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Research Paper

# Transcriptional networks of progressive diabetic peripheral neuropathy in the *db/db* mouse model of type 2 diabetes: An inflammatory story

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ABSTRACT

Diabetic peripheral neuropathy is the most common complication of diabetes and a source of considerable morbidity. Numerous molecular pathways are linked to neuropathic progression, but it is unclear whether these pathways are altered throughout the course of disease. Moreover, the methods by which these molecular pathways are analyzed can produce significantly different results; as such it is often unclear whether previously published pathways are viable targets for novel therapeutic approaches. In the current study we examine changes in gene expression patterns in the sciatic nerve (SCN) and dorsal root ganglia (DRG) of *db/db* diabetic mice at 8, 16, and 24 weeks of age using microarray analysis. Following the collection and verification of gene expression data, we utilized both self-organizing map (SOM) analysis and differentially expressed gene (DEG) analysis to detect pathways that were altered at all time points. Though there was some variability between SOM and DEG analyses, we consistently detected altered immune pathways in both the SCN and DRG over the course of disease. To support these results, we further used multiplex analysis to assess protein changes in the SCN of diabetic mice; we found that multiple immune molecules were upregulated at both early and later stages of disease. In particular, we found that matrix metalloproteinase-12 was highly upregulated in microarray and multiplex data sets suggesting it may play a role in disease progression.

## 1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder which arises primarily from obesity and is characterized by hyperglycemia, hyperlipidemia, and impaired insulin signaling. As T2D progresses, these factors lead to microvascular complications which significantly increase both the morbidity and the mortality of afflicted patients (Kim et al., 2011; Edwards et al., 2008). In particular, diabetic peripheral neuropathy (DPN) is one of the most common diabetic complications, with 50% of T2D patients developing DPN (Callaghan et al., 2015). DPN is characterized by progressive loss of sensation in the limbs, pain, and allodynia. It also increases infection risk and the rate of foot ulcers that can lead to amputation (Callaghan et al., 2015).

Despite the prevalence of T2D and the morbidity associated with DPN, the mechanisms of neurodegeneration are poorly understood. Numerous pathways, including inflammation (Pop-Busui et al., 2016), reactive oxygen species (ROS) formation (Hur et al., 2010; Hinder et al., 2013), mitochondrial dysfunction (Hinder et al., 2014), and endoplasmic reticulum stress (O'Brien et al., 2014) are implicated and may play a role in disease progression. To elucidate potential mechanisms, previous studies have utilized either microarray or RNA-Seq analyses to identify genes which are up- or downregulated in the peripheral nervous system during DPN (Hur et al., 2015; Wiggin et al., 2008; Pande et al., 2011; Hur et al., 2016; Hur et al., 2011; Hinder et al., 2017a). However, these studies have focused on specific time frames and do not examine changes over the course of disease; pathways that are upregulated early or late in disease may not necessarily play an important role over the entirety of the disease course.

Using microarrays as a transcriptomic platform, we identified DPN-associated pathways in the sciatic nerve (SCN) and dorsal root ganglia (DRG) at 8, 16, and 24 weeks of age in the well characterized *db/db* mouse model of T2D and DPN. Previously published 16 and 24 week transcriptomic datasets (Hur et al., 2015; Pande et al., 2011) were preprocessed with new datasets using a unified analysis pipeline. Two forms of data analyses were then used in parallel to identify altered molecular pathways in DPN. First, using Self-Organizing Map (SOM)

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analysis, pathways of interest were organized into distinct clusters which followed similar kinetic patterns over the course of disease. Next, Differential Expression Analysis was used to identify up- and down-regulated differentially expressed genes (DEGs) at all three time points. By comparing both sets of analyses, we found that pathways associated with inflammation and immune regulation were altered at all time points in both the SCN and the DRG. To validate these data, multiplex analysis was used to assess protein changes in the SCN during early (8 week) and late (24 week) stages of DPN. Several pro- and anti-inflammatory markers, including interferon (IFN)-γ and interleukin (IL)-10, were upregulated over the course of disease. Also, consistent with our previous observations (Hinder et al., 2017a; O'Brien et al., 2015), we found that matrix metalloproteinase-12 (MMP-12) was highly upregulated in DPN. Together, these data suggest that the immune system plays a key role during DPN and that aspects of the immune system may represent a viable therapeutic target.

## 2. Methods

### 2.1. Animals

Three cohorts of C57BLKS (BKS·Cg-m +/+ Lepr$^{db}$/J; stock number 000642, Jackson Laboratory, Bar Harbor, ME) *db/db* mice, a well-characterized murine model of T2D, were used for the study. Control (*db/+*) and diabetic (*db/db*) mice were fed a standard diet (5LOD, 13.4% kcal fat for 8/24 week; or AIN76A, 11.5% kcal fat for 16 week; Research Diets, New Brunswick, NJ). Mice were cared for in a pathogen-free environment by the University of Michigan Unit for Laboratory Animal Medicine. Mice were, euthanized at 8, 16 or 24 weeks of age using a lethal injection of pentobarbital (Vortech, Dearborn, MI). Animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals.

