Early retinal pigment epithelium dysfunction is concomitant with hyperglycemia in mouse models of Type 1 and Type 2 diabetes.

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Running head: Early-onset RPE dysfunction in type 1 and 2 diabetes
Abstract:

In the diabetic retina, cellular changes in the retinal pigment epithelium (RPE) and neurons occur before vision loss or diabetic retinopathy can be identified clinically. The precise etiologies of retinal pathology are poorly defined and it remains unclear if the onset and progression of cellular dysfunction differs between type 1 and type 2 diabetes. Three mouse models were used to compare the time course of RPE involvement in type 1 and type 2 diabetes. *C57Bl/6J* mice injected with streptozotocin modeled type 1 diabetes, while *Lepr* \( ^{db/db} \) mice on both BKS and B6.BKS background strains modeled type 2 diabetes. Electroretinogram (ERG)-based techniques measured light-evoked responses of the RPE (dc-ERG) and the neural retina (a-wave, b-wave). Following onset of hyperglycemia, a-wave and b-wave amplitudes of STZ mice declined progressively and by equivalent degrees. Components of the dc-ERG were also altered, with the largest reduction seen in the c-wave. *Lepr* \( ^{db/db} \) mice on the BKS strain (BKS.Lepr) displayed sustained hyperglycemia and a small increase in insulin, while *Lepr* \( ^{db/db} \) mice on the B6.BKS background (B6.BKS.Lepr) were transiently hyperglycemic and displayed severe hyperinsulinemia. BKS.Lepr mice exhibited sustained reductions in the dc-ERG c-wave, fast oscillation and off response that were not attributable to reduced photoreceptor activity; B6.BKS.Lepr mice displayed transient reductions in the c-wave and fast oscillation which correlated with hyperglycemia and magnitude of photoreceptor activity. In summary, all mouse models displayed altered RPE function concomitant with the onset of hyperglycemia. These results suggest that RPE
function is directly reduced by elevated blood glucose levels. That RPE
dysfunction was reversible and mitigated in hyperinsulinemic B6.BKS.Lepr mice
provides insight into the underlying mechanism.

**Abbreviations:** cd, candela; CNTL, control; dc-ERG, direct-current coupled
ERG; DM, diabetes mellitus; DR, diabetic retinopathy; EOG, electro-oculogram;
ERG, electroretinogram; OP, oscillatory potential; RPE, retinal pigment
epithelium; SDOCT, spectral domain optical coherence tomography; SLO,
scanning laser ophthalmoscopy; STZ, streptozotocin; T1D, type 1 diabetes; T2D,
type 2 diabetes

**Key Words**
1. retinal pigment epithelium
2. electroretinogram
3. hyperglycemia


INTRODUCTION

Diabetic retinopathy (DR) is one of the most severe complications of diabetes and is the most common cause of blindness in working age adults (Klein et al. 1995). The prevalence of DR in patients with type 1 diabetes (T1D) is 40% and 20% for patients with type 2 diabetes (T2D). Within each cohort, prevalence increases with duration of diabetes. After 20 years of disease, 90% of T1D patients and 60% of T2D patients experience some level of clinical retinopathy (Klein 2007). Therapies, including laser photocoagulation and intraocular injection of VEGF inhibitors, have been successful at improving vision in patients with diabetic macular edema; however, these therapies do not reduce the risk of developing vision loss (Cheung et al. 2010; Robinson et al. 2012; Salam et al. 2011; Wilkinson-Berka and Miller 2008). Development of early detection procedures and identification of early indicators of dysfunction within the retina remains a significant challenge.

Patients with early stages of diabetes and no sign of DR have decreased light sensitivity and delayed dark adaptation recovery, indicative of outer retina dysfunction (Arden et al. 1998; Holopigian et al. 1997). Altered outer retinal neuron function has been documented in diabetic patients via electroretinography (ERG), even in the absence of clinical retinopathy (Bresnick and Palta 1987; Gardner et al. 2002; Juen and Kieselbach 1990; Parisi and Uccioli 2001; Yamamoto et al. 1996), and delays in multifocal electroretinography
(mfERG) implicit times can predict the location of microaneurysm development (Bearse et al. 2006; Fortune et al. 1999; Harrison et al. 2011; Ng et al. 2008) and are found at very early times in adolescents with either T1D or T2D (Bronson-Castain et al. 2012).

