001 vs WT; $^{\#}$*P*<0.05, or $^{\#\#\#\#}$ *P*<0.0001 vs KO.

The effect of Nox4 deletion on PN progression: After 12 weeks of high-fat feeding, thermal sensitivity was improved in KO mice relative to HFD mice (**Figure 6A**). However, this effect was abolished after longer periods of high-fat feeding (**Figure 6D**). On the other hand, Nox4 deletion had no effect on delayed SNCV and MNCV at early (**Figure 6B, C)** and later (**Figure 6E, F)** disease stages.

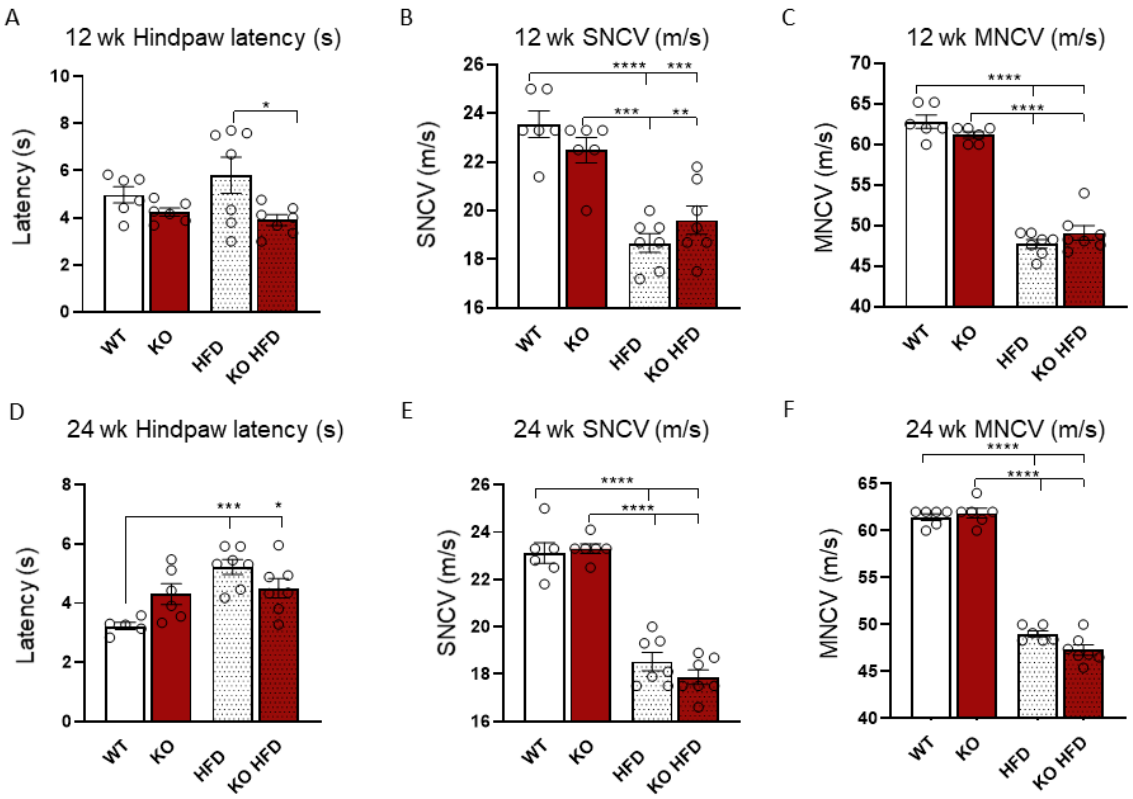

Figure 6. The effect of Nox4 deletion on the neuropathy phenotype. Data are mean±s.e.m. One-way ANOVA with Tukey's multiple comparisons test, **P*<0.05, ***P*<0.01, ****P*<0.001, or *****P*<0.0001.

The effect of Nox4 deletion on other Nox isoforms: We then investigated whether Nox4 deletion triggered a compensatory induction of other Nox isoforms that may affect nerve function. There was no significant change in Nox1 protein expression in the sciatic nerve across all experimental groups (**Figure 7A**). However, we found that similar to HFD, Nox2 protein expression is significantly increased in KO mice relative to WT mice. These results suggest that the increase in Nox2 expression may mediate HFD-induced nerve dysfunction in the absence of Nox4.

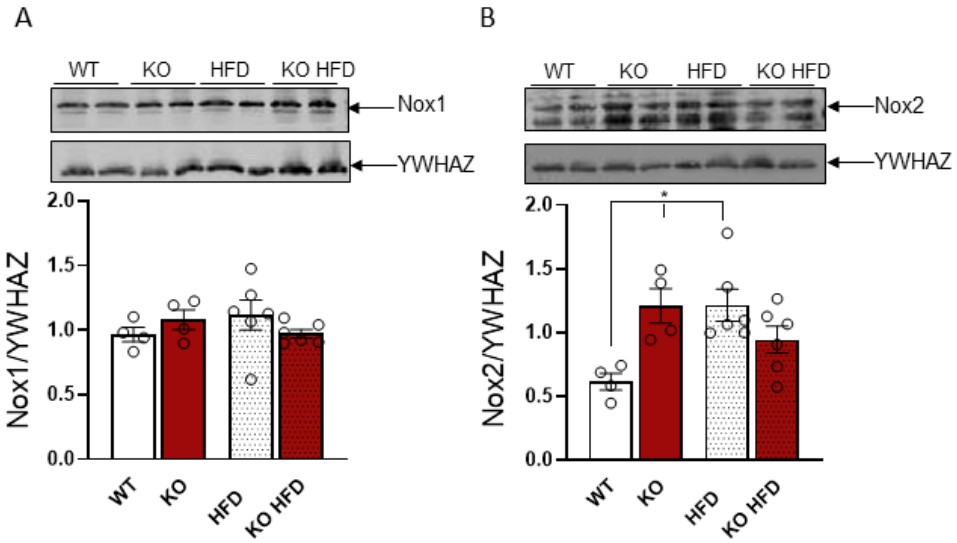

Figure 7. The effect of Nox4 deletion on Nox1 and 2 protein expression in sciatic nerves. Data are mean ± s.e.m. One-way ANOVA with Tukey's multiple comparisons test, **P*<0.05.

Conclusions and additional studies: Although Nox4 is a major ROS source in PN, our results show that total Nox4 deletion does not afford neuroprotection following high-fat feeding. These results further support our view that maintaining redox homeostasis is required for physiological peripheral nerve function and that completely abolishing a ROS source, such as Nox4, can result in off-target effects, like a compensatory increase in the Nox2 isoform. Subsequently, the use of GKT, the Nox inhibitor and the dose administered in our future studies will be crucial as we aim for normalizing Nox levels rather than completely silencing them. Additionally, ongoing studies in our laboratory will determine whether Nox5 expression will affect Nox4 and Nox2 levels in $Tg^{SC\text{-}Nox5}$, as well as GKT efficacy on PN progression.