Animal Models of Diabetic Complications Consortium
(U01 DK076160)

Annual Report
(2009)

"Mitochondrial SOD as a Target for Diabetic Neuropathy"

University of Michigan

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Part A:

Principal Investigator's Summary

1. Program Accomplishments:

Hypothesis:

We continue to investigate the damaging effects of diabetes-induced oxidative stress on the nervous system. Our primary goal is examining the onset and progression of diabetic polyneuropathy (DPN). Recent data collected from our archived human samples indicates that elevated triglycerides predict the progression of DPN in human patients (1). We have confirmed the damaging effects of oxidized lipids on neuronal oxidative stress and DPN progression in an animal model of type 2 diabetes, high fat fed C57Bl/6J mice (2). We are also investigating the role of diabetes induced oxidative stress, dyslipidemia and insulin resistance on neurons of the central nervous system with regard to the pathology of Alzheimer's disease. The results of these two studies are detailed below.

Progress Towards Stated Milestones:

Dyslipidemia-Induced Neuropathy in Mice

We investigated the impact of a high fat diet on the induction of insulin resistance, dyslipidemia and neuropathy in C57Bl/6J mice. This work was presented at the Peripheral Nerve meeting in Wurtzburg Germany in July of 2009 and recently published in Diabetes (2).

Emerging data indicates that dyslipidemia contributes to the development of DPN (7; 8). Lipid profiles are commonly abnormal early in the course of type 2 diabetes in a temporal pattern that correlates with the presence of DPN and we recently reported that elevated triglyceride levels predict a more rapid disease course (9; 10). We employed high fat feeding in the C57/Bl6 mouse strain using a 45 kcal% fat (mostly from lard) diet and demonstrate morphological and functional evidence of DPN prior to loss of glucose regulation in agreement with clinical findings (10; 23; 24).

High Fat Fed Mice

C57/Bl6 mice (Jackson Laboratories, Bar Harbor, Maine) at 3 wk age were placed on either control AIN5003 (10%kcal%fat) or high fat (45%kcal%fat) chow D12451i from Research Diets (New Brunswick, NJ), with 10 mice/group. Chows were matched for protein and carbohydrate content. Blood glucose was tested every 4 wk following a 6 h fast. One drop of tail blood was analyzed using a standard glucometer (One Touch Profile, LIFESCAN, Inc. Milpitas, CA, #6 strips). Glucose tolerance tests were performed by measuring blood glucose 5, 15, 30, 60, and 120 min after gavage administration of a glucose bolus. Nerve conduction velocity (NCV) studies were performed after 12 and 34 wk, and neuropathy phenotyping (see below) at termination at 34 wk. Glycated hemoglobin (GHb) was measured using the Helena Laboratories Test Kit, Glyco-Tek Affinity Column Method (Catalog #5351) as previously described (25; 26). Insulin was measured by radioimmunoassay in the MDRTC Chemistry Core.

Measures of DPN and Insulin Resistance

Following 12 weeks on a high fat diet, mice were modestly heavier than mice on a control diet (28.9$\pm$1.4 g compared to 25.4$\pm$0.5 g) (Fig. 1A) but did not have increased fasting blood glucose (Fig. 1B). Despite the lack of evidence for glucose intolerance, the mice displayed evidence of neuropathy. The latency of hind paw response to a heat stimulus was significantly increased (Fig. 1C; $p<0.05$) and sciatic NCV was slowed in the high fat mice (Fig. 1D; $p<0.05$).

By 34 wk, mice displayed glucose intolerance with significantly higher blood glucose levels 15 min after applying a glucose bolus. This difference remained at 2 h (Fig. 2A). Body weight, plasma insulin, and glycated hemoglobin (GHb) were all significantly increased in the high fat group compared to control diet (Fig. 2B-D), indicating frank diabetes in the high fat mice. NCV measures in sural ($p<0.05$) and sciatic ($p<0.01$) nerves were both decreased in the high fat compared to control diet mice (Fig. 2E-F). Sensory neuropathy was evident in the high fat mice through decreased response to a heat stimulus on the hind paw (Fig. 2G) and decreased intraepidermal nerve fiber density (IENFD) in the hind paw skin (Fig. 2H-I).

This study demonstrates that dyslipidemia produces dorsal root ganglion (DRG) neuron injury and supports emerging clinical evidence that dyslipidemia is an independent risk factor for DPN. The data suggest that glycemic control alone is insufficient to prevent complications in type 2 diabetes and argue for combination therapies targeting multiple metabolic imbalances and receptor-mediated signaling that leads to oxidative injury.

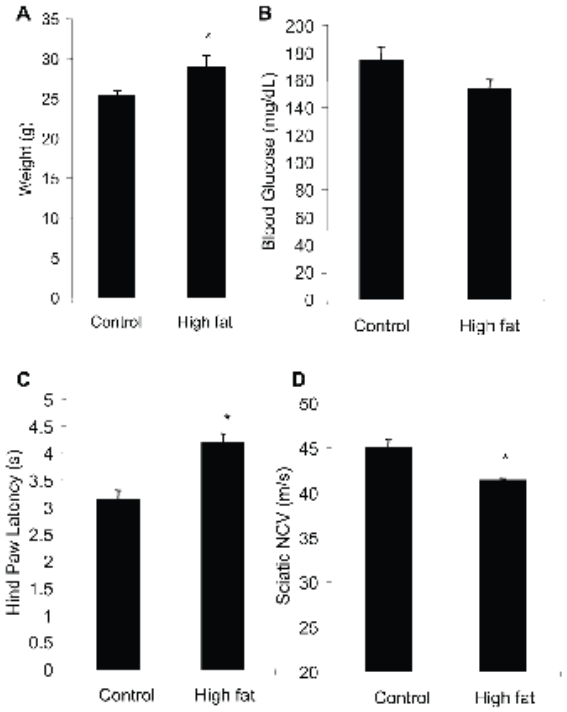

Figure 1. Mild neuropathy after 12 wk high fat diet. Following 12 wk of high fat or control diet, weight (A), blood glucose (B), hind paw withdrawal latency (C) and sciatic NCV (D) were assessd. n = 10/group. *p<0.05 compared to control diet.

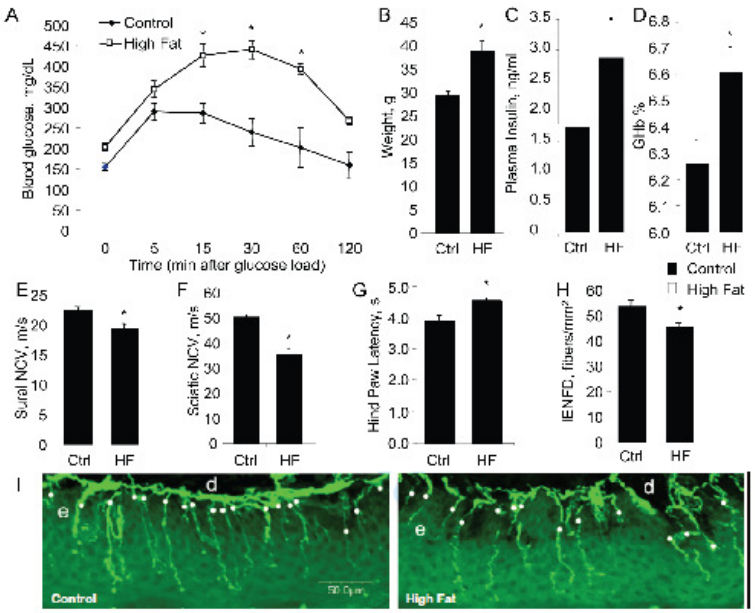

Figure 2. Neuropathy and impaired glucose tolerance after 34 wk high fat diet. Complete phenotyping after 34 wk on control (Ctrl) or high fat (HF) diet was performed. The figure displays the glucose tolerance test (A), weight (B), plasma insulin (C), glycated hemoglobin (D), sural NCV (E), sciatic NCV (F), hind paw withdrawal latency (G), and intraepidermal nerve fiber density (IENFD) (H). *p<0.05 compared to the control diet group. In all panels, n=10. (I) Representative IENFD images from one control and one high fat fed sample. Bar = 50 um, d=dermis, e=epidermis. White dots indicate nerve fibers counted.

