Animal Models of Diabetic Complications Consortium
(U01 U01 DK60994)

Annual Report
(2005)

"Mouse models of diabetic nephropathy and neuropathy"
Universities of Michigan and Chicago

Principal Investigator
Frank C. Brosius

Address: 1560 MSRB II, 1150 W. Medical Center Dr.
Department of Internal Medicine
Division of Nephrology
Ann Arbor, MI 48109-0676
Phone: 734-936-5645
E-mail: fbrosius@umich.edu

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Principal Investigator's Summary

1. Program Accomplishments:

The overall goal of our center is to develop improved mouse models of diabetic complications especially for nephropathy and neuropathy. Our main strategy is to enhance diabetic injury by increasing glucose uptake and/or oxidative stress in podocytes in the kidney glomerulus and in peripheral neurons in order to augment diabetic injury. Therefore, we are focusing on models with genetic alterations that should change glucose transporter expression or increase oxidative stress in glomerular podocytes and in peripheral neurons.

Since we are investigating both complications, we have developed 2-4 models for each complication. These have not turned out to be the same for each complication. Four models are briefly presented for nephropathy because the second model was a direct derivative from the first and the data on the 4th model are recent confirmations and extensions of previous work by two other AMDCC investigators, Drs. Breyer and Coffman.

Nephropathy models: 1) GLUT1 tg C57BL/6J db/db "high fat" diet
2) Nphs2 GLUT1 tg C57BLKSdb/db
3) GLUT4 -/- STZ or Akita
4) DbA/2J

Neuropathy models: 1) C57BLKS db/db
2) Nestin SOD2 -/- STZ diabetic.

.**Major achievements have been:**

Nephropathy Models:

1) *GLUT1 tg C57BL/6J mice ± db/db high fat diet*

The GLUT1 transgenic model was developed by Dr. Heilig at the University of Chicago and has been analyzed as part of our AMDCC unit. In these mice, GLUT1 expression was driven by a modified β-actin promoter and was expressed in many tissues including glomerular mesangial cells. These animals developed albuminuria (**Fig. 1**) and mesangial expansion on a C57BL/6J background in the absence of diabetes. In addition these animals developed substantial increases in glomerular fibronectin and type IV collagen accumulation (**Fig. 2**) as well as marked glomerular enlargement (**Fig. 3**). Finally, there appears to be a gradual increase of HPLC determined serum creatinine and concomitant deterioration of glomerular filtration in these animals (from 0.63 to 0.88), suggesting for the first time that increased glucose uptake by glomerular cells could lead directly to glomerular enlargement, nephrosclerosis, and some decrement in renal function or alternatively significant hyperfiltration which gradually diminishes. These animals have additional phenotypes which suggest that GLUT1 participates in enhancing diabetic pathology in a

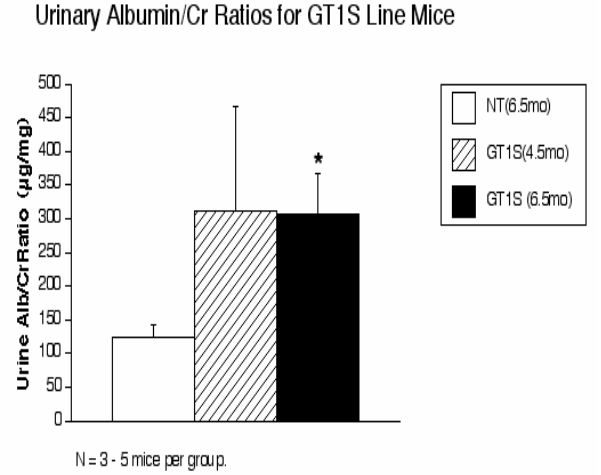
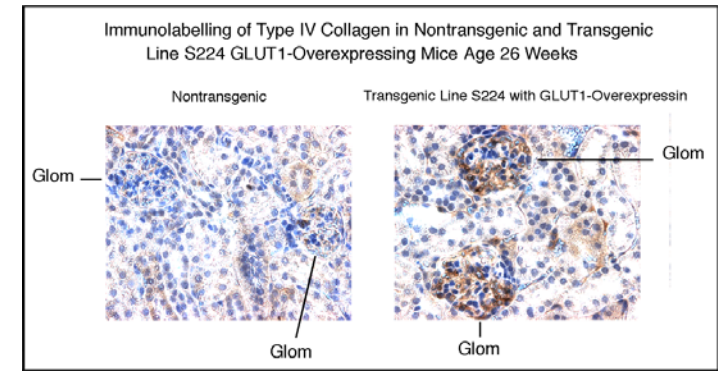


Fig. 1. Nondiabetic GLUT1 tg mice developed a greater than 2-fold increase in albuminuria by 4.5 months of age.