ERGs measure the massed electrical response of the retina to light stimuli and demonstrate the function of multiple retinal cell types through analysis of the individual waveform components. ERGs recorded in response to strobe-flashes allow for evaluation of the short latency components generated by the neural retina (see Fig 1D). The cornea-negative a-wave is generated by the light-induced decline in the dark current around the rod photoreceptor outer segments (Hood and Birch 1990; Lamb 1996; Penn and Hagins 1969). The positive polarity b-wave is generated by the activity of rod depolarizing bipolar cells and is dependent upon output of the photoreceptor (Hood and Birch 1996; Kofuji et al. 2000; Robson and Frishman 1995). The higher frequency oscillatory potentials (OPs) superimposed on the b-wave reflect amacrine cell activity (Wachtmeister 1998). ERG protocols using longer duration stimuli and dc-amplification (dc-ERG) allow the slower responses of non-neuronal cell types to be examined. In the dc-ERG waveform (see Fig 2E), the positive-polarity c-wave represents the summation of a [K⁺]-decline induced hyperpolarization of the RPE apical membrane and a negative Kir4.1-mediated slow PIlll response of the Müller glia cells (Kofuji et al. 2000; Oakley and Green 1976; Schmidt and Steinberg 1971; Steinberg and Miller 1973; Steinberg et al. 1970; Witkovsky et al. 1975; Wu et al. 1998).
The c-wave is followed by the negative-polarity fast oscillation that reflects recovery of the subretinal [K\(^{+}\)] and a [Cl\(^{-}\)] dependent hyperpolarization of the basal RPE membrane (Griff and Steinberg 1984; Linsenmeier and Steinberg 1982). The positive-polarity light peak is induced by depolarization of the basal membrane of the RPE due to increased Cl\(^{-}\) permeability and is followed by the off-response (Fujii et al. 1992; Gallemore and Steinberg 1989; 1993; Linsenmeier and Steinberg 1982; Wu et al. 2004b). None of the dc-ERG components are a direct response to light, but instead reflect the summed RPE/Müller cell responses generated subsequent to the response of rod photoreceptors. These components thus provide non-invasive measures of RPE function, although their interpretation must take into account the status of rod photoreceptor function (Samuels et al. 2010).

ERGs performed on diabetic patients demonstrate reductions in b-wave amplitude and increases in the implicit time of the high frequency OPs which are also reduced in amplitude (Shirao and Kawasaki 1998). These changes become more pronounced with disease progression and the a-wave also becomes involved at later disease states (Bresnick et al. 1984; Holopigian et al. 1997; Pardue et al. 2014). Reports from patient studies and analysis of animal models indicate that the RPE is an early site of dysfunction (Kirber et al. 1980; Klein et al. 1980; Krupin et al. 1982; Tso et al. 1980; Vinores et al. 1989). The electro-oculogram (EOG) can be used to measure function of the RPE in patients, and the fast oscillation component of the EOG is reduced in diabetic patients both...
with or without DR (Schneck et al. 2008). After 6 months of diabetes, T1D mice
display reductions in amplitude of several components of the dc-ERG, including
the c-wave, fast oscillation and off response (Samuels et al. 2012). Importantly,
reductions in ERG components generated by the RPE are noted as early as 2
weeks post onset of diabetes (Pautler and Ennis 1980), earlier than the reduction
in OPs or b-wave, which appear 4 weeks post onset of diabetes (Aung et al.
2013; Kohzaki et al. 2008; Li et al. 2002; Pardue et al. 2014; Phipps et al. 2004;
Phipps et al. 2006; Shinoda et al. 2007). In comparison to animal models of
T1D, models of T2D have been less well studied, although reductions in a-wave,
b-wave and OP amplitude, and increased OP latencies have been documented
at 16 weeks of age in \textit{Lepr}^{db/db} mice (Bogdanov et al. 2014; Simo and Hernandez
2014). This work does not, however, define the time course over which retinal
and RPE function develops.

