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Gender-specific differences in diabetic neuropathy in BTBR *ob/ob* mice

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Abstract

Aims—To identify a female mouse model of diabetic peripheral neuropathy (DPN), we characterized DPN in female BTBR *ob/ob* mice and compared their phenotype to non-diabetic and gender-matched controls. We also identified dysregulated genes and pathways in sciatic nerve (SCN) and dorsal root ganglia (DRG) of female BTBR *ob/ob* mice to determine potential DPN mechanisms.

Methods—Terminal neuropathy phenotyping consisted of examining latency to heat stimuli, sciatic motor and sural sensory nerve conduction velocities (NCV), and intraepidermal nerve fiber (IENF) density. For gene expression profiling, DRG and SCN were dissected, RNA was isolated and processed using microarray technology and differentially expressed genes were identified.

Results—Similar motor and sensory NCV deficits were observed in male and female BTBR *ob/ob* mice at study termination; however, IENF density was greater in female *ob/ob* mice than their male counterparts. Male and female *ob/ob* mice exhibited similar weight gain, hyperglycemia, and hyperinsulinemia compared to non-diabetic controls, although triglycerides were elevated more so in males than in females. Transcriptional profiling of nerve tissue from female mice identified dysregulation of pathways related to inflammation.

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The authors are deeply saddened by the passing of our dear friend and colleague Lisa L. McLean. Lisa died on July 5, 2015 from a sudden illness. Her selfless dedication facilitated many studies in the field of diabetic complications. She will be deeply missed by everyone who knew her.

Conclusions—Similar to males, female BTBR *ob/ob* mice display robust DPN, and pathways related to inflammation are dysregulated in peripheral nerve.

Keywords

Type 2 Diabetes; Animal Models; Diabetic Peripheral Neuropathy; Dyslipidemia; Gene Expression

1. Introduction

The diabetes epidemic is a major medical concern of the 21st century, affecting over 387 million people globally (IDF Diabetes Atlas 2014). The majority of cases (>90%) have type 2 diabetes (T2D) and display insulin resistance that is typically acquired from poor lifestyle choices combined with genetic susceptibility. T2D is associated with increased morbidity, and the debilitating nature of this disease stems from an array of macrovascular and microvascular complications. Diabetic peripheral neuropathy (DPN) is one such complication that presents in approximately 50% of diabetic patients (Edwards, Vincent et al. 2008) and is a leading cause of diabetes-related hospital admissions and non-traumatic foot amputations in the USA (CDC 2011).

Current therapeutic options for DPN rely on controlling and treating T2D; however, significant gender dimorphisms in the responsiveness of patients to anti-diabetic drugs have been reported (Kim, Cha et al. 2005, Donnelly, Doney et al. 2006, Osterbrand, Fahlen et al. 2007). These observations highlight the importance of elucidating gender-specific differences in diabetes disease manifestation; a decree which has been iterated by the National Institutes of Health (Clayton and Collins 2014). To date, clinical studies investigating the role of gender in relation to obesity and diabetes are limited (Gale and Gillespie 2001) but demonstrate that although T2D does not discriminate between gender, typically affecting males and females alike, young males are at a higher risk of developing insulin resistance (Geer and Shen 2009, Macotela, Boucher et al. 2009) and T2D (Wild, Roglic et al. 2004, Ding, Song et al. 2006). Clearly, with differences in prevalence and drug-responses between males and females, treatments tailored to gender-type are needed. Moreover, pre-clinical research utilizing both male and female models of diabetes is required, as the varying responses of gender to anti-diabetic drugs is a likely consequence of pre-clinical studies primarily utilizing male mouse models to avoid the distinct differences in female physiology and metabolism. Although female mouse models of diabetes are readily available, reports on the gender-specific differences observed in diabetic complications are either limited, or in the case of DPN, absent. Thus, characterization of DPN in female mice is important to establish the differences in physiological profiles between the sexes, including time of onset, degree of severity, and response to disease modifying agents.

In the current study, effects of gender were investigated in female and male BTBR *ob/ob* mice, with an emphasis on identifying differences in DPN severity in the context of comprehensive diabetes phenotyping. Both male and female BTBR *ob/ob* mice present with a condition similar to T2D (Clee, Nadler et al. 2005), and we recently confirmed that male BTBR *ob/ob* mice display a robust neuropathic phenotype as early as 9 weeks (O'Brien, Hur

et al. 2014). Previous examination of diabetes phenotypes in male and female BTBR *ob/ob* mice have revealed marked deficits in metabolic homeostasis between gender, with more severe metabolic perturbations in males that include increased hyperglycemia, hypertriglyceridemia, insulin resistance, and dyslipidemia (Clee, Nadler et al. 2005, Hudkins, Pichaiwong et al. 2010). Thus, as these components of the metabolic syndrome are known to be involved in DPN pathogenesis, we hypothesized that females would display a milder neuropathic phenotype, similar to observations seen in the human population (Aaberg, Burch et al. 2008). As this was the first instance of DPN characterization in a female model, we also performed gene expression profiling on dorsal root ganglia (DRG) and sciatic nerve (SCN) of female mice to identify differentially expressed genes (DEGs) that contribute to DPN in female mice and may provide insight into underlying disease mechanisms.