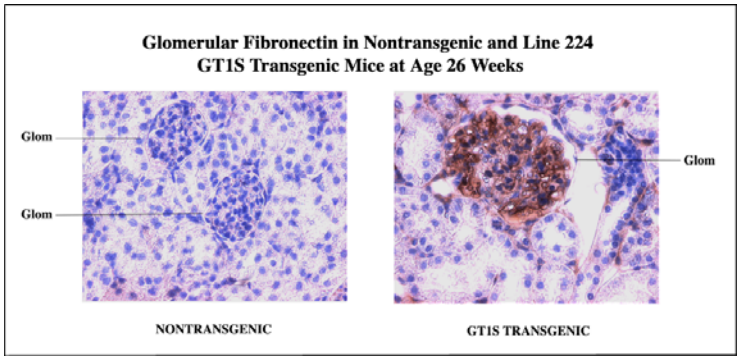


Fig. 2. Immunohistochemical staining of nondiabetic GLUT1 tg mice and nontransgenic mice showing substantially increased glomerular collagen IV and fibronectin accumulation in the GLUT1 tg mice.

number of tissues. For example, left ventricular mass and volume are increased in hearts from these mice (not shown.

Curiously, when bred into a db/db C57BL/6J line, the nephropathy was not significantly augmented in the diabetic mice compared to the nondiabetic ones. The diabetic mice were put on a chow with mildly elevated fat content (~6.5%) compared to normal rodent chow (~4.5%). This promoted a substantial increase in hyperglycemia, glycosylated hemoglobins and indices of nephropathy (**Figs. 4**). Data from models surveyed earlier in the AMDCC funding period indicated that the db/db (leptin receptor) mutation resulted in only modest and transient hyperglycemia and minimal nephropathy. Thus, the institution of a somewhat higher fat diet resulted in substantial worsening of the diabetes

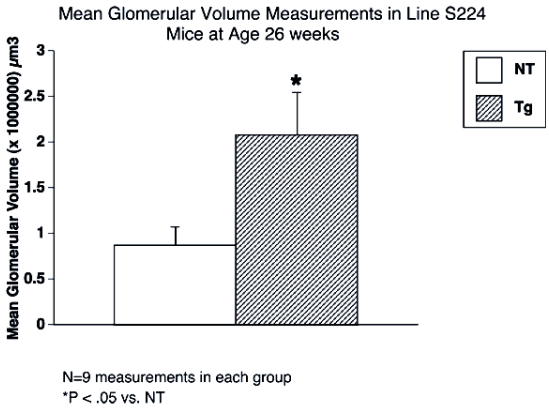


Fig. 3. Determination of glomerular volume by 2-serial profile method[1] showing a greater than 2 fold increase in glomerular volume in GLUT1 tg mice.

and a major increase in albuminuria and mesangial expansion. However, by contrast, the GLUT1 tg bred into this model failed to augment nephropathy parameters (**Fig. 4**), despite confirmation of robust overexpression of GLUT1 in glomerular extracts from these mice. It is not certain why the GLUT1 transgene which was fully expressed in glomerular cells in this model, failed to augment nephropathy. Possible explanations