Systemic glucose concentration is the only known predictor for DR (1993; King et
al. 1999). The purpose of the present study is to identify and differentiate the
earliest effects of hyperglycemia on the function of the RPE and outer retina in
mouse models of T1D and T2D. Herein, we demonstrate reductions in the light-
evoked electrical responses of the RPE in a well-established mouse model of
T1D (STZ-injection) and two strains of \textit{Lepr}^{db/db} mutant mice which model T2D
(Lai and Lo 2013). In all models, RPE abnormalities appeared concomitantly
with hyperglycemia and before or at the same time that photoreceptor
dysfunction commenced. Moreover, low doses of insulin appear to delay RPE
dysfunction, but not neuronal dysfunction. Our data indicate that RPE dysfunction is an early hallmark of T1 and T2D, and may be predictive for development of clinical retinopathy.

METHODS:

Ethical Approval

Treatment of animals was in compliance with the ARVO Resolution on Treatment of Animals in Research, and all animal procedures were approved by the Institutional Animal Care and Use Committees of the Louis Stokes Cleveland VA Medical Center and the Cleveland Clinic.

Mice

Mouse model of T1D: C57BL/6J mice from The Jackson Laboratory (Bar Harbor, ME) were bred, and 4-8 week old littermates were randomly assigned to diabetic (STZ) or non-diabetic control (CNTL) groups. Diabetes was induced by three sequential daily intraperitoneal injections of a freshly prepared solution of STZ in 0.1 M citrate buffer (pH 4.4) at 30 mg/kg body weight. Insulin was given to STZ-injected mice by intraperitoneal injection every other day beginning at the onset of hyperglycemia, to prevent ketosis without preventing hyperglycemia and glucosuria (0–0.2 units of neutral protamine Hagedorn (NPH) Humulin N, Eli Lilly and Co., Indianapolis, IN). This was typical for all STZ mice to maintain body
weight and hydration. CNTL-injected mice received citrate buffer only and did
not receive insulin.

**Mouse models of T2D:** *Lepr*\(^{db/db}\) breeders were purchased from The Jackson
Laboratory. Mice homozygous for the *Lepr*\(^{db/db}\) allele on the B6.BKS background
(000697) will be referred to as “B6.BKS.*Lepr*”, whereas *Lepr*\(^{db/db}\) mice on the pure
BKS background (000642) will be referred to as “BKS.*Lepr*”. For both strains,
*Lepr*\(^{+/db}\) animals were bred to generate *Lepr*\(^{db/db}\) diabetic mice and control *Lepr*\(^{+/+}\)
or *Lepr*\(^{+/db}\) littermates.

Mice were genotyped for the *Lepr*\(^{db}\) allele by PCR amplification by one of two
sets of primers, depending on the experiment. For: ‘db one’ (20 uM), -
AGAACGGACACTCTTTGAAGTCTC- and ‘db two’ (20 uM), -
CATTCAAACCATATTTAGGTTTGTGT- PCR amplification was performed for 35
cycles at 94°C for 30 s, 52°C for 45 s, and 72°C for 45 s, followed by 2 min at
72°C. PCR products were digested with Rsa1 overnight at 37°C and separated
on a 2% agarose gel. For: ‘db forward’ (10 uM) -ACCAACTTCCCAACAGTCCA-
and ‘db reverse’ (10 uM) -TGATGCCCTGAAAATCAAGC- PCR amplification was
carried out for 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s followed by
3 min at 72°C and then direct sequencing with Big Dye Terminator v1.1 (Applied
Biosystems, Grand Island, NY).

Body weight and non-fasting blood glucose were measured at least monthly and
at the time of electroretinography using a One-Touch Ultra Glucometer (Bio-Rad
Laboratories, Hercules, CA) and plasma insulin concentrations were measured using the Ultra-sensitive rat insulin ELISA kit with mouse insulin standards (Crystal Chem Inc, Downer's Grove, IL). No T2D mice received insulin.