2. Materials and Methods

2.1 Animals

Male and female BTBR *ob/+* and *ob/ob* mice (n=4; BTBR.Cg-Lep^{ob}/WiscJ, Jackson Laboratory, Bar Harbor, ME) were fed a standard diet (5LOD; 13.4% kcal fat; Research Diets, NJ). All procedures complied with protocols established by the Diabetic Complications Consortium (DCC) (Sullivan, Lentz et al. 2008) and approved by the University of Michigan (U-M) University Committee on Use and Care of Animals (UCUCA). Daily monitoring and maintenance of mice was provided by the U-M Unit for Laboratory Animal Medicine (ULAM).

2.2 Metabolic and Neuropathic Phenotyping

Male and female BTBR *ob*/+ and *ob/ob* mouse phenotyping included both metabolic and neurological measures at ~24 wks. Terminal body weights and fasting blood glucose (FBG; 4 hr fast) were measured. Percent glycosylated hemoglobin (%GHb) was measured by the Chemistry Core at the Michigan Diabetes Research and Training Center (MDRTC), while plasma insulin, cholesterol and triglyceride measurements were performed by the National Mouse Metabolic Phenotyping Center (MMPC; Vanderbilt, TN and University of Washington, WA). Nerve conduction velocities (NCVs) were measured according to published protocols (Sullivan, Hayes et al. 2007, Vincent, Hayes et al. 2009), and at study termination, intraepidermal nerve fiber (IENF) density profiles were determined as previously described (Sullivan, Hayes et al. 2007).

2.3 Affymetrix Microarray

RNA isolated from DRG and SCN of five female BTBR *ob/ob* and BTBR *ob/+* mice was used for microarray hybridization. Total RNA (75 ng) from each sample was amplified and biotin-labeled using the Ovation™ Biotin-RNA Amplification and Labeling System (NuGEN Technologies Inc., San Carlos, CA) according to the manufacturer's protocol. Amplification and hybridization was performed at the University of Michigan DNA Sequencing Core's Affymetrix and Microarray Core Group (Ann Arbor, MI) using the Affymetrix GeneChip Mouse Genome 430 2.0 Array. To validate microarray data, DEGs were ranked by fold-change (Tables 1 and 2) and several of the most highly altered DEGs

were analyzed by real time RT-PCR (RT-qPCR) using *Ywhaz* as the endogenous reference gene as previously described (O'Brien, Hur et al. 2014). The genes chosen for validation along with fold-change compared to controls are provided (Supplemental Table 1). Primers were designed in house, optimized, and purchased from Integrated DNA Technologies (Supplemental Table 2).

2.4 Data and Microarray Analyses

Body weight, blood glucose and %GHb levels, plasma cholesterol and triglyceride levels, thermal latency measures, IENF density, and NCVs of BTBR *ob/+* and *ob/ob* mice for each gender were compared using two-tailed T-test in GraphPad Prism version 6 for Windows (San Diego, California).

Microarray data were analyzed using our established in-house microarray data analysis pipeline (Hur, Sullivan et al. 2011, Pande, Hur et al. 2011, O'Brien, Hur et al. 2014). Briefly, Affymetrix raw data files (CEL files) were processed using a local copy of GenePattern, a bioinformatics platform from the Broad Institute (Reich, Liefeld et al. 2006). The samples were Robust Multi-array Average (RMA) normalized using the BrainArray Custom Chip Definition File (CDF) ENTREZG version 16 (Dai, Wang et al. 2005). The raw and processed microarray data have been deposited into the NCBI Gene Expression Omnibus data repository (http://www.ncbi.nlm.nih.gov/geo, accession # GSE70852). Intensity-Based Moderated T-test (IBMT) (Sartor, Tomlinson et al. 2006) identified DEGs using a false discovery rate (FDR) < 5% cutoff. DEGs were obtained between control (*ob*/+) and diabetic mice (*ob*/*ob*) in DRG and SCN. Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Huang da, Sherman et al. 2009, Huang da, Sherman et al. 2009) identified significantly enriched biological functions among the DEGs in terms of Gene Ontology (GO; http://www.geneontology.org/) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathways. Heat-maps were generated using the top 10 most enriched biological functions in each DEG set based on significance values (log-transformed Benjamini-Hochberg (BH)-corrected P-values) to visually represent the overall similarity and differences between the DEG sets. DEGs from our previously published 5- and 13-week BTBR *ob/ob* mice were included in the comparison identify commonly dysregulated pathways between male and female DPN models.