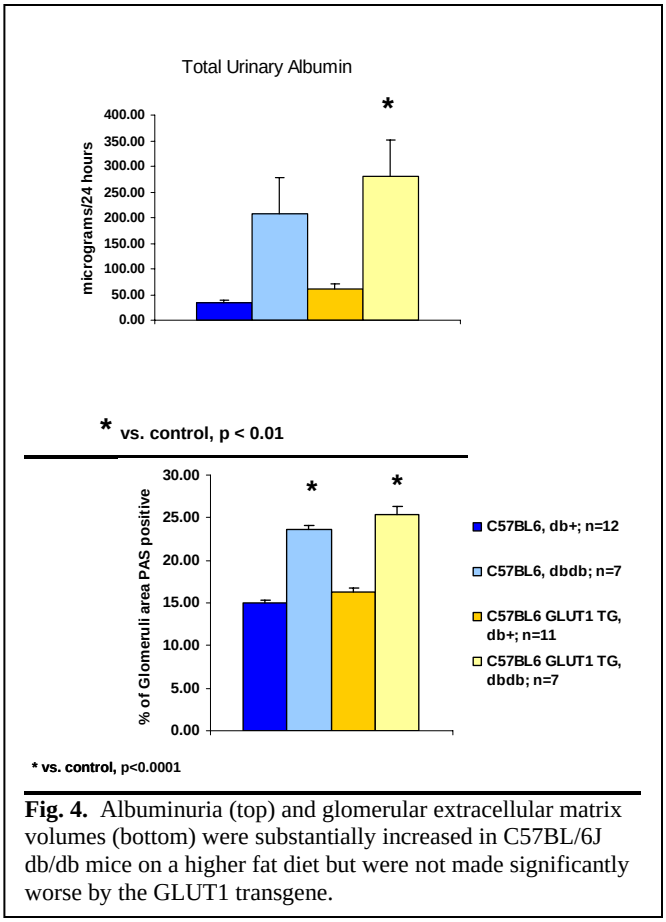


Fig. 4. Albuminuria (top) and glomerular extracellular matrix volumes (bottom) were substantially increased in C57BL/6J db/db mice on a higher fat diet but were not made significantly worse by the GLUT1 transgene.

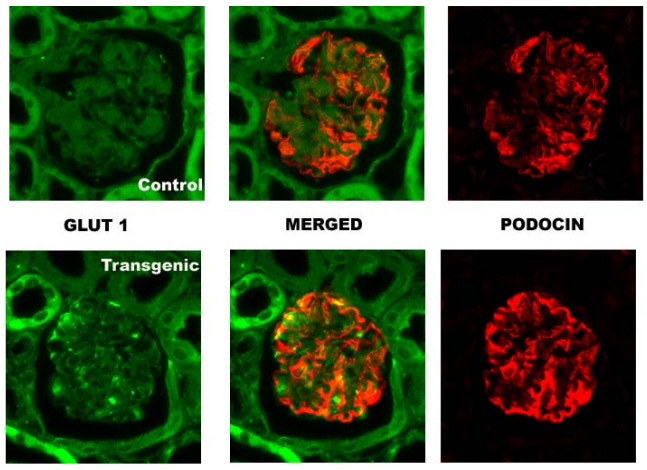


Fig. 5. GLUT1 and podocin immunofluorescence on serial sections of nphs2 GLUT1 C57BLKS transgenic mice (Bottom row) and wild-type mice (Top Row) showing increased GLUT1 expression in a podocyte distribution in the transgenic mice.

include the masking of GLUT1 effects by the diabetic and high fat milieu, the effect of high fat feeding and hyperglycemia on glomerular cell glucose handling despite high GLUT1 levels, or even a potential effect of the leptin receptor mutation on the response of glomerular cells to GLUT1. One possible result that could have led to lack of augmented injury in these db/db GLUT1 tg "high fat" group is the possible increased podocyte expression of GLUT1. This is discussed in the next subsection.

2. Nphs2 GLUT1 tg C57BLKSdb/db

Because of the preliminary data suggesting the development of substantial nephropathy in the nondiabetic GLUT1 tg mice, we felt that specific overexpression of GLUT1 in podocytes would likely lead to augmented glucose toxicity, including ROS generation, and subsequent podocyte damage, effacement and loss, leading to changes characteristic of severe if early diabetic nephropathy. These animals were developed at the University of Michigan by Dr. Thom Saunders (Transgenic Core Director and AMDCC investigator), Dr. Chuck Heilig (Director of University of Chicago AMDCC group) and Dr. Brosius. These tg mice have podocyte specific overexpression of GLUT1 on the C57BLKS db/m background. We obtained 3 strains (one of which was lost) that show substantial overexpression of GLUT1 in podocytes alone (**Fig. 5**). Surprisingly, the podocyte specific expression led to a minor decrement in albuminuria in the nondiabetic (db/+) animals, but led to a profound reduction in albuminuria in the diabetic (db/db) animals (**Fig. 6**). Analysis of glomerular morphometry in these animals is now ongoing. The mechanism by which GLUT1 protects podocytes from injury or dysfunction is entirely unknown at present. However, we have preliminary data suggesting that GLUT1 interacts with the important foot process protein, podocin, and that nephrin levels may be somewhat higher in the podocyte specific GLUT1 tg