Tau Modification in Experimental Diabetes

As mentioned at the beginning of the Program Accomplishments, we are examining how hyperglycemia and dyslipidemia inter-relate with regard to the pathology of Alzheimer's Disease. We examined both type 1 and 2 models of experimental diabetes. We discovered differences in tau biology between the two models indicating that diabetes may accelerate Alzheimer's Disease by activating multiple pathways. This work was recently accepted for publication in *Endocrinology*.

Alzheimer's disease (AD) and type 2 diabetes are two age-related diseases with high morbidity and mortality. Hyperglycemia is associated with impaired cognitive performance and an increased number of mental subtraction errors in individuals with diabetes (3). Type 1 and type 2 diabetic patients demonstrate cognitive changes in learning and memory, mental flexibility, and mental speed (4; 5). In parallel, a recent study of the Mayo Clinic AD Patient Registry reveals that 80% of AD patients have either type 2 diabetes or impaired fasting glucose (6). Many features of the metabolic syndrome, including obesity, dyslipidemia, and high blood pressure, are risk factors not only for diabetes and cardiovascular disease, but also AD (7).

The majority of the human studies focus on the connection between type 2 diabetes and AD (8; 9); however, most animal studies utilize streptozotocin (STZ)-injected animals, a model of type 1 diabetes (10; 11). We hypothesize that tau modification (hyperphosphorylation and cleavage) may serve an important connection between diabetes and AD and examined this possibility in mouse models of both type 1 (STZ injected mice) and type 2 diabetes. The BKS.Cg-m +/+ Leprdb/J mouse (commonly known as db/db) is the best-characterized genetic model of type 2 diabetes (12; 13).

Induction of Diabetes

BKS-db/db and db+ mice (BKS.Cg-m +/+ Leprdb/J, JAX Mice stock # 000642) were purchased from Jackson Laboratory (Bar Harbor, ME) and used as a model of type 2 diabetes. Db/db mice displayed elevated plasma insulin levels by 2 wk of age; hyperglycemia developed between 3-4 wk of age and frank diabetes was always present by 5 wk of age. At least 6 animals were tested for each age group for both db^+ and db/db except 16 wk (n=4).

Type 1 diabetes was induced by STZ injection when mice (C57Bl/6J, JAX Mice #000664) reached a weight of 25 g (~12 wk old). STZ was injected at the concentration of 50 mg/kg for 5 consecutive days (http://www.amdcc.org/shared/Protocols.aspx) (low dose STZ) or 150 mg/kg once (high dose STZ). At least 6 animals were used for each group. Mice were euthanized at 24 wk of age (12 wk diabetes). At least 10 animals were examined per group. Fasting blood glucose levels were measured every 4 wk to document the persistence of diabetes. After a 6-h fast, one drop of tail blood was analyzed using a standard Glucometer (OneTouch; LifeScan Inc., Milpitas, CA). Insulin level was determined as previously described by ELISA (14)
Mouse brain preparation:

Tau Phosphorylation

We measured tau phosphorylation in the cortex and hippocampus of BKS-db/db and db^+ mice at 2, 8, 16, and 24 wk of age. These ages approximately represent prediabetic (2 wk), and diabetes duration of 4, 12, and 20 wk. Tau phosphorylation of Ser199/202, Thr231, and Ser396 was measured using polyclonal antibodies directed against phosphorylated status of these residues. Total tau level was measured using Tau5 monoclonal antibody.

In 2 wk old BKS-db/db mouse brain, there was no detectable difference in tau phosphorylation between db^+ and db/db (Fig. 3A); however, as diabetes progressed (at 8 and 16 wk), tau phosphorylation increased in db/db mice, but not in db^+ mice (Fig. 3). At 24 wk, the phosphorylation levels of tau from the cortex and hippocampus of db/db mice were greatly increased at all residues examined compared to db^+ mice (Fig. 3B).