3. Results

3.1 Female and Male BTBR *ob/ob* Mice Develop Robust DPN

We recently reported that male BTBR *ob/ob* mice rapidly develop considerable peripheral nerve deficits after diabetes onset (O'Brien, Hur et al. 2014). Thus, our initial goal for this study was to assess phenotypic differences associated with gender using this robust mouse model of diabetes with established DPN. Male mice display signs of extreme morbidity sooner than females; therefore, male and female mice were sacrificed at 22 wk and 26 wk, respectively. At terminal stages, electrophysiological testing and quantification of IENF densities confirmed the presence of neuropathy in both obese male and female mice compared to gender-matched non-diabetic controls (Figure 1). NCVs of the sciatic nerve

(motor; MNCV) and sural nerve (sensory; SNCV) were both significantly decreased (Figure 1A,B), with obese male mice demonstrating a ~1.7-fold decrease in MNCV and obese females displaying a ~1.5-fold decrease compared to their respective controls. Similar SNCV deficits were also observed; males exhibited a ~1.3-fold decrease while females exhibited a ~1.4-fold decrease relative to lean control mice. Morphological analysis was performed to assess IENF innervation (Supplementary Figure 1) and quantification of IENF densities revealed significantly lower IENF levels in diabetic mice compared to the respective non-diabetic controls (Figure 1C). Further assessment indicated that IENF loss was greater in obese males (~2.7-fold decrease) than females (~1.4-fold decrease). Additional behavioral testing of nerve function confirmed a loss of sensation in female *ob/ob* mice, as evidenced by decreased sensitivity to thermal stimuli in tail flick and hind-paw testing (Supplementary Figure 2A,B). Combined, these physiological and anatomical DPN measures confirm a peripheral neuropathic phenotype in both male and female *ob/ob* mice that is similar to that seen in T2D patients, although female BTBR *ob/ob* mice display a less severe loss of IENFs despite being 4 wk older than males.

3.2 Female and Male BTBR *ob/ob* Mice Share a Similar, Robust Diabetic Phenotype

Both male and female BTBR *ob/ob* mice exhibit the key physical and metabolic features of obesity and T2D (Figure 2). At study termination, both male and female BTBR *ob/ob* mice were severely obese (68.1±4.04 and 61.8±3.56 g, respectively), exhibiting a ~1.8- and 1.4-fold increase in body weight compared to non-diabetic controls, respectively (Figure 2A). Likewise, %GHb was 1.9-fold higher in males and 1.65-fold higher in female obese mice relative to controls, reflecting severe hyperglycemia (Figure 2B). Early during the course of diabetes, male BTBR *ob/ob* mice have increased FBG compared to females (unpublished observations), but by 24 wk both males and females display similar levels of hyperglycemia, in agreement with previous studies (Hudkins, Pichaiwong et al. 2010). Fasting plasma lipids were also measured, as dyslipidemia has previously been strongly associated with DPN progression (Vincent, Hayes et al. 2009, Hur, Sullivan et al. 2011). As expected, total plasma triglycerides were elevated in obese BTBR *ob/ob* mice compared to controls (Figure. 2C). Male BTBR *ob/ob* exhibited a higher level of hypertriglyceridemia than their female counterparts (256±17.6 vs. 119±45.5 mg/dL, respectively), however both males and females had a similar relative fold-change increase in respect to non-diabetic controls (3.9- and 4.4-fold increase, respectively). Measures of fasting plasma insulin in both male and female BTBR *ob/ob* mice further confirmed severe hyperinsulinemia compared to control mice (female *ob*/+ = 1.32 ± 0.13 S.E.M.; male *ob*/+ mice = 1.28± 0.16 S.E.M.), with levels exceeding the threshold of detection (maximum threshold = 10 ng/ml).

3.3 Gene Set Enrichment Analysis Indicates Inflammation as a Prominent Dysregulated Pathway in the Peripheral Nerve of Female BTBR *ob/ob* Mice

To complement our neuropathy phenotyping, microarray analysis was performed on RNA isolated from SCN and DRG to identify dysregulated genes in female BTBR *ob/ob* mice. Using a 5% FDR cutoff, 584 and 1,105 DEGs were identified in SCN and DRG, respectively. The 10 most up-regulated and down-regulated DEGs identified in DRG and SCN tissues are listed in Tables 1 & 2, respectively. Subsequently, gene set enrichment analysis identified numerous biological functions dysregulated in female mice with

established DPN. Overrepresented pathways in female BTBR *ob/ob* mice included functions related to inflammation and the immune response (Figure 3), similar to our findings in 13 wk male BTBR *ob/ob* mice (O'Brien, Hur et al. 2014). There were 131 common DEGs between the DRG and SCN sets, 124 of which show concordant changes in gene expression. The common DEGs are listed in Supplementary Table 3, while Supplementary Figure 3 illustrates the most over-represented (enriched) biological functions among these DEGs. We found numerous significantly enriched terms among the concordant DEGs, including *complement activation*, *humoral immune response*, and *inflammatory response* (Supplementary Figure 3), suggesting that inflammatory processes occur within both tissues.