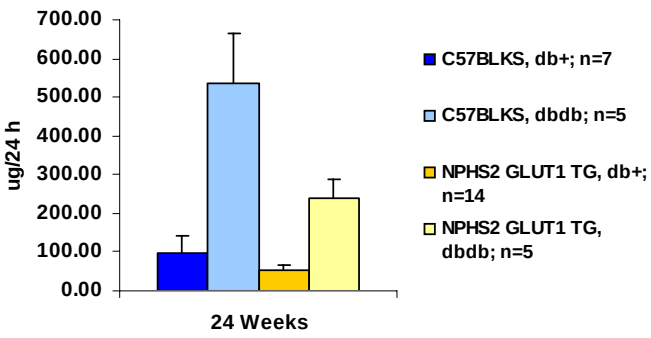


Fig. 6. Albuminuria was markedly increased at 24 wks of age in the db/db C57BLKS mice but this increase was substantially and surprisingly prevented by the podocyte-specific GLUT1 transgene.

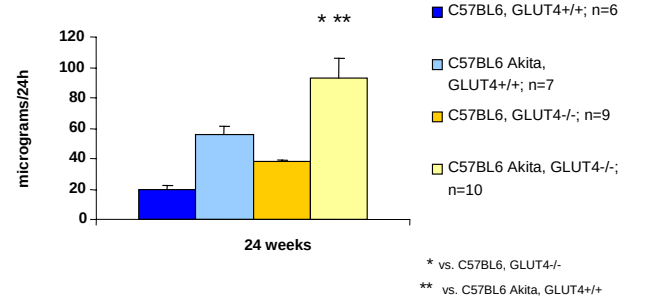


Fig. 7. 24 hour albuminuria was doubled in the GLUT4 -/- Akita mice at 24 weeks of age. As other AMDCC investigators have found, the levels of albuminuria are generally low in this model (compare to Figs. 4 and 6).

animals than in the controls. Low nephrin levels have been associated with podocyte damage and nephropathy in diabetes[2].

3) *GLUT4 -/- STZ or Akita*

As previously reported, the total body GLUT4 -/- STZ diabetic mice developed a two-fold higher albuminuria and a significant reduction in podocyte number compared to wild-type STZ diabetic mice (both on a C57BL/6J background). Similar and slightly more robust changes were seen in GLUT4 -/- mice bred into Akita diabetic C57BL/6J mice (**Fig. 7**).

4) *DbA/2J*.

Although these studies were not performed to develop a new mouse model of diabetic nephropathy, we have examined the effects of STZ diabetes in the DbA/2J mouse strain. This strain had been examined previously by both Dr. Breyer's and Coffman's groups in their strain analysis of STZ-diabetes[3, 4]. Both groups had found that this strain developed much more robust nephropathy in terms of albuminuria and mesangial expansion, though in neither group was there substantial decrement in glomerular filtration rate. We have found even more profound albuminuria in this strain in response to the conventional low dose STZ regimen (**Fig. 8**) (we reduced the daily dose to 40 mg/kg instead of the standard 50 mg/kg, based on Dr. Breyer's experience with this model). In this trial, sponsored by the JDRF, we have found profound reduction in albuminuria by treatment with rosiglitazone (3mg/kg/day) starting 2 weeks after completion of the STZ injections (**Fig. 8**).

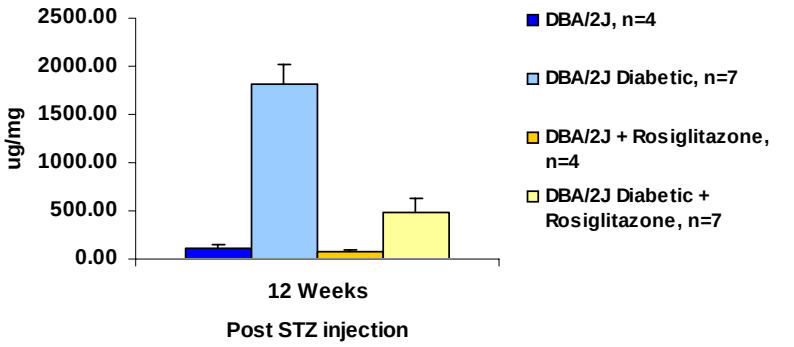


Fig. 8. Albumin/creatinine ratios (ACRs) were increased 16 fold in the DbA/2J mice 12 weeks after completion of STZ injections. These are the highest levels of albuminuria we have found. This increase was largely reduced by treatment with rosiglitazone beginning 2 weeks after STZ injections.