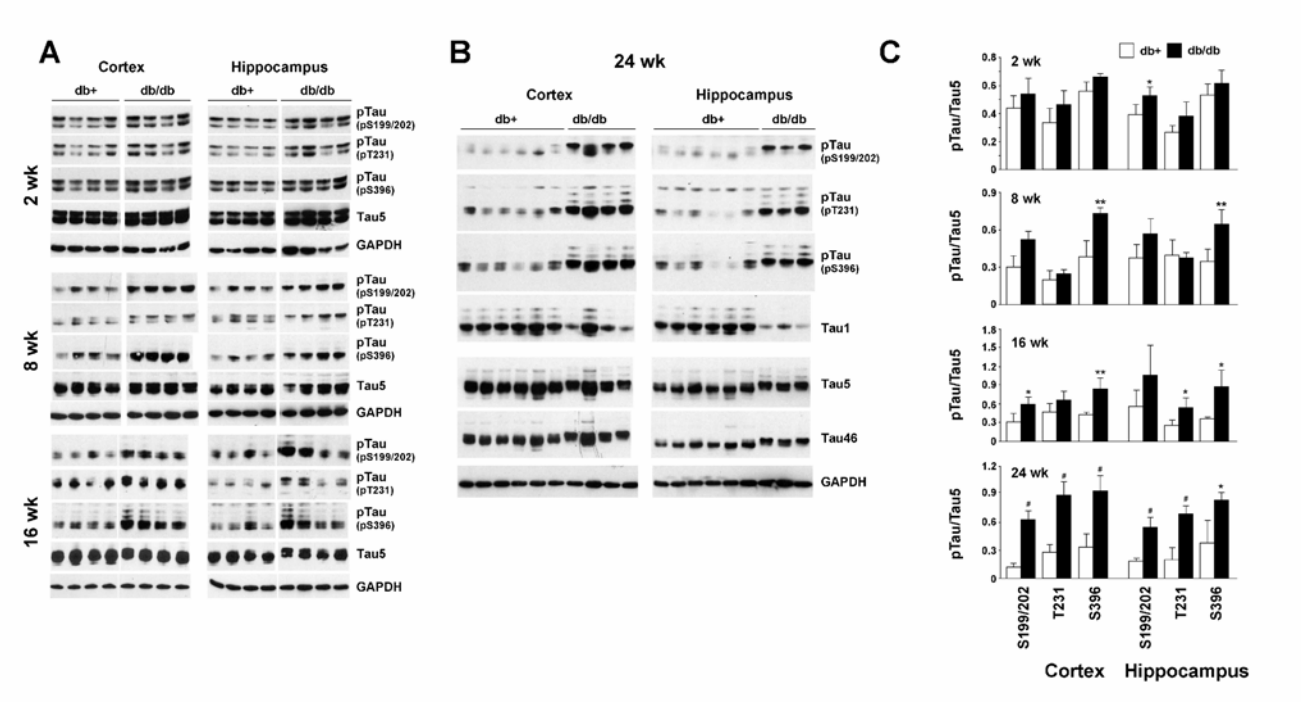

Figure 3. Age-dependent increase of tau phosphorylation in db/db mouse brain. Cortex and hippocampus from 2, 8, 16 (A) and 24 (B) wk old db+ and db/db mouse brains were homogenized in T-PER buffer. (A, B) The lysates are immunoblotted with the indicated antibodies. (C) The relative density of phosphorylated tau over total tau (Tau5) from the same mouse was measured after immunoblotting. *, p<0.05; **, p<0.01; and #, p<0.001 by *t*-test.

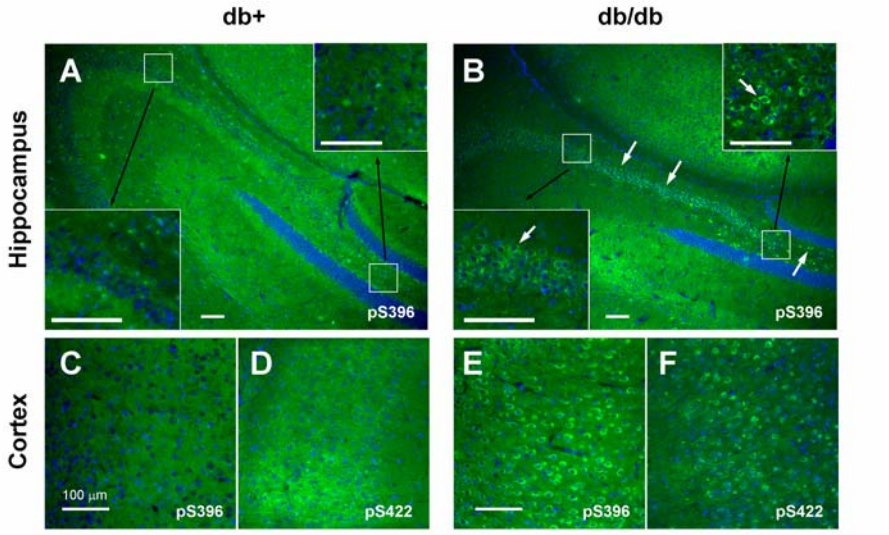

Figure 4. Tau phosphorylation is increased at multiple sites in 24 wk old db/db mouse brain. Brain slices from db+ (A, C &D) and db/db (B, E & F) mouse are stained with antibodies against phosphorylated tau (green) at Ser396 (A, B, C & E) or Ser422 (D & F) and DAPI for nuclear staining (blue). Arrows indicate the increased tau staining in db/db brains in both hippocampus (A & B) and cortex (C – F). Bar = 100 µm.

Similar results were obtained with immunohistochemistry (IHC). At 24 weeks, IHC of brain sections with anti-tau phosphorylated at Ser396 (pS396) clearly demonstrated increased phosphotau immunoreactivity in db/db (Fig 4B, arrows) hippocampus compared to db^{+} (Fig. 4A). We also detected a similar increase in tau phosphorylation in the cortex of 24 wk db/db mice using phosphotau antibodies against pS396 and pS422 (Fig 4C-2F).

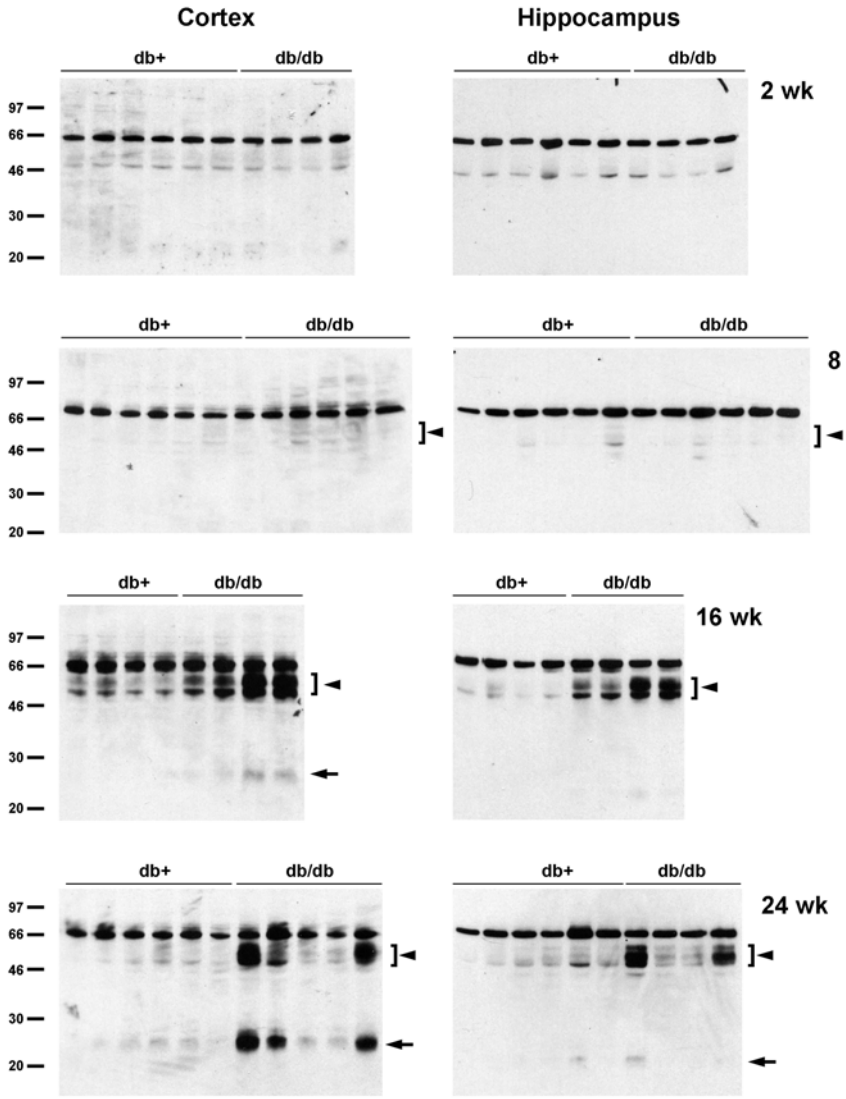

Figure 5. Increased expression of cleaved tau in older db/db mouse brain. The lysates of cortex or hippocampus from db+ and db/db mouse brains were immunoblotted with TauC3 antibody. There is an age-dependent increase of cleaved tau expression (arrowheads), including a smaller fragment around 25 kDa (arrows), in db/db mouse brains.