4. Discussion

The rising interest in gender-based differences in diabetes (Clayton and Collins 2014) has highlighted the need for further study into the effects of sex on T2D pathogenesis and the development of diabetic complications. We report here the first instance of a female T2D mouse model presenting with a neuropathic phenotype. Similar to male BTBR *ob/ob* mice, female BTBR *ob/ob* mice exhibit robust peripheral neuropathy, including decreased IENF density, impaired motor and sensory NCVs, and thermal hypoalgesia. Moreover, application of our established microarray and gene set enrichment analysis approach (Hur, Sullivan et al. 2011, Pande, Hur et al. 2011, O'Brien, Hur et al. 2014, Hur, Dauch et al. 2015) to DRG and SCN of female BTBR *ob/ob* mice identified genes and biological functions related to inflammation and the immune response. These functions are similar to our previously reported findings in male BTBR *ob/ob* mice (O'Brien, Hur et al. 2014), providing further evidence that these pathways are implicated in DPN pathogenesis.

We are the first to report that female BTBR *ob/ob* mice display significant deficits in nerve function as well as decreased IENF, thus indicating a similar neuropathic phenotype to that observed in T2D patients. With the general consensus that male BTBR *ob/ob* mice are at greater risk for development of hyperglycemia, insulin resistance, and related metabolic abnormalities (Clee, Nadler et al. 2005, Hudkins, Pichaiwong et al. 2010) whereas females display mild protection (Gale and Gillespie 2001), we anticipated a more robust DPN phenotype in males compared to females. However, upon examination of terminal electrophysiological measures, we observed no noticeable differences in MNCV and SNCV between genders at study termination (Figure 1, A,B), suggesting that electrophysiological functions are similarly impaired across gender. It is unknown, however, whether metabolic imbalances between genders at disease onset may impact nerve function earlier than 26 wk. Thus, further assessment of sex-dimorphisms in peripheral nerve function is warranted, as differences may be identified during early stages of DPN development. Future studies will include side-by-side NCV assessments earlier in the disease course to determine if gender-related differences are present. Although no discernable nerve electrophysiology differences were observed, assessment of IENF density, considered the most accurate measure of small fiber neuropathy (Pittenger, Ray et al. 2004), revealed that male BTBR *ob/ob* mice exhibited greater IENF loss than females (Figure 1C). These findings suggest that the greater small fiber loss in male BTBR *ob/ob* mice may possibly be a result of more robust increased metabolic perturbations (described below). In support of this idea, small fiber neuropathy in

humans is associated with obesity and dyslipidemia, as seen in the male BTBR *ob/ob* mice, more than hyperglycemia (Smith and Singleton 2013).

Our terminal metabolic phenotyping involved measuring weight, %GHb, plasma triglycerides, and fasting plasma insulin (Figure 2), hallmarks typically increased in diabetes and obesity. BTBR *ob/ob* mice were profoundly heavier than their controls (Figure 2A) and had hyperglycemia and poor glycemic control (Figure 2B), hypertriglyceridemia (Figure 2C), and hyperinsulinemia. %GHb, a marker of glucotoxicity and a hallmark of diabetes, was only mildly more elevated in male than in female BTBR *ob/ob* mice at study termination when comparing fold-change in relation to non-diabetic controls (Figure 2B). This mild increase in plasma glucotoxicity was expected due to the increased FBG typically found in males and is consistent with previous reports in this model (Clee, Nadler et al. 2005, Askari, Wietecha et al. 2014).

As dyslipidemia has recently been identified as a contributory factor in DPN progression in both humans and mice (Vincent, Hinder et al. 2009), plasma triglycerides were measured in male and female BTBR *ob/ob* mice. Although male and female BTBR *ob/ob* mice similarly display an abnormal lipid profile compared to controls (Figure 2), male mice exhibited a greater degree of hypertriglyceridemia which agrees with previous reports (Hudkins, Pichaiwong et al. 2010). As the increase in triglycerides correlates with decreased IENF, which was more profound than the difference in %GHb between genders, our findings are in line with the hypothesis that lipids, and not just glycemia, may contribute to DPN progression. A recent Cochrane review investigating blood glucose control in the prevention and treatment of DPN in man found that targeting hyperglycemia has little effect on neuropathy outcomes in T2D (Callaghan, Little et al. 2012). The observed sex-based dimorphism in our study was anticipated due to greater metabolic imbalances seen in male mice that, as a consequence, would promote DPN and greater IENF loss. Indeed, studies have reported that elevated plasma triglycerides significantly correlate with both decreased sural nerve fiber density in T2D patients (Wiggin, Sullivan et al. 2009) and a loss of small unmyelinated nerve fibers (Smith and Singleton 2013). Although the morphology of sural nerves was not measured, our findings support that decreased IENFs in ob/ob mice may similarly be the result of lipotoxicity and elevated triglycerides (Figure 2C), and that lipid lowering therapies may therefore halt DPN progression. Indeed evidence suggests that such therapies may alter the progression of diabetic complications, including diabetic neuropathy (Vincent, Hinder et al. 2009). Results from the FIELD trial demonstrates that fibrates are effective treating diabetic nephropathy and retinopathy (Keech, Mitchell et al. 2007, Forsblom, Hiukka et al. 2010), while a report from the Fremantle Diabetes Study suggests that fibrates and statins may protect against the development of sensory neuropathy in patients with type 2 diabetes (Davis, Yeap et al. 2008). Furthermore, we have recently published data from C57BKS db/db mice treated with pioglitazone, a PPAR-γ agonist, in which decreased plasma triglycerides are associated reduced small fiber neuropathy, as determined by IENF density and latency to thermal response (Hur, Dauch et al. 2015). We contend that insulin resistance in peripheral nerves is another potential contributor to DPN (Grote, Groover et al. 2013). Our results demonstrate that both male and female mice have profound hyperinsulinemia, as fasting plasma insulin exceeded the threshold for detection