**Electroretinography**

After overnight dark adaptation, mice were anesthetized (65 mg/kg sodium pentobarbital), the cornea was anesthetized (1% proparacaine hydrochloride), and the pupils were dilated (1% tropicamide, 2.5% phenylephrine hydrochloride, and 1% cyclopentolate). Mice were placed on a temperature-regulated heating pad throughout each recording session. Responses of the outer retina were recorded on an Espion E3 ColorDome Full field Ganzfeld (Diagnosys, LLC, Lowell, MA) with a Ag/AgCl electrode referenced to a Ag/AgCl pellet electrode placed in the mouth of the mouse in response to strobe-flash stimuli presented in the dark. Ten steps of increasing flash luminance (-3.6 to 2.1 log candela (cd) s/m²) were presented in order of increasing flash strength and the number of successive trials averaged together decreased from 20 for low-level flashes to 2 for the highest flash stimuli. The duration of the interstimulus interval increased from 4 s for low luminance flashes to 90 s for the highest stimuli. The amplitude of the a-wave was measured 6.6 ms after flash onset from the pre-stimulus baseline. The amplitude of the b-wave was measured from the a-wave amplitude at 6.6 ms to the peak of the b-wave.
Immediately following the dark adapted strobe-flash stimuli, components of the
direct-current coupled (dc)–ERG, generated by the RPE, were recorded in
response to a 5 cd/m² stimulus presented for 7 min. The amplitude of the c-wave
was measured from the pre-stimulus baseline to the peak of the c-wave. The
amplitude of the fast oscillation was measured from the c-wave peak to the
trough of the fast oscillation. The amplitude of the light peak was measured from
the fast oscillation trough to the asymptotic value. The amplitude of the off-
response was measured from the light peak asymptote to the peak of the off-
response.

Immediately after the conclusion of the dc-ERG recording, a steady 20 cd/m²
adapting field was presented in the ganzfeld bowl. After 4 min of light adaptation,
cone ERGs were recorded to strobe flash stimuli (-1 to 2 log cd s/m²)
superimposed upon the adapting field. The amplitude of the cone ERG was
measured from the pre-stimulus baseline to the positive peak of the waveform.

**Statistical analysis**

For all analyses, data was compiled as average ±SEM and statistics were
performed using Two-way ANOVA with Tukey post-hoc analysis or the Student’s
paired t-test. To normalize the dc-ERG responses to that of the a-wave, the
amplitude of each individual animal was compared to the averaged control
response and calculated as the relative amplitude. Relative amplitudes were then
averaged and compared to the control average for statistical analysis. At least 3
animals per genotype per time point were used for all experiments.

Scanning Laser Ophthalmoscopy and Spectral-Domain Optical Coherence
Tomography

Mice were anesthetized with 65 mg/kg of sodium pentobarbital. Mydriasis was
induced by administration of 1 \( \mu \)L of 0.5% Mydrin-P (tropicamide-phenylephrine
combination) drops (Santen Pharmaceutical Co., Ltd., Osaka, Japan). The drops
were gently massaged into the eye by manually blinking the eyelids. Eyes were
then immediately covered with Systane Ultra artificial tears (Alcon Laboratories,
Inc., Ft. Worth, TX). Mice were then placed in a warmed, humidified, oxygenated
acrylic plastic sheet chamber for a minimum of 5 min to permit time for pupil
dilation. Mice were then removed for imaging by scanning laser ophthalmoscopy
(SLO) (model HRA2; Heidelberg Engineering, Inc., Vista, CA) and spectral-
domain optical coherence tomography (SDOCT) (model Envisu SDOIS;
Bioptigen, Inc., Research Triangle Park, NC). The SLO imaging involved
collection of different imaging modalities, including dark-field reflectance and
autofluorescent images with both blue (488 nm) and infrared (795 nm and 830
nm) illumination wavelengths. Using a wide-field objective lens with a 55° field of
view (FOV), retinal images were collected with the optic nerve centrally
positioned. Additional views of the peripheral regions were obtained to further
investigate the nasal, temporal, superior, and inferior quadrants. Eyes were
occasionally rehydrated with balanced salt solution or Systane Ultra artificial ears
(Alcon Laboratories) and mechanically massaged to simulate blinking as needed. After SLO imaging, the mouse was transferred to the Envisu SDOIS system (Bioptigen, Inc.) for SDOCT imaging. The SDOCT volumetric scans (250 a-scans per b-scan, x 250 b-scans per volume) were obtained with the optic nerve centrally located within the FOV. Using a 50° objective lens, the SDOIS system afforded a retinal FOV of approximately 1.5 mm, with an axial, in-depth resolution of approximately 6 μm. After imaging, both eyes received bacitracin zinc and polymyxin B sulfate ophthalmic ointment (Bausch & Lomb, Inc., Tampa, FL) to prevent corneal dehydration. During recovery, mice were placed in a bottom-warmed (33–36°C), oxygenated (21%–60%) acrylic plastic sheet chamber until they fully recovered from general anesthesia.