Tau Cleavage in db/db Mouse Brains

Tau cleavage as well as tau hyperphosphorylation plays an important role in the progression of AD (15-17). Therefore, we next examined the possibility of tau cleavage in db/db mice. Lysates from cortex and hippocampus of db/db mice were immunoblotted using a TauC3 antibody that detects tau cleaved at Asp421. We observed an increase in tau cleavage in db/db mice, as early as 8 wk (Fig. 5). At 16 and 24 wk, we also observed a smaller cleavage product around 25 kDa in the db/db mice. These cleaved bands, however, were not detected at 2 wk. IHC at 24 wk demonstrated an increase in TauC3 immunoreactivity in the hippocampal CA2/CA3 region of db/db mice (Fig 6).

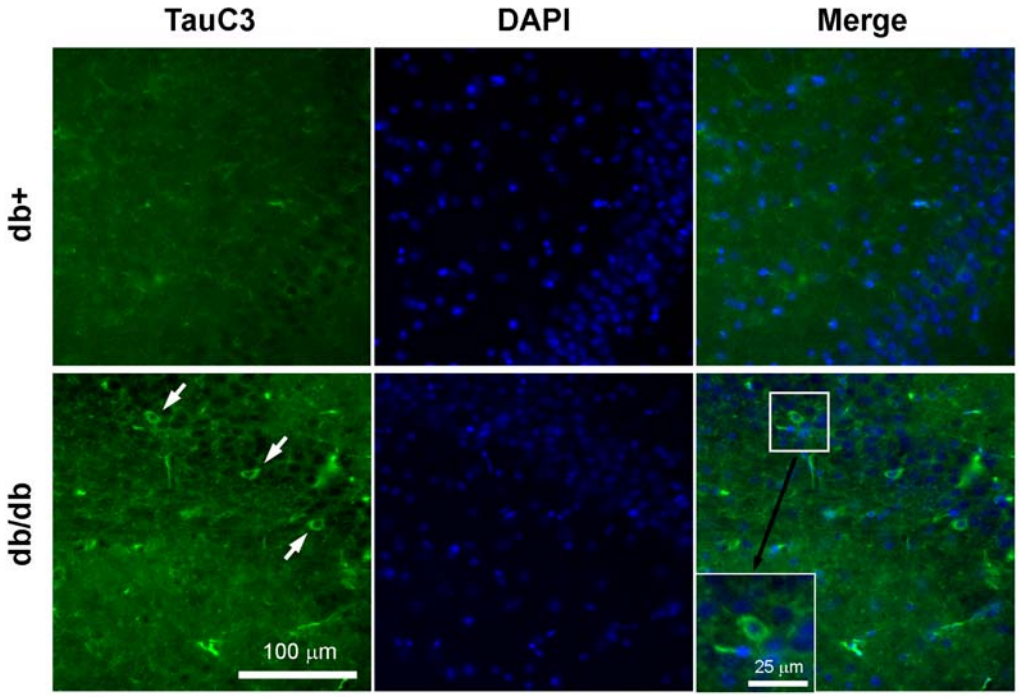

Figure 6. Increased TauC3 immunostaining in 24 wk old db/db mouse hippocampus. Brain slices from 24 wk old db+ and db/db mice were processed for immunohistochemistry using TauC3 antibody detecting cleaved tau (green). Nuclei are stained with DAPI (blue). Arrows indicate TauC3 immunopositive cells in db/db mouse hippocampus. Bar = 100 μm

Tau Phosphorylation in STZ-Injected Mice

We next examined whether similar changes in tau occur in type 1 diabetic mice. C57Bl/6J mice were injected with STZ at 12 wk of age and euthanized 12 wks later. A low dose group was injected with 50 mg/kg STZ for 5 consecutive days and a high dose group received a single injection of 150 mg/kg STZ. At 24 wk both low (25.21 + 1.4 g) and high dose STZ (25.19 + 3.7 g) groups demonstrated decreased body weight compared to control (29.17 + 2.5 g).

We detected increased tau phosphorylation in the high dose STZ group at residues of Thr231 and Ser199/202 (Fig. 7A & 5B). In spite of increased glucose levels and frank diabetes, tau phosphorylation was not increased in low dose STZ group. The difference in tau phosphorylation at Ser396 did not reach statistical significance between control and high dose STZ group. IHC indicated increased tau phosphorylation only in high dose STZ group, but not in control or low dose STZ group (Fig. 7C). Tau phosphorylation in the db/db mice was accompanied with the increased tau cleavage. In contrast, we did not detect tau cleavage even in high dose STZ group (Fig. 7D) which displayed increased tau phosphorylation. These results suggest that the

increased incidence of AD in type 1 and type 2 diabetes may be mediated by different mechanisms.

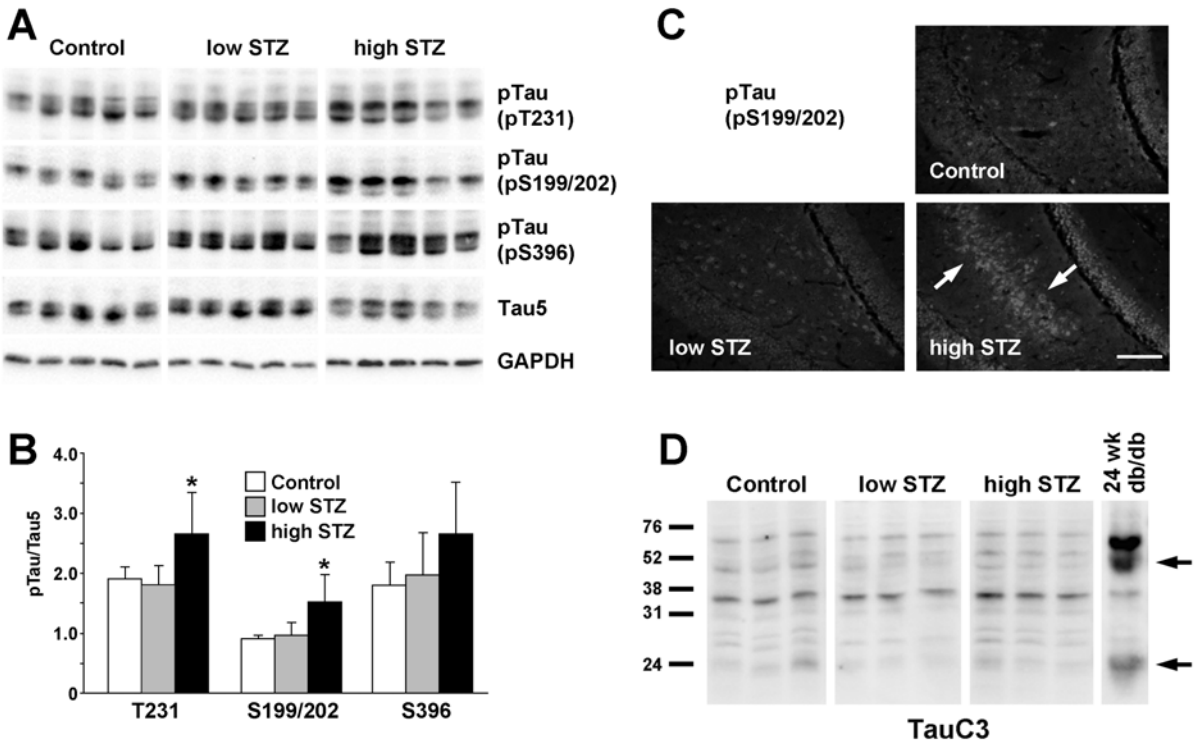

Figure 7. Tau phosphorylation, but not cleavage, is increased in STZ-injected animals. Mice are injected with STZ at 12 wk of age and sacrificed at 24 wk (12 wks diabetes). Low STZ group received 50 mg/kg STZ for 5 consecutive days and high STZ group received one injection of 150 mg/kg. (A) Cortex lysates are immunoblotted for the phosphorylated and total tau. (B) The densitometric analysis indicates significant increase in tau phosphorylation at pT231 and pS199/202 for the high STZ group compared to control or low STZ. *, p<0.01. (C) Brain slices are stained with antibody against phosphorylated tau at Ser199/202. Arrows indicate the increased tau staining in high dose STZ brains. Bar = 50 m. (D) Cortex lysates are immunbloted with TauC3 antibody to detect cleaved tau (arrows).