Neuropathy models:

1) *C57BLKS db/db*

AMDCC data over the past 3 years have amply demonstrated that C57Bl/6J mice are relatively resistant to developing neuropathy. Although db/db C7BL/6J mice are more susceptible to microvascular complications than the STZ C57Bl/6J animals, this model remained generally resistant to progressive nephropathy and neuropathy despite genetic modifications. In continued efforts to find the best genetically modified mouse for further neuropathy studies, we completed screening and advanced phenotyping of C57BLKS db/db mice, a genetic model of type 2 diabetes. The time of withdrawal (latency) from a heat stimulus applied to the tail (tail flick) or the hind paw was increased in db/db animals, reflecting loss of thermal sensation (**Fig. 9B and C**). Tail distal motor latencies were increased (**Fig. 9E**) while tail sensory and sciatic motor conduction velocities were decreased in db/db compared to db+ control mice (**Fig. 9D and F**), a pattern similar to diabetic neuropathy in humans.

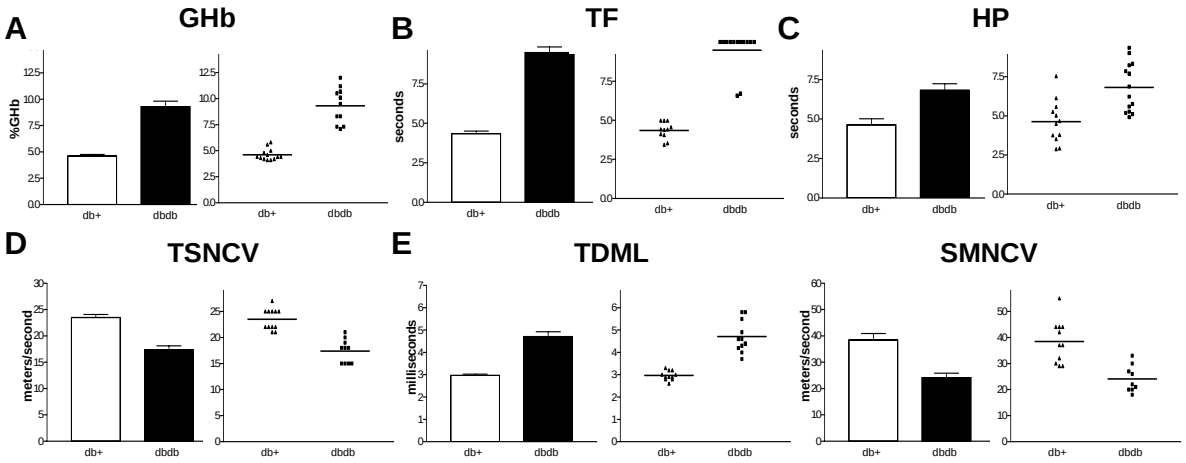


Fig. 9. Neuropathy phenotyping measures were used to assess nerve function in control (db+, open bars) versus diabetic (db/db, black bars) C57BLKS mice. In each lettered panel, the average reading ± standard error of the mean (SEM) is presented on the left and the individual animal measurements on the right. A) db/db mice had increased glycated hemoglobin (GHb), B) tail flick latency (TF) and C) hind paw latencies (HP). D) Tail sensory nerve conduction velocity (TSNCV) was decreased, E) tail distal motor latency (TDML) was increased, and F) sciatic motor nerve conduction velocity (SMNCV) was decreased in db/db mice.