RESULTS:

STZ model of T1D

We modeled T1D in mice by injection of STZ, which kills pancreatic β cells, leading to loss of insulin production (Rossini et al. 1977). Using this paradigm, mice become hyperglycemic within three days of STZ injection and remain hyperglycemic for the duration of their lives. We assessed outer retina function at 1, 2 and 4 weeks post onset of hyperglycemia (Fig. 1A) and measured blood glucose levels to confirm that all STZ-injected mice were significantly hyperglycemic (Fig. 1B). STZ mice were treated with exogenous insulin, at levels which do not normalize blood glucose (Fig. 1B) or serum insulin (Fig. 1C) but do prevent ketosis and dehydration.
STZ mice displayed significant reductions in a- and b-wave amplitudes beginning at two weeks of diabetes (Fig. 1D-F). When the relative b-wave is plotted against the relative a-wave amplitude for each time point, it was evident that the amplitude reduction in these two parameters was equivalent, indicating that the decreased photoreceptor response accounts for the loss of bipolar cell activity (Fig. 1G).

Assessment of RPE function via the dc-ERG demonstrated that STZ induces similar reductions in each of the major components of this waveform (c-wave, fast oscillation, light peak and off response) at 2 and 4 weeks (Fig. 2A-E). Both the c-wave and fast oscillation were significantly reduced by 40% and 35%, respectively, at 2 weeks (Fig. 1A-B), localizing dysfunction to both the apical and basal RPE membranes. At four weeks, reductions were not as overt in these components, but remained significant and the off response was found to be significantly lower in the STZ mice as well. The light peak is the most variable component of the dc-ERG due to the duration of the stimulus and the requirement to maintain stability for the entirety of the recording period. Although we observed a reduction in light peak amplitude at all times, these reductions were not significant.

We did not find any signs of retinopathy via histology or SDOCT, nor did we find any evidence of RPE damage by SLO at any time point of analysis (data not
shown). It is well documented that few or no abnormalities occur in the retina/RPE at these early time points in either STZ-treated mice or rats (Enzsoly et al. 2014; Robinson et al. 2012), and our data agree with these findings. We compared the time course over which reductions to photoreceptor (a-wave) and RPE (dc-ERG components) appear by normalizing the amplitude of each dc-ERG component from each STZ-treated animal to the control average of the corresponding dc-ERG component and then assessing that relative value in relation to the relative a-wave amplitude for individual animals. Figure 2F-H displays the average relative amplitude of the c-wave (Fig. 2F), fast oscillation (Fig. 2G) and off response (Fig. 2H) as a function of the relative a-wave amplitude for the time points examined. At two weeks of hyperglycemia, the c-wave was significantly affected to a greater extent than the reduction in photoreceptor activity (Fig. 2F, p<0.05); the two week data point falls significantly below the diagonal line. At 1 and 4 weeks, the data points fall along or near the line, which indicates an equivalent reduction of a- and c-wave amplitudes. When we measured the fast oscillation (Fig. 2G) and off response (Fig. 2H), the data also fell close to the diagonal line, indicating that these amplitude reductions were equivalent to the reduced photoreceptor response underlying the a-wave. Given that RPE components are generated secondary to photoreceptor activity (Berman 1991; Samuels et al. 2010; Wu et al. 2004b), it is possible that the reduction in the dc-ERG components reflects photoreceptor dysfunction, with the exception of the 2 week c-wave.
Lepr model of T2D