In this report, we demonstrate for the first time that increased tau phosphorylation in type 2 diabetic mouse brain is accompanied with enhanced tau cleavage. Mounting evidence suggests that not only the hyperphosphorylation, but also the cleavage of tau plays an important role in the progression of AD (15-17). Our results from db/db mice, suggest a temporal sequence of hyperinsulinemia/hyperglycemia, tau hyperphosphorylation, and eventual tau cleavage in diabetic animals. We also notice that tau cleavage in 24 wk db/db mice demonstrated high variability whereas tau phosphorylation is more constantly observed. These results suggest that tau cleavage may not be fully active at this stage resulting in more variability. Tau cleavage was not observed in STZ-injected mice and displayed lower levels of tau hyperphosphorylation compared to db/db mice. Therefore tau hyperphosphorylation combined with cleavage may contribute to the more severe tau pathology in type 2 diabetes animal brains.

In summary, our results suggest a new mechanistic link between increased susceptibility to AD and diabetes. We demonstrate using type 1 and type 2 diabetic mice that hyperglycemia (diabetic pathology) and tau modifications (AD pathology) are tightly correlated. In type 2

diabetes the combined effect of tau hyperphosphorylation and cleavage may contribute to more prominent AD-like progression.

Current Animal Models of Diabetes and Diabetic Neuropathy

Considerable controversy has existed over the development of DPN in the C57Bl/6J mouse and may be related to variations on the method of STZ administration. We examined this issue by using low dose (50 mg/kg for 5 consecutive days) versus high dose (a single dose of 150 mg/kg) STZ treatment. Our results indicate that in the animals receiving low dose STZ, a small amount of insulin remains and may be neuroprotective.

Table 1: AMDCC Low Dose STZ Induction Versus Single High Dose Induction

	Blood Glucose Mg/dL	Weight g	Hind Paw Latency sec	Sural Sensory NCV m/sec
Control n = 5	136 ± 44	30 ± 2.3	4 ± 0.6	18.8 ±2.4
Low Dose STZ n = 5	400 ± 114	25.5 ± 1.7	4 ± 0.4	18.0 ± 3.0
High Dose STZ n = 5	486 ± 78	25.2 ± 3.7	7.2 ± 2	14 ± 1.5*

*p<0.05 compared to control and low dose STZ administration

An area of concern, especially with diabetic mice, is repeated anesthesia. We compared the effects of four typically used methods of anesthesia, isofluorane, ketamine, Nembutal (sodium pentobarbital) and Avertin (2,2,2-tribromoethanol) in adult C57/Bl6 12 week old mice, 6 animals per group. None of the four agents significantly affected sensory nerve conduction but motor conduction velocity was depressed by ketamine, Avertin and Nembutal. The animals were also better able to maintain core body temperature and respiratory rate under isoflurane anesthesia. Finally, they also recovered more quickly from isoflurane. This method of anesthesia will be added to the AMDCC protocols.

Table 2: Anesthesia Effects Motor NCV in C57/Bl6 Mice

	Isoflurane	Ketamine	Avertin	Nembutal
Sciatic Motor NCV m/sec	63.0 ± 6.0	52.4 ± 3.4	50.0 ± 5.2	50.0 ± 4.6
Rectal Temperature ^{o}C	35.0 ± 0.5	30.0 ± 2.0	31.0 ± 0.7	31.0 ± 1.0

2. Plans for the Upcoming Year:

We are currently pursuing two new animal models to investigate our novel hypothesis that increased levels of plasma lipids and oxidative stress act in concert with glucose to produce injury leading to diabetic neuropathy. Our goal is to establish the role of dyslipidemia and the relative contributions of dyslipidemia and hyperglycemia to the development of neuropathy in

mouse models. The mice we are using for this study were developed by Murielle Veniant-Ellison and co-workers at Amgen (can provide MTA upon request). They developed triple knockout (3 KO) mice that are deficient in either ApoE or in the low density lipoprotein receptor Ldlr and are both additionally lacking leptin (ob/ob) and apolipoprotein B-48. Both lines develop obesity, hyperinsulinemia, hyperlipidemia, hypertension, and atherosclerosis. However, only ApoE 3KO mice are hyperglycemic and glucose intolerant - they are more obese than Ldlr 3KO mice. By comparing neuropathy phenotypes and biochemical changes between these lines we will determine the contribution of dyslipidemia to the onset and progression of neuropathy. Our goal is to phenotype mice for the onset and progression of neuropathy over time and, at 6 months of age, perform terminal neuropathy phenotyping, including metabolic profiling of lipids and oxidative modifications in plasma, DRG, and sciatic nerve and explore the biochemical alterations in mitochondria, NAD(P)H oxidase, and the oxidative stress pathways. A plan for our work is presented in Table 3. We are currently completing the 12 week phenotyping.

The mice will be maintained on the same chow as the original studies , the 22/5 Rodent Diet from Harlan Teklad (Indianapolis, IN), containing 22% protein, 5% fat, and 4.5% fiber. Mice will be assigned to groups of 12 mice each as follows: ApoE 3KO, Ldlr 3KO and C57BL/6 wt. These 3 groups will be maintained up to 24 wk age. Phenotyping will be performed according to the schedule in Table 3. Eight animals per group is powered to detect a 2 msec change in hind paw withdrawal and 2 m/sec change in motor nerve conduction velocity (44) so 12 animals per group should provide sufficient numbers to complete the study.

Table 3. Neuropathy Phenotyping Schedule for 3 KO Mice and Controls.

Evaluation	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24
Weight	X	X	X	X	X	X
Glucose	X	X	X	X	X	X
HbA1C						X
Glucose Tolerance Test			X			X
Lipids, oxLDL, oxidative stress markers						X
Tail blood pressure	X		X			X
Thermal sensitivity testing	X	X	X	X		X
Nerve conductions			X			X
Von Frey mechanical sensation	X	X	X	X	X	X
Intraepidermal nerve fiber density & skin microvessel analysis						X
IHC markers						X
DRG TUNEL						X
Tissue Harvest						X

We are also investigating the role of dyslipidemia and neuropathy in two other animal models. For a type 1 model, we treated the DBA2J mice with fenofibrate, but the animals became ill. We are currently repeating this study with rosiglitazone. For another type 2 model (other than the 3KO mice described above), we have crossed the ApoE knockout mice onto a db/db (leptin deficient) background. Our goal is to demonstrate that further perturbations in plasma lipids will compound the progression of diabetic neuropathy in these mice. We will complete phenotyping on this animals at 12 and 24 weeks with sufficient numbers in each genotype by the end of September 2009 and will present these data at the annual AMDCC meetings. The 12 weeks animals show a trend to slower hind paw withdrawal and sciatic motor nerve conduction velocity with loss of leptin expression ahead of the development of insulin resistance. Db+ mice do not

develop diabetes, but we observe a trend to increase neuropathy in ApoE-/- db+ compared to ApoE-/- wild type (ApoE wt).