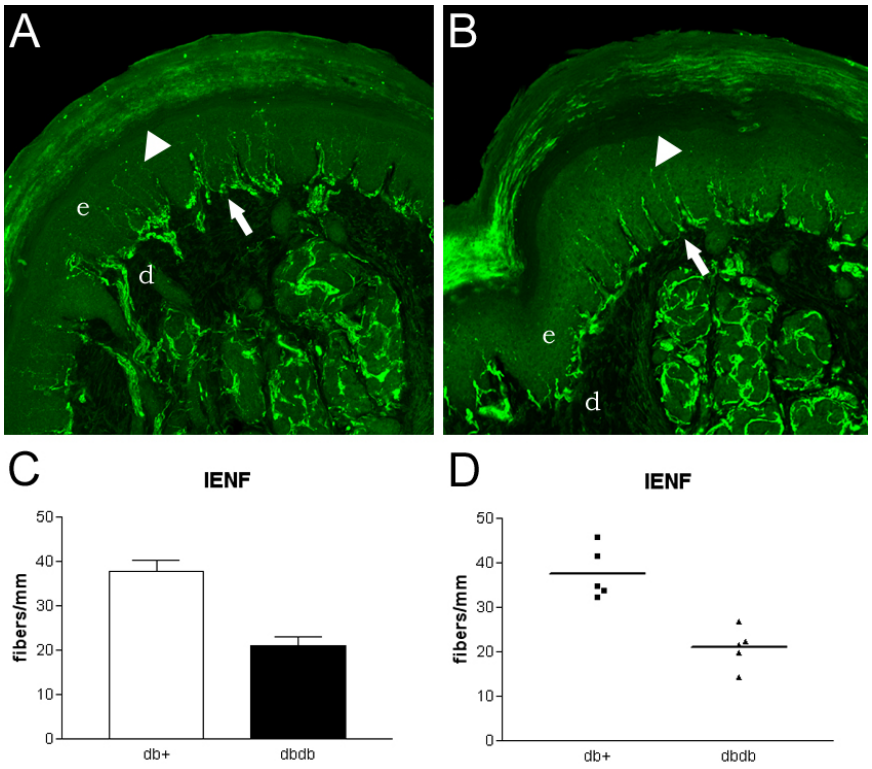


Fig. 10. IENF densities were assessed using PGP9.5 IHC. A) IHC of a control db+ foot pad. B) IHC of a db/db foot pad. Quantitation of foot pad densities is presented as fiber number/mm of epidermis in C) the average reading ± standard error of the mean (SEM) and D) the individual animal measurements. d = dermis, e = epidermis, arrows = intraepidermal nerve fiber, arrowheads = dermal fiber bundles. [Methods in Neuropathy Manual online at www.amdcc.org.]

Anatomical evidence of nerve fiber loss in the C57BLKS db/db mice was measured in the third screening component by quantitative assessment of IENF in the footpad. The hind paws of db+ control mice were normally innervated with a full component of PGP9.5 immunoreactive fibers (**Fig. 10A and C**) while db/db mice had nerve fiber loss and decreased intraepidermal nerve fiber (IENF) density (**Fig. 10B and C**). These differences in IENF numbers between diabetic and control mice are in agreement with published reports in human patients [5, 6]and experimental rat models of DN[7]. More advanced neuropathy phenotyping on this model is included in the Neuropathy Core progress report.

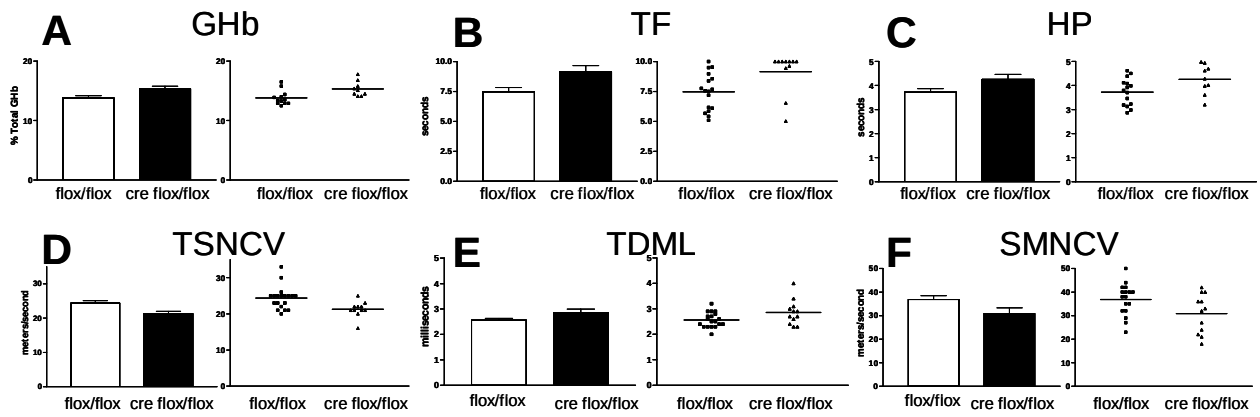


Fig. 11. Neuropathy phenotyping measures were used to assess nerve function in diabetic C57Bl/6J SOD2 loxP/loxP (open bars) vs. diabetic C57BL/6J Cre loxP/loxP (with neuronal specific disruption of SOD2, black bars) mice. In each lettered panel, the average reading ± standard error of the mean (SEM) is presented on the left and the individual animal measurements on the right. A) Both sets of mice had increased glycated hemoglobin (GHb); B) tail flick latency (TF) and C) hind paw latencies (HP) were greater in the knockout mice; D) tail sensory nerve conduction velocity (TSNCV) was decreased, E) Tail distal motor latency (TDML) was increased, and F) sciatic motor nerve conduction velocity (SMNCV) was decreased to a greater extent in the knockout mice. These difference were not yet statistically significant given the small numbers and short duration of diabetes.