(10 ng/ml). A comprehensive assessment of insulin resistance in peripheral nerves was beyond the scope of this study; however, as men are at greater risk of developing systemic insulin resistance (Gale and Gillespie 2001, Geer and Shen 2009), confirmed in the BTBR *ob/ob* mouse model (Clee, Nadler et al. 2005), this sex dimorphism would suggest a similar situation in peripheral nerves where insulin resistance is more pronounced in males, thus promoting greater nerve dysfunction. Furthermore, the increased risk of developing insulin resistance in males may explain the higher fold-change of fasting plasma triglycerides when compared to females. As a consequence of systemic insulin resistance, impaired insulin action on adipocytes results in decreased uptake of circulating triglycerides (Guilherme, Virbasius et al. 2008), an important feature in maintaining lipid homeostasis. Interestingly, female BTBR *ob/ob* mice retain adipose tissue insulin sensitivity (Clee, Nadler et al. 2005). Thus, our findings demonstrating higher levels of circulating triglycerides in male mice may be due to increased adipose tissue insulin resistance, thus contributing even further to nerve dysfunction that is brought upon by peripheral nerve insulin resistance.

We previously performed gene expression profiling on male BTBR *ob/ob* mice which resulted in the identification of numerous biological functions dysregulated in the peripheral nerve of diabetic mice compared to controls (O'Brien, Hur et al. 2014); therefore, we performed an identical analysis on SCN and DRG from 26 wk old female BTBR mice. We identified numerous DEGs with large fold changes in female BTBR *ob/ob* mice compared to controls (Tables 1 and 2). Among the DEGs identified in DRG tissue, several encode proteins implicated in inflammatory signaling pathways, including *Crh* (corticotropin releasing hormone), which promotes macrophage foam cell formation (Cho, Kang et al. 2015), and *Stfa3* (stefin A3), a cysteine protease inhibitor upregulated in lipopolysaccharide-stimulated glial cells (Hosoi, Suzuki et al. 2005) that has a role in protecting cells from inappropriate proteolysis. *Saa3* (serum amyloid A3) is an acute phase protein that was also highly overexpressed and is association with diabetic complications (Hamano, Saito et al. 2004). In addition, SAA3 is also a mediator in diabetic kidney disease (Anderberg, Meek et al. 2015), and was previously identified as overexpressed in male BTBR *ob/ob* mouse SCN (O'Brien, Hur et al. 2014).

Using the publically available DNMKB database (a repository of our completed gene expression profiling in nerves of diabetic animals; http://jdrf.neurology.med.umich.edu/DNMKB/) to compare the 26 wk old female DEG set with the 13 wk male DEG set, a post-hoc comparison identified 161 commonly dysregulated DEGs between the two genders (Supplementary Table 3). Similar to our findings in female BTBR *ob/ob* SCN, those with the greatest fold change for both genders included several immunoglobulin family members (*Ighg1, Ighg2c and Ighg2b), Mmp12* (matrix metallopeptidase 12), and *Ucp1*. In addition, *S100a8, S1009, Pon1* (paraoxonase 1), and *Pmp2* were also similarly dysregulated in both genders. While our current analyses examined gene expression changes in females later in the disease course than our male mouse analyses, we similarly identified dysregulation of biological functions related to inflammation in both the DRG and SCN of female BTBR *ob/ob* mice, suggesting a common mechanism of nerve injury in both genders (Figure 3). Although several branches of inflammatory response are likely to be involved in the peripheral nerve environment, collectively, our microarray data suggest an increase in

peripheral nerve antigens in BTBR *ob/ob* mice. As antibodies to neural myelin antigens have been identified in demyelinating diseases (Allen, Giannopoulos et al. 2005), an increase of IgG immunoglobulins in SCN may similarly due to an increase in neural autoantigens. For example, neuropeptide y (*Npy*), a widely expressed protein in the peripheral nervous system that is increased in response to nerve injury (Ji, Zhang et al. 1994) is a DEG in our female BTBR *ob/ob* mice and is a known autoantigen in T1D and T2D patients (Skarstrand, Dahlin et al. 2013). Interestingly, *Mmp12* and *S100a8* are involved in inflammation, tissue remodeling, and injury. MMP12 is an extracellular matrix protease involved in collagen degradation and tissue destruction that is produced by Schwann cells (Hughes, Wells et al. 2002). S100 proteins are calcium binding proteins expressed in neural tissues, increased in patients with T2D (Krisp, Jacobsen et al. 2013), that stimulate a local inflammatory response through biding to RAGE receptors causing a release in proinflammatory cytokines.