2. <u>Collaboration:</u>

We are continuing our ongoing collaboration with the Kretzler and Brosius laboratories for cross tissue comparisons of genes regulated by diabetes. To obtain insight into the processes leading to DPN and nephropathy (DN), gene expression profiles of human sural nerve and kidney biopsies from diabetic patients were surveyed by microarray analysis. A total of 4,680 and 4,630 genes were differentially regulated in nerve (between progressive and non-progressive DPN) and kidney (between albuminuric and non-albuminuric DN), respectively. A cross-tissue comparison of transcriptional networks using a suboptimal graph matching tool allowed the identification of a core network of 91 genes (Fig. 8). This shared network includes well-studied diabetes-related genes such as peroxisome proliferator-activated receptor gamma (PPARG) and leptin receptor (LEPR). Identification of these key genes confirms the validity of the current approach of pursuing a shared transcriptional network. This conserved gene network is expected to lead us to a better understanding of disease progression and will serve as a starting point to define therapeutic strategies targeting micro-vascular complication of DM independent of organ manifestation.

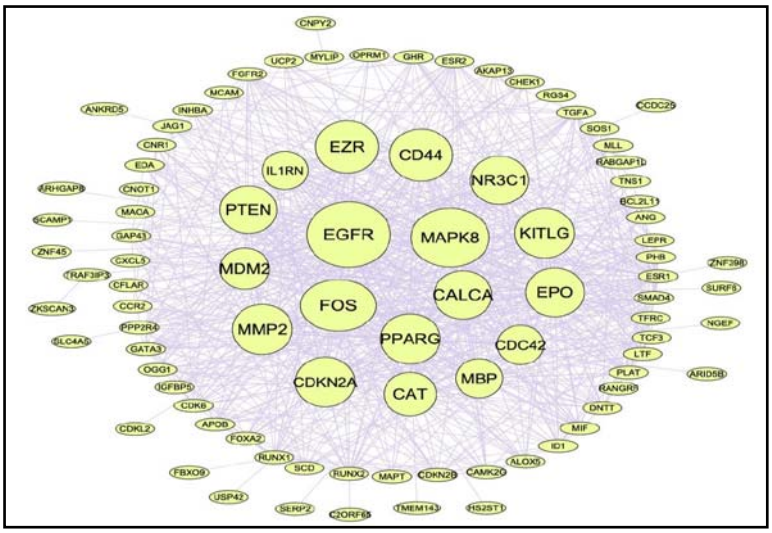

Figure 8. Gene-gene network shared by neuropathy and nephropathy TALE identified 91 shared core genes and their network. The size of each node is representing the number of interactions with other genes. Well-studied diabetes-related genes such as peroxisome proliferator-activated receptor gamma (PPARG) and leptin receptor (LEPR) are included in the shared network, confirming the validity of the current approach.

<u>Dr. Moshe Levi</u>

We provided neuropathy phenotyping for Dr. Moshe Levi in August 2009. He has a unique colony of mice, now 5-6 months of age, treated with low or high fat diets plus or minus agonists for Farnesoid X Receptor or the G protein-coupled receptor for bile acids, TGR5. His studies are aimed at understanding these diets and receptors in diabetic nephropathy. This study compliments and extends our current studies where we are assessing the role of hyperlipidemia in diabetic neuropathy (see our paper in press in *Diabetes* PMID: 19592619). We will perform neuropathy and plasma lipid profiling in these mice. This is the first full neuropathy phenotying in this diabetic mouse model, as well as the first examination of the FXR and TGR5 in neuropathy. The study was completed last week and data analyses are pending and will be provided at the AMDCC annual meeting. We will harvest plasma, sciatic nerves, spinal cord, dorsal root ganglia, and foot pads.

JAX Laboratory Update

Stock No.7210 - BKS.Cg- Lepr<db> Sod2<tm1Shs> – SNP typing of the N5 generation identified 2 females and 1 male with segregating markers in the congenic interval only. These mice were intercrossed to expand the family and identify Sod2<floxed> homozygous mice. JAX now has 6 het x hom and 4 hom x hom (Sod2<floxed allele> and heterozygous for db) matings set up. Mice homozygous for the SOD2 mutation have been set up to expand the colony and ultimately have a colony homozygous for the SOD2 mutation and segregating for the Lepr<db> mutation. JAX currently has a small quantity of homozygous Sod2<tm1Shs>/+, Lepr<db>/+ in mating. Pups have been weaned and JAX is waiting for genotyping results. This stock is almost established to supply mice homozygous for Sod2<tm1Shs> and segregating for Lepr<db>.

Stock#7219- BKS.Cg- Lepr<db Tg(Nes-cre)1Kln /J – JAX has identified Lepr<db> Tg(Nes-cre) mice with no segregating SNP markers outside of the congenic region. These mice have been mated to BKS-db to expand the family (waiting for genotyping results of weaned pups) and matings have been set up with stock 7210- BKS.Cg- Lepr<db> Sod2<tm1Shs>

Stock 7210 has been mated to stock 7219 to produce mice heterozygous for Lepr<db>, Sod2<tm1Shs>, and Tg(Nes-cre)1Kln. The first litter is being genotyped and this colony will need expansion to provide breeder mice heterozygous for Lepr<db>, Sod2<tm1Shs> and Tg(Nes-cre)1Kln and BKS.Cg- Lepr<db> /+ Sod2<tm1Shs> homozygous for shipment to establish a breeding colony to produce experimental animals homozygous for Lepr<db>, Sod2<tm1Shs> and Tg(Nes-cre)1Kln hemizygous. As mice homozygous Tg(Nes-cre) on the B6 background are runted and in poor health, JAX is not planning to attempt to make the BKS. Lepr<db>, Tg(Nes-cre) homozygous for either allele.

JAX is hoping to be able to provide breeder mice so that we can produce the experimental mice in the next 2-4 months.

With the MMPCs

We are currently sending samples to the Seattle Mouse Metabolic Phenotyping Center (MMPC) Housed at the University of Washington for total cholesterol and triglyceride analyses and pooled FPLC. An example of data from a new low fat versus high fat feeding study in C57Bl/6J mice is presented in Figure 9. We have sent over 100 samples to the MMPC.