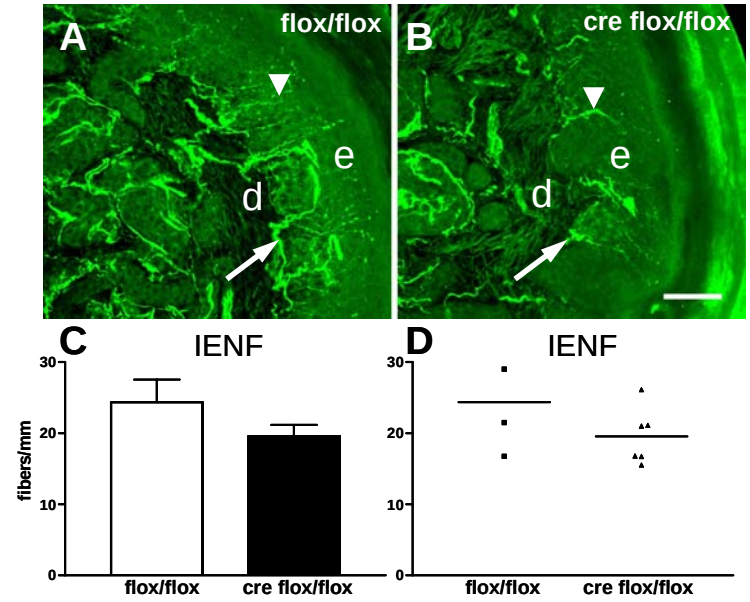


Fig. 12. IENF densities were assessed using PGP9.5 immunohistochemistry (IHC). A) IHC of a flox/flox foot pad. B) IHC of a cre flox/flox foot pad. C) Quantitation of foot pad densities is presented as fiber number/mm of epidermis. D) Individual animal measurements. Arrowheads = intraepidermal nerve fibers, arrows = dermal fiber bundles. e = epidermis, d = dermis

2) *Nestin SOD2 -/- STZ diabetic.* As we have previously reported, there was evidence of a mildly increased neuropathic phenotype in the total body SOD2 +/- db/db animals compared to the db/db animals alone after 24 weeks. These data led us to pursue tissue specific, targeted disruption of SOD2 by crossing nestin Cre with SOD2 loxP/loxP mice, to generate relatively specific neuronal SOD2 knockout mice (total body SOD2 -/- mice are neonatal lethals). Initial data suggest that knockout of SOD2 in neurons results in a more pronounced neuropathy phenotype. After 16 weeks of diabetes, both tail flick and hind paw latencies were increased to a greater extent in the nestin SOD2 -/- diabetic mice than in the floxed SOD2 diabetic mice with normal SOD2 expression (**Fig. 11B and C**). Tail distal motor latencies were also increased (**Fig. 11E**) while tail sensory and sciatic motor conduction velocities were decreased (**Fig. 11D and F**) in the knockout mice

In addition, the diabetic nestin SOD2 -/- diabetic mice had a more severe loss of decreased intraepidermal nerve fiber (IENF) density when compared to the floxed SOD2 mice after only 16 weeks of diabetes (**Fig. 12**).

2. Collaboration within your group:

We have coordinated all trials and models with the neuropathy group. All AMDCC personnel at the University of Michigan meet monthly to discuss models, timing, data and new approaches. All decisions are made as a group. The mouse colony manager works closely with both nephropathy and neuropathy phenotyping personnel to coordinate phenotyping and tissue harvest. Dr. Heilig's group from the University of Chicago has been instrumental in developing the initial generalized and podocyte specific GLUT1tg animals and performed initial and ongoing assessments of these models.