One notable downregulated shared DEG identified was *Pon1* (similarly decreased in BKS *db/db* mice (Pande, Hur et al. 2011)). PON1 is an anti-atherosclerotic component of high-density lipoprotein (HDL) and plays a role in the prevention of lipid peroxidation. The PON1 gene is activated by PPARγ, whose expression itself has been found to be deceased in the sural nerve of patients with progressive DPN (Hur, Sullivan et al. 2011). *Pmp2* was another common DEG decreased in male and female BTBR *ob/ob* SCN. Pmp2 is one of the most abundant myelin proteins in the peripheral nervous system, with 15% of myelin comprising of Pmp2, and it is predominantly expressed in myelinated Schwann cells where it has a role as a lipid binding protein and is thought to mediate lipid transport. PMP2-deficient mice exhibit decreased NCVs (Zenker, Stettner et al. 2014), so decreased expression of *Pmp2* in our mouse models of DPN is not surprising, as they too display decreased NCV.

We acknowledge that this current study has some caveats. First, while there are multiple concerns with leptin-deficient models in diabetes research, these models remain favorable to high fat diet (HFD)-fed models for generating a robust diabetes with a predictable and extensively characterized neuropathic phenotype (O'Brien, Sakowski et al. 2014). However, due to the rapid onset of a severe neuropathic phenotype in BTBR *ob/ob* mice (O'Brien, Hur et al. 2014), this model may not be suitable for understanding the early subtle changes that occur in the peripheral nerve as a result of metabolic imbalances. In addition, our non-diabetic control animals consisted only of heterozygote BTBR ob/+ mice. As these mice are known to exhibit subtle metabolic differences to wild-type mice (Hudkins, Pichaiwong et al. 2010) that may also manifest in mild neurological differences, the inclusion of wild-type BTBR mice in future in vivo studies is warranted. Second, for reasons related to study design and execution, side-by-side comparisons between gender at exact ages could not be performed. The primary reason for this is that male BTBR *ob/ob* mice exhibited signs of ill health at 22 weeks requiring study termination, while female mice remained healthy until the prescribed 26 week termination. Lastly, as this was a preliminary investigation, only a small cohort of animals was phenotyped. Despite these caveats, these analyses support the use of female BTBR *ob/ob* mice as a novel model for evaluating the effects of gender on DPN mechanisms and treatments.

With the global incidence of T2D on the rise and an increase in the aging population predicted, the number of men and women with diabetes and diabetic complications is set to increase. This inevitable crisis highlights the need for further investigation into how gender influences the development of diabetic complications. Though marginal, there is a higher prevalence of T2D in young men than women (Wild, Roglic et al. 2004, Ding, Song et al. 2006), likely attributed to the fact that males are more susceptible to insulin resistance than females (van Genugten, Utzschneider et al. 2006). Studies have also demonstrated that (i) the insulin analogue glargine causes a significantly greater decrease in HbA1c in males than females (Osterbrand, Fahlen et al. 2007) and that (ii) males respond better to sulfonylureas than females (Donnelly, Doney et al. 2006), while (iii) females respond more favorably to rosiglitazone than males (Kim, Cha et al. 2005). Our understanding of sex dimorphisms in diabetes is compounded by underlying physiological differences which are numerous and include differences in glucose control and energy homeostasis (Basu, Dalla Man et al. 2006), insulin disposal and clearance (Jensen, Nielsen et al. 2012), regional fat disposition (Geer and Shen 2009, Macotela, Boucher et al. 2009), and sex steroid hormones (Shi and Clegg 2009). For instance, high levels of estrogen confer protection against diabetes development in women (Margolis, Bonds et al. 2004, Le May, Chu et al. 2006, Shi and Clegg 2009, Tiano and Mauvais-Jarvis 2012), and the decreased estrogen production along with increased longevity in post-menopausal women promotes a greater incidence of T2D in this population compared to males, which is also due to increased longevity in this sex (Gale and Gillespie 2001). Evidence has shown that the incidence of diabetes in females is similar to males prior to puberty or after onset of menopause, suggesting that protection is conferred by female hormone, estrogen. Indeed, estrogen prevents β cell failure in most rodent models of diabetes, demonstrating protection through various pathways (Tiano and Mauvais-Jarvis 2012). Similar to T2D incidence, DPN appears to be more prevalent in men than women. The impact of gender on peripheral nerve function is evidenced by nerve conduction studies and quantitative sensory testing that demonstrate earlier development of DPN in male patients with either type 1 diabetes (T1D) and T2D relative to their female counterparts (Aaberg, Burch et al. 2008). Further studies are needed to verify whether this DPN-predominance persists in men with T2D, as males with T1D are more susceptible to DPN (Gale and Gillespie 2001).