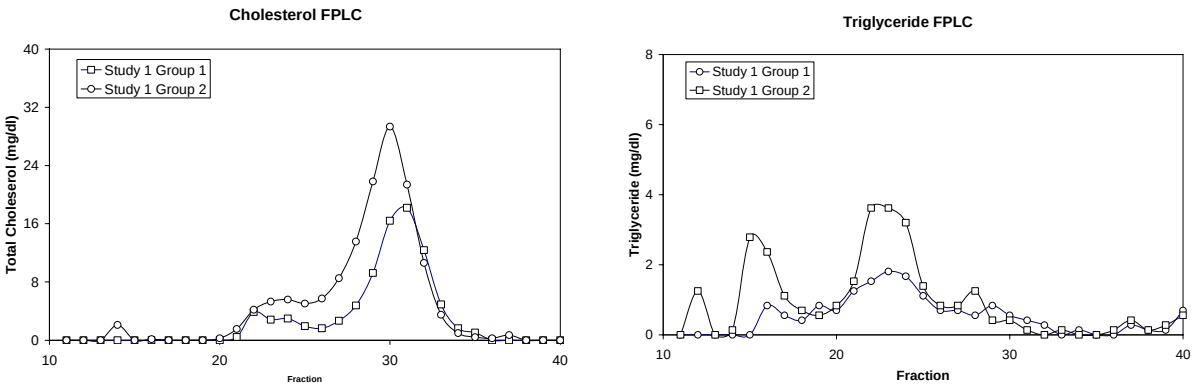

Figure 9. Cholesterol and triglyceride levels are affected by diet. Cholesterol (left panel) and triglycerides (right panel) are elevated in C57Bl/6J mice receiving a high fat diet for 35 weeks (group 2) compared to controls (group 1).

3. Address Previous EAC Comments

Feldman

1. The organization of the NeuroDiab satellite meeting with the goal of arriving at validation criteria for neuropathy is an excellent plan.

The meeting took place in Orvieto in September of 2008. It was a lively and productive series of discussions resulting in a Consensus Statement. This document has been circulated for comment and is currently undergoing final edits prior to publication.

NeuroDiab Satellite Program 2008 Participants

Kristin Abraham – NIH/NIDDK
Barbera Araneo - JDRF
Geert-Jan Biessels – University Medical Center Utrecht
Vera Bril - University of Toronto
Norman Cameron - University of Aberdeen
Nigel Calcutt – University of California, San Diego
Mary Cotter - University of Aberdeen
Rick Dobrowsky - University of Kansas
Eva Feldman – University of Michigan
Paul Fernyhough - University of Manchester
Helen Nickerson - JDRF
Johannes Jakobsen - Århus Kommunehospital
Chris Ketchum – NIH/NIDDK
Rayaz Malik - University of Manchester
Andrew Mizisin - University of California, San Diego
Peter Oates - Pfizer
Irina Obrosova - Pennington Biomedical Research Center/Louisiana State University System
Rodica Pop-Busui - University of Michigan
James Russell – University of Maryland
Anders Sima – Wayne State University
Martin Stevens - University of Michigan
Solomon Tesfaye - Sheffield Teaching Hospitals NHS Foundation Trust Diabetes UK
David Tomlinson - University of Manchester
Aris Veves - Beth Israel Deaconess Medical Center, Harvard Medical School
Arthur Vinik - Eastern Virginia Medical School
Douglas Wright - The University of Kansas Medical Center
Soroku Yagihashi - Hirosaki University
Mark Yorek - The University of Iowa
Dan Ziegler - German Diabetes Center DDZ
Doug Zochodne - University of Calgary

2. DBA2 expression studies have been completed; it may be useful to eventually compare these data to Davis' expression QTL to better define the QTL. The BKS expression is underway and comparing these to the DBA data could be fruitful since BKS is a recombinant congenic between B6 and DBA. Comparing the expression data between tissues (nerve and Kidney) and to Kretzler's human data is a good idea.

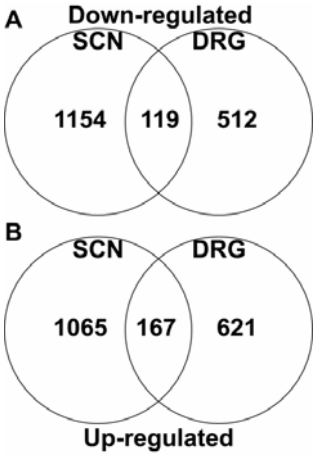

Figure 10. A comparison of up- and down-regulated genes in the DRG and sciatic nerve of BKS-db/db mice. Microarray analyses were performed 20 weeks post onset of diabetes. Each tissue displays a unique set of regulated genes; however, there are regulated genes common to both tissues

We are currently analyzing microarray data from dorsal root ganglia (DRG) and sciatic nerves dissected from BKS-db/db and db^{+} mice harvested at 24 weeks of age (20 weeks of diabetes). Our initial analyses reveal uniquely regulated genes in both tissues; however, there is considerable overlap as illustrated in Figure 10. Further analyses are underway to confirm a subset of these genes with RT-PCR and to examine their resulting proteins with western immunoblotting and immunolocalization.

With regard to our human data, we compared the results of gene expression analyses between archived sural nerve and kidney biopsies. The sural nerve samples were classified as progressing or non-progressing diabetic neuropathy (DPN). The kidney biopsies were classified as albuminuric vs. non-albuminuric. Two transcriptional networks were generated by the literature-mining tool BiblioSphere using 4680 DPN and 4630 DN DEGs respectively. TALE, the Tool for Approximate Large Graph Matching, was used to identify sub-networks shared by the two networks. Figure 8 of our progress report illustrates the identified shared sub-network of 91 nodes. Well-known diabetes-related genes such as peroxisome proliferator-activated receptor gamma (PPARG) and leptin receptor (LEPR) are included in this sub-network.

3. Backcrossing of the SOD allele onto BKS-db seems to be progressing well.

This work is being conducted by JAX.

4. The Jax need specialized training in nerve dissection. Should we post Nigel's video tutorials on the web under protocols? Are there other protocols that would benefit from video tutorials?

We would be happy to create videos of fresh or fixed DRG dissection, nerve conduction studies or any procedure deemed useful by the AMDCC investigators.

5. We still cannot measure nerve blood flow in mice. How do we push the technology? Next MMPC P&F program (http://www.mmpc.org/shared/pilotFeasibility.aspx)?

This is not within the Feldman laboratory's area of expertise; however, we would be happy to work with investigators interested in this measure.

6. Good progress. Longer term models are very welcome.

Thank you.

7. Two excellent review articles. Dr. Sullivan is a major asset/resource for this group.

Thank you.

8. Data tends to be "sciatic" centric. Encourage the use of sural nerves, and more extensive analysis and use of the DRG samples-for mRNAs, WBs, array work and for quantitative morphometry and immunohistochemistry.

We agree and are currently dissecting the sural nerve for morphometric analyses. The sural nerve in mice is very small but RT-PCR measures should be possible. Western blotting may require pooling of nerves from several animals to obtain enough protein for reliable measurements. Radioimmunoassay (RIA) or ELISA may be useful for some proteins. If pooling of multiple animals proves necessary, they would be grouped based on multiple factors including degree of hyperglycemia (glycated hemoglobin) and nerve conduction velocity.

Analysis of DRG mRNA is underway and our initial results are included above (comment 2)

9. More morphometric analysis needed e.g. on distal sural samples.

We are currently harvesting proximal and distal sciatic and sural nerves. This is especially relevant now that we are beginning to assess mitochondrial numbers and morphology. Subtle changes in these organelles are likely to occur in a distal to proximal gradient.