3. Collaboration with other AMDCC groups:

Since our center incorporates the Neuropathy Core as part of its operations, all eligible animals are phenotyped for neuropathy. In addition, at tissue harvest we routinely harvest eyes and bladders for the Retinopathy and Uropathy Cores. Dr. Daneshgari continues to measure bladder elasticity of selected animal models. Eyes from relevant models are routinely sent to Dr. Kern for retinal analysis. These collaborations led to an application to form a Mouse Metabolic Phenotyping Center between University of Michigan (Feldman and Brosius), Cleveland Clinic (Danshgari) and Case Western (Kern).

The Neuropathy Core has been especially active in phenotyping models from the other AMDCC investigators. A number of these collaborations are highlighted in the Neuropathy Core report and will not be repeated here.

Other collaborations have been made with several of the AMDCC groups. These include: 1) the generation and phenotyping of the Nphs2 GLUT4 knockout model with Dr. Dale Abel (University of Utah); 2) Nphs2 SOD2 knockout model with Drs. Harris and Breyer (Vanderbilt University). 3) Total body GLUT4 -/- knockout mice with Dr. Charron (Albert Einstein College of Medicine) and Dr. Erwin Bottinger (Mt. Sinai School of Medicine); 4) nphs2 PPARγ knockout project with Drs. Hsueh and Nichols (UCLA). Finally, any models used for terminal neuropathy phenotyping that have not had kidney phenotyping will have spot urine albumin/creatinine ratios determined and the kidneys will be assessed for morphological changes.

4. Pertinent non-AMDCC Collaboration:

1) JDRF Center of Excellence in the study of diabetic complications. This center encompasses a number of collaborative projects exploring the role of oxidative stress in diabetic complications. It also includes a clinical project testing antioxidants and other agents in the treatment of diabetic complications. The Center Director is Dr. Feldman (AMDCC Neuropathy Core Director) and the co-Director is Dr. Brosius (PI of the AMDCC UM/UChicago unit). Drs. Russell and Stevens have projects in this center. The renewal of this project is based on the effects of thiazolidinediones in microvascular complications and will lead to an immediate clinical trial in type 1 diabetic patients.

2) Dr. Feldman is PI for several collaborative NIH grants investigating the etiology, pathogenesis and treatment of diabetic polyneuropathy. Dr. Russell is a co-investigator on several of these grants.

3) Dr. Feldman is an investigator in neuropathy aspects of the multi-institutional Epidemiology of Diabetes Interventions and Complications (EDIC) study.

4) Dr. Brosius is PI on NIH grants investigating the role of glucose transporters in vascular disease. Collaborators include several vascular biologists.

5) Dr. Heilig is the PI on several funded and pending NIH proposals for the study of GLUT1 in diabetic and nondiabetic nephropathy and diabetic embryopathy as well as GLUT1 haplodeficiency syndromes. Dr. Brosius is a collaborator and consultant on these proposals.

6) Dr. Matthias Kretzler has just joined the faculty at the University of Michigan. He has developed an international renal biopsy data bank for functional genomics of renal diseases and has a strong interest in diabetic nephropathy. He has worked closely with Drs. Brosius in the proposed AMDCC renewal project which will allow matching of mouse models of diabetic complications with human disease by comparing transcriptomic profiles and improving mouse models by recapitulating patterns found in humans.

5. Address previous EAC comments:

There was only one comment from the fall, 2005 EAC report directed specifically toward our unit:

"The C57BL/6 tgGLUT, db/db model is of special interest from the standpoint of retinopathy because it presents itself as a candidate model for pure glucotoxicity. In order to interpret in an informative way whichever degree of retinopathy may be present in these animals, it will be important to document in which retinal cells the transgene is expressed. Reliable data can be obtained by immunohistochemistry on retinal sections for the localization to ganglion cells, Muller glial cells, astrocytes, and large groups of neurons. The precise attribution to pericytes vs. endothelial cells in the vessels may require a combination of studies in isolated retinal microvessels and dispersed retinal cells (please see Diabetes 2004; 53: 2404-2411)."

We agree and are harvesting eyes from these mice and forwarding them to Dr. Kern for evaluation.

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Action Items

1. Please email a copy of your final 2005 Annual Report to me no later than *February 24, 2006*.

Christian J. Ketchum, Ph.D.
Division of Kidney, Urologic, and Hematologic Diseases
National Institute of Diabetes and Digestive and Kidney Diseases
National Institutes of Health
Two Democracy Plaza, Room #647
6707 Democracy Blvd., MSC 5458
Bethesda, MD 20892-5458

Phone: 301-402-1411
Fax: 301-480-3510
Email: chrisketchum@nih.gov