Our findings demonstrate that female BTBR *ob/ob* mice exhibit a robust DPN phenotype. Although terminal measures of body weight, hyperglycemia, and hyperinsulinemia were relatively similar at the study conclusion irrespective of gender, male mice exhibited a greater degree of dyslipidemia. The hypertriglyceridemia in male BTBR *ob/ob* mice highlights the presence of gender-specific differences in this T2D mouse model, and based on recent studies, identifies a feature which may explain why male BTBR *ob/ob* mice exhibit a greater decrease in IENF densities suggesting a more robust small fiber neuropathy in males compared to females. Although we have provided preliminary data into how sex dimorphisms in diabetes may influence DPN progression, further investigation is required to identify the biological components that confer male susceptibility/female resistance so that tailored gender-specific therapeutic strategies can be devised and implemented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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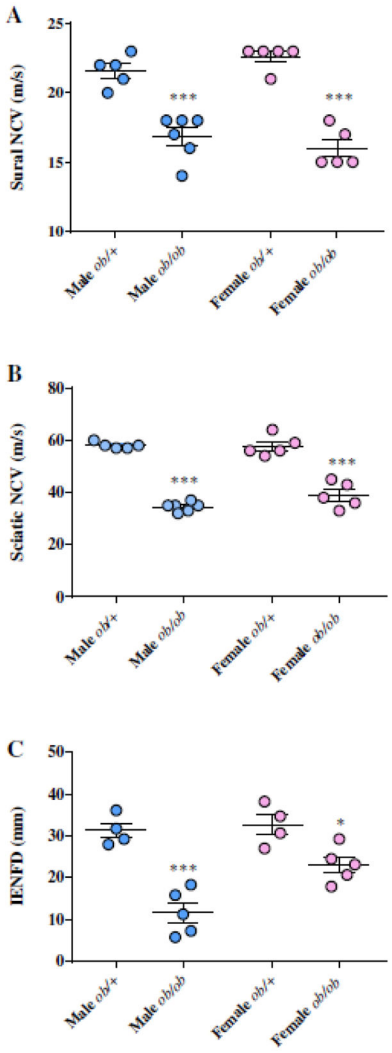

Figure 1. Female BTBR *ob/ob* mice display robust neuropathy characterized by electrophysiological and morphological deficits

Analysis of sciatic (A; Motor NCV) and sural (B; Sensory NCV) nerve conduction velocity in male and female BTBR *ob/+* and *ob/ob* mice ~24 weeks of age. **C.** Quantification of IENF density in male and female BTBR *ob/+* and *ob/ob* mice. Male BTBR mice are represented by blue filled circles while female mice are shown in pink filled circles. Means ± SEM, n = 4–5 per group. * $P < 0.05$; *** $P < 0.0001$ vs. gender-matched, non-diabetic *ob/+* mice.

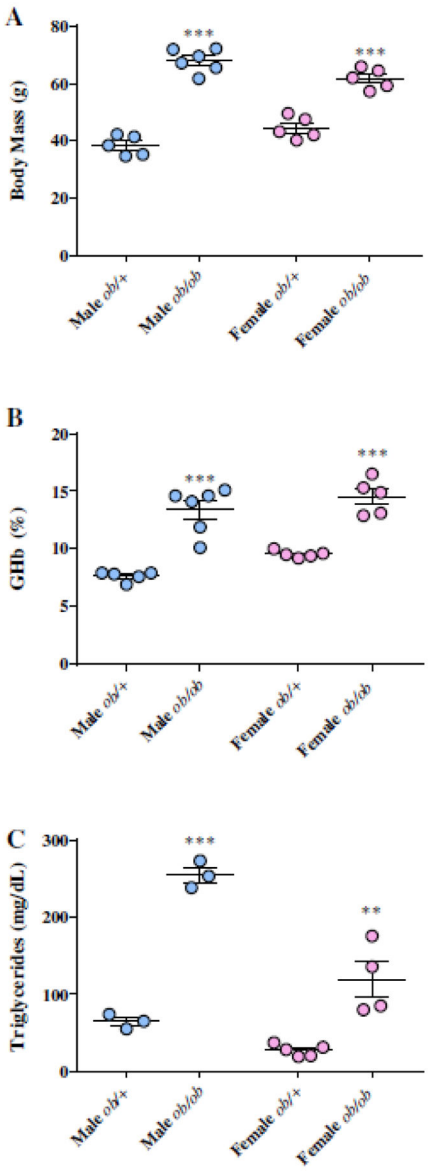

Figure 2. Diabetic phenotype of BTBR *ob/ob* mice

Terminal body weights (A) of male and female BTBR *ob/+* and *ob/ob* mice. At study completion, either blood or plasma of fasting mice was analyzed for glycosylated hemoglobin (%GHb) and (B) triglycerides (C), respectively. Means ± SEM, n = 4–5 per group. Male BTBR mice are represented by blue filled circles while female mice are shown in pink filled circles. *** $P < 0.0001$ vs. non-diabetic *ob/+* mice.