### 2.2. Metabolic and neuropathy phenotyping

Control *db/+* and *db/db* mice were phenotyped for body weight, fasting blood glucose (FBG), and glycated hemoglobin (GHb) levels (n = 5–6 for each group per time point). FBG levels were measured with an AlphaTrak Glucometer (Abbott Laboratories, Abbott Park, IL) for 8 and 16 week mice and a standard Glucometer (OneTouch; LifeScan Inc., Milpitas, CA) for 24 week mice. GHb levels were determined using a Glyco-Tek Affinity column (Helena Laboratories, Beaumont, TX) at the Michigan Diabetes Research and Training Center Chemistry Core. Peripheral nerve function was assessed at 8, 16, and 24 week according to Diabetic Complications Consortium guidelines (https://www.diacomp.org/shared/protocols.aspx). Motor (sciatic) and sensory (sural) nerve conduction velocities (NCVs), hind-paw withdrawal latency from a thermal stimulus, and intraepidermal nerve fiber density were measured for large and small fiber nerve function, and small fiber loss using our previously published protocols (Oh et al., 2010; Cheng et al., 2009; Cheng et al., 2012; Hinder et al., 2017b). Data from 16 week and 24 week cohorts are previously published (Hur et al., 2015; Pande et al., 2011).

### 2.3. Microarray data analyses

Lumbar DRG were harvested, and nerve roots removed under a dissecting microscope. A 15 mm segment of SCN was harvested immediately proximal to the sural/tibial/peroneal trifurcation, and adherent fat and connective tissue removed under a dissecting microscope. Total RNA was collected from SCN and DRG at 8 week (*db/+*, n = 5; *db/db*, n = 6), 16 week (*db/+*, n = 6; *db/db*, n = 6), and 24 week (*db/+*, n = 6; *db/db*, n = 6) using the silica gel-based isolation protocol RNeasy Mini Kit (QIAGEN, Valencia, CA) at each time point. RNA quality was measured using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples meeting RNA quality criteria were analyzed by microarray as previously described (Pande et al., 2011). In brief, total RNA (75 ng) was amplified and biotin-labeled using the Ovation Biotin-RNA Amplification System (NuGEN Technologies Inc., San Carlos, CA) per the manufacturer's protocol; amplification and hybridization were performed by the University of Michigan DNA Sequencing Core using the Affymetrix GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA). These studies include previously published transcriptomics datasets (16 week SCN and DRG (Hur et al., 2015); 24 week SCN (Pande et al., 2011)).

### 2.4. Quality control

The quality of the microarray CEL files were assessed using the affyAnalysisQC R package (http://arrayanalysis.org) with Bioconductor (www.bioconductor.org). The Robust Multi-array Average method with the BrainArray Custom Chip Definition File version 19 (Dai et al., 2005) was applied to normalize each group of microarray data using GenePattern (http://genepattern.broadinstitute.org/gp/).

### 2.5. Self-organizing map analysis

Patterns of gene expression were identified during DPN progression in SCN and DRG in an unbiased manner using SOM analysis. To compare the gene expression changes across the groups, fold-change (FC) values of all genes at 8, 16, and 24 week for each tissue were calculated. Due to biological and technical variation resulting from different harvest time points and microarray batches, a $\log_2$-transformed fold-change ratio between *db/+* and *db/db* was used for SOM input data. To enhance the visualization of SOM algorithms, Melikerion (Version 1.2.1) software (http://www.finndiane.fi/software/melikerion/) was used with a 7 × 7 grid structure (Makinen et al., 2008). Genes having a similar FC pattern across the groups were gathered together in cells called “modules”. Functional enrichment analysis using DAVID (http://david.abcc.ncifcrf.gov) based on Gene Ontology with a Benjamini-Hochberg (BH) corrected p-value (< 0.05) identified the enriched functions in each module (Huang et al., 2009). Pathway enrichment analysis was then performed to identify significantly enriched canonical pathways with a BH corrected p-value (< 0.05) for the clusters of interest using Ingenuity Pathway Analysis software (www.qiagen.com/ingenuity).

### 2.6. Differential expression analysis

Conventional differential analysis was performed by pairwise comparisons between groups (*db/+* vs. *db/db*) for each group in SCN and DRG. DEGs were determined using the Significance Analysis of Microarrays tool (Tusher et al., 2001) with an estimated false discovery rate of < 0.05 on the basis of 1000 permutations. Next, DEG sets were identified at each time point to identify unique and common DEG sets between time points. To functionally characterize DEG sets common between all three time points, pathway enrichment analysis was then performed to identify significantly enriched canonical pathways with a BH corrected p-value (< 0.05) using Ingenuity Pathway Analysis software (www.qiagen.com/ingenuity).

### 2.7. Multiplex analysis

Cytokine levels were analyzed in SCN from an additional cohort of 8- (n = 6 per group) and 24-week (n = 6 per group) *db/+* and *db/db* mice as previously described (O'Brien et al., 2015). Briefly, SCN samples were homogenized and cytokine concentrations in the lysate were analyzed using MILLIPLEX xMAP magnetic bead technology (Millipore, Billerica, MA) with an MMP-12 (MMMP3MAG-79K) and custom cytokine/chemokine panel (MCYTOMAG-70K). Multiplex assays were run on a Bio- Plex200 multiplex array system with Bio-Plex Manager™ 6.0 software (BioRad, Hercules, CA) according to the manufacturer's