10. Serial studies-electrophysiology, behavior very important.

We strongly agree and are documenting behavioral changes at multiple time points across the experimental period. Great care must be applied to nerve conductions measures. It is possible for a "near nerve" recording to become an actual nerve recording. Puncture of the nerve results in damage and scarring that may impact subsequent measures. It is possible to measure the right side early in the experiment and the left nerve at study end assuming that all conductions are normal at study onset. The one caveat of this approach remains the potential of nerve puncture that may confound anatomical measures. To avoid this type of problem, the nerve measured last could be used for anatomical measures as a "fresh" puncture wound could be identified during tissue harvest.

We have also addressed issues of repeated anesthesia, see Progress Report

11. Important to stay skeptical of the microarray data - if the fold changes are less than 2 fold, hard to know if it is truly significant.

We agree and consistently perform both technical and biological controls to validate microarray data. Differences in chips and analysis techniques (Cyber T versus Chip Inspector) identify genes as "regulated." These techniques are useful tools for examining broad changes in gene expression and identifying potential novel gene regulation. Only be confirming the biological

relevance of identified genes through Q-PCR, i.e., a separate set of samples from a parallel group of animals, are we able to assess their true significance. Other informatics tools are useful in placing identified genes into known pathways and exploring the literature for parallels in other diseases or tissue types.

12. Glial (SC or satellite cell) vs. neuronal content of specific transcripts not considered. Investigators should give some thought as to which cell type is responsible for specific changes and why.

We agree and are currently comparing regulated gene expression in DRG and sciatic nerve. Both tissues contain glia; however we anticipate an enrichment of neuronal genes in the microarray data derived from DRG. Many of the informatics databases may be sorted by cell and tissue type. This type of data filtering is always performed when analyzing whole tissue experiments. We are also able to compare/confirm cell specific expression by performing Q-PCR mRNA collected from enriched DRG or Schwann cell cultures. Other cell types that we consider are fibroblasts and endothelial cells.

13. RT-PCR of sciatic nerves must be viewed with caution. Most of the mRNAs will be non-neuronal in this sample and its meaning interpreted accordingly. Comparing human nerve samples with mouse samples will have the same issues.

See above.

14. Overall emphasis here is on creating new mouse models with specific mitochondrial abnormalities. While these models will give important information about this aspect of diabetes neuropathic complications, the concept is narrow and assumes that this is the only direction worth pursuing. There is a very much larger neuroscience picture of neurodegeneration here with a wide range of other players and mechanisms that should be exploited given the terrific advantages of having the AMDCC mechanism. It will be very important to retain a very open and inclusive mind about what mechanisms promote neuropathy given the history of many blind directions in this field in the past.

We agree that the exact etiology of DPN has yet to be determined and it is highly likely that multiple mechanisms play a role. Our laboratory is particularly interested in mitochondrial biology. We would be extremely pleased to collaborate and share models and our expertise with other investigators (AMDCC and otherwise) who are expert in the areas of advanced glycation endproducts, axonal transport or other pathways. We have phenotyped neuropathy in other models of diabetic complications that are not related to mitochondrial biology; however, we do not have the person power to fully characterize these models but hope other interested investigators do so.

15. Overall there are many other AMDCC models that could be highly relevant to neuropathy e.g. eNOS knockout, bradykinin knockouts, etc.

As listed in our 2008 Progress Report we have performed neuropathy phenotyping on bradykinin/akita mice supplied by the Smithies laboratory via JAX. In collaboration with the Abel laboratory we phenotyped Swiss Webster mice, Swiss Webster mice, made diabetic with STZ and treated with netrin and CD1A mice. As this report is being written, my technical staff is coordinating a trip to Denver to phenotype mice in the Levi laboratory. The results of this analysis will be reported to the AMDCC as soon as it is completed.

16. Minor items: Too many significant digits in nerve conduction and behavioral data (Tables) i.e. two decimal places in the mean and two in the SEM. 12.67+0.38 should read 12.7+0.4. Some of the sural NCV values seem very low-has near nerve temperature (not just rectal) been monitored and controlled? Page 17 top paragraph-do you mean neurturin or netrin?

We have modified the data tables included in this Progress Report. Page 17, netrin.

4. <u>Publications:</u>

Figueroa-Romero C, Sadidi M, **Feldman EL.** Mechanisms of disease: The oxidative stress theory of diabetic neuropathy. *Reviews in Endocrine and Metabolic Disorders*, 9, 301-314, 2008. PMID:18709457

Wiggin TD, **Kretzler M**, Pennathur S, Sullivan KA, **Brosius FC, Feldman EL.** Rosiglitazone treatment reduces diabetic neuropathy in STZ treated DBA/2J mice. *Endocrinology*, 149, 4928-4937, 2008. PMC2582925

Hur J., Schuyler A.D., States D.J., **Feldman E.L.** SciMiner: Web-based literature mining tool for target identification and functional enrichment analysis. *Bioinformatics*, 25, 838-40, 2009. PMID: 19188191

Peltier A., Smith A.G., Russell J.W., Sheikh K., Bixby B., Howard J., Goldstein J., Song Y., Wang L., **Feldman E.L.**, Singleton J.R. Reliability of quantitative sudomotor axon reflex testing and quantitative sensory testing in neuropathy of impaired glucose regulation. *Muscle Nerve,* 39, 529-535, 2009. PMID: 19260066

Pop-Busui R., Low P.A., Waberski B.H., Martin C.L., Albers J.W., **Feldman E.L.**, Sommer C., Cleary P.A., Lachin J.M., Herman W.H.; for the DCCT/EDIC Research Group. Effects of prior intensive insulin therapy on cardiac autonomic nervous system function in Type 1 diabetes: The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC). *Circulation*, 119, 2886-2893, 2009. PMID: 19470886

Schuyler A.D., Carlson H.A. and **Feldman E.L**. Computational methods for predicting sites of functionally important dynamics. *Journal of Physical Chemistry*, 113, 6613-6622, 2009. PMID: 19378962

Vincent A.M., Hayes J.M., McLean L.L., Vivekanandan-Giri A., Pennathur S., **Feldman E.L**. Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1. *Diabetes* Jul 10 [Epub ahead of print] 2009 PMID: 19592619

Currently in press, in revision or submitted:

Edwards J.L., Quattrini A., Lentz S.I., Figueroa-Romero C., Cerri F., Backus C., Hong Y., **Feldman E.L**. Diabetes regulates mitochondrial biogenesis and fission in neurons. *Diabetologia* in revision

Kim B., Backus C., Oh S.S., Hayes J.M., **Feldman E.L.** Increased Tau Phosphorylation And Cleavage In Mouse Models of Type 1 And Type 2 Diabetes. *Endocrinology* 2009 in press

Lentz S.I., Edwards J.L., Backus C., McLean L.L., Haines K.M., **Feldman E.L.** Mitochondrial DNA (mtDNA) Biogenesis: Enhanced Visualization of BrdU and EdU Incorporation in Newly Synthesized mtDNA In Vitro. *Journal of Histochemistry and Cytochemistry* submitted

Pop-Busui R., Roberts L., Pennathur S., Kretzler M., Brosius F., **Feldman E.L.** The management of diabetic neuropathy in chronic kidney disease and dialysis patients. *American Journal of Kidney Diseases* in revision

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