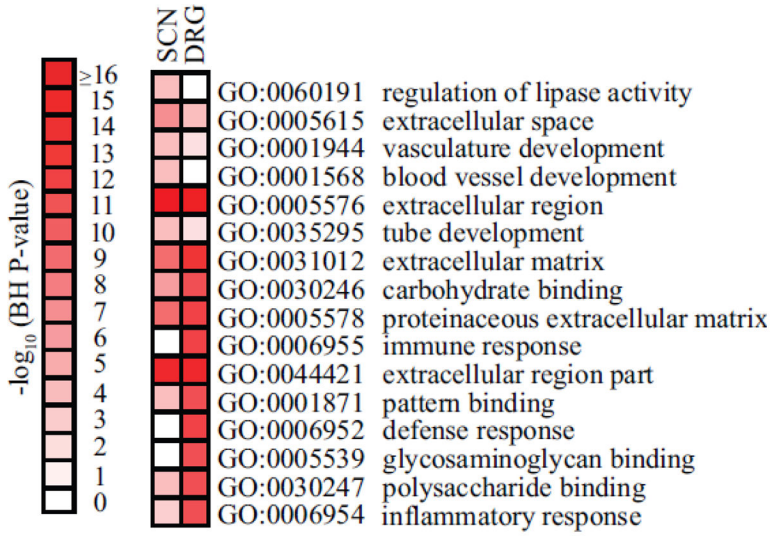

Figure 3. Gene expression analysis of nerve tissue isolated from 26 wk female BTBR *ob/ob* mice

Functional enrichment analysis was performed using DAVID on each of the DEG data sets from either DRG or SCN. Benjamini-Hochberg (BH)-corrected P-values of the top 10 most significant functional terms are represented by heat-map with a $\log_{10}$-based color and number index.

Table 1

DRG DEGs

Description	Gene Symbol	Gene ID#	Fold Change
corticotropin releasing hormone	*Crh*	12918	22.43
stefin A3	*Stfa3*	20863	20.86
small proline-rich protein 1A	*Sprr1a*	20753	17.29
solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	*Slc6a4*	15567	10.57
Wilms tumor 1 homolog	*Wt1*	22431	10.36
cholecystokinin B receptor	*Cckbr*	12426	10.18
stefin A2 like 1	*Stfa2l1*	268885	10.05
endothelin converting enzyme-like 1	*Ecel1*	13599	8.68
neuropeptide Y	*Npy*	109648	7.62
serum amyloid A 3	*Saa3*	20210	7.54
mast cell protease 4	*Mcpt4*	17227	−3.33
carboxylesterase 1D	*Ces1d*	104158	−3.54
complement factor D (adipsin)	*Cfd*	11537	−3.54
cytochrome P450, family 2, subfamily e, polypeptide 1	*Cyp2e1*	13106	−3.82
cytochrome c oxidase subunit VIIIb	*Cox8b*	12869	−3.89
somatostatin	*Sst*	20604	−4.25
growth differentiation factor 10	*Gdf10*	14560	−4.32
peripheral myelin protein 2	*Pmp2*	18857	−4.79
mast cell protease 2	*Mcpt2*	17225	−4.80
cytochrome P450, family 2, subfamily f, polypeptide 2	*Cyp2f2*	13107	−5.04

Table 2

SCN DEGs

Description	Gene Symbol	Gene ID#	Fold Change
immunoglobulin heavy constant gamma 1 (G1m marker)	*Ighg1*	16017	24.97
immunoglobulin heavy constant gamma 2C	*Ighg2c*	404711	19.75
immunoglobulin heavy constant gamma 2B	*Ighg2b*	16016	18.85
matrix metallopeptidase 12	*Mmp12*	17381	18.40
chitinase-like 3	*Chil3*	12655	11.96
uncoupling protein 1 (mitochondrial, proton carrier)	*Ucp1*	22227	11.55
S100 calcium binding protein A9 (calgranulin B)	*S100a9*	20202	9.63
S100 calcium binding protein A8 (calgranulin A)	*S100a8*	20201	8.36
brain expressed gene 1	*Bex1*	19716	7.78
pentraxin related gene	*Ptx3*	19288	7.39
apelin receptor	*Aplnr*	23796	−3.17
melan-A	*Mlana*	77836	−3.17
enoyl-Coenzyme A delta isomerase 3	*Eci3*	69123	−3.21
Ras association (RalGDS/AF-6) domain family member 6	*Rassf6*	73246	−3.28
macrophage galactose N-acetyl-galactosamine specific lectin 2	*Mgl2*	216864	−3.30
dickkopf homolog 2 (Xenopus laevis)	*Dkk2*	56811	−3.35
spondin 1, (f-spondin) extracellular matrix protein	*Spon1*	233744	−3.91
paraoxonase 1	*Pon1*	18979	−11.67
aldo-keto reductase family 1, member C-like	*Akr1cl*	70861	−17.46
cytochrome P450, family 2, subfamily f, polypeptide 2	*Cyp2f2*	13107	−21.13