DR occurs in T2D patients with only a slightly lower incidence than that of T1D patients (Klein 2007). Therefore, we conducted parallel studies in genetic mouse models of T2D over a 6 month time course, assessing mice at 4 week intervals through 16 weeks with endpoint testing at 24 weeks (Fig. 3A). Mice harboring a homozygous G to T transversion mutation in the leptin receptor (Lepr\textsuperscript{db/db}) display abnormal splicing and termination of the gene, and loss of leptin-mediated signal transduction (Barinaga 1996; Chen et al. 1996; Lee et al. 1996). These mice recapitulate a subset of T2D characteristics including overt obesity (Fig. 3B), hyperglycemia (Fig. 3C) and increased insulin production (Fig. 3D). The Lepr\textsuperscript{db/db} phenotype is influenced by the background strain on which the mutation is expressed (Coleman and Hummel 1973; Leiter et al. 1987) (Fig. 3). Homozygous Lepr\textsuperscript{db/db} mice on a pure C57BLKS/J background, “BKS.Lepr”, are significantly and chronically hyperglycemic by 8 weeks of age (Fig 3C, right). In contrast, the BKS.Lepr\textsuperscript{db/db} mutant mice bred onto the C57Bl/6J background, “B6.BKS.Lepr”, display overt hyperglycemia as early as 4 weeks of age but their blood glucose levels fall to normal concentrations by 12 weeks of age (Fig. 3C, right). Furthermore, B6.BKS.Lepr mice display significant hyperinsulinemia compared to BKS.Lepr mice (Fig 3D; note the difference in scales). These two distinct strains of the Lepr\textsuperscript{db/db} mutants permit ERG analyses that test whether differences in obesity, insulin levels and/or hyperglycemia affect the function of the outer retina and RPE of mice sharing the same Lepr\textsuperscript{db} mutation.
BKS.Lepr mice

The BKS.Lepr mice displayed significant reductions in a-wave and b-wave amplitudes at 24- and 16-weeks of age, respectively (Figs. 4A-C). Both parameters declined with age, with the b-wave falling severely at 24 weeks. At all ages, the extent of b-wave reduction was more pronounced than that of the a-wave (Fig. 4D).

When RPE function was examined in BKS.Lepr mice, a small and non-significant reduction in amplitude of the c-wave was observed at 4 weeks (Fig 5A, E), when glucose levels were only slightly elevated (Fig. 3C). This reduction became more pronounced and was significant at each of the older ages (Fig. 5A, E, F) which correspond to the chronic hyperglycemia noted in this model (gray boxes, Fig 3C). A similar progressive reduction was noted for the fast oscillation and off response, beginning at 8 and 12 weeks of age, respectively (Fig 5B, E). These reductions correlated with that of the ERG b-wave (Fig. 4C). The dc-ERG light peak also tended to be reduced in BKS.Lepr mice at 4 and 8 weeks as compared to control littermates. Due to variability of the light peak component, statistical significance was not met. At later ages, the light peak amplitudes of control BKS.Lepr mice were abnormally low in comparison to other control mouse lines (Fig. 5C, E).

To determine how dc-ERG defects and photoreceptor dysfunction correlated in BKS.Lepr mice, we plotted the normalized average amplitudes of the c-wave,
fast oscillation and off response, and the a-wave, as a function of age. If each dc-ERG component was affected to the same extent as the a-wave, the lines would superimpose. Instead, we found that the c-wave and fast oscillation were reduced to a greater extent than that of the a-wave at each time point through 12 and 16 weeks, respectively (Fig. 5F). These data indicate that RPE dysfunction in BKS.Lepr mice cannot be attributed to a loss of photoreceptor function alone, and must include an additional mechanism.

**B6.BKS.Lepr mice**

B6.BKS.Lepr mice were also examined by ERG. B6.BKS.Lepr mice exhibited an initial reduction in b-wave amplitude that developed over a similar time frame as that seen in BKS.Lepr mice. Significant reductions were observed at 8 weeks of age (Fig. 6A, C), following four weeks of hyperglycemia (Fig. 3C). At later ages, the b-wave reduction became progressively more pronounced (Fig 6C). In comparison, statistically significant reductions in the a-wave were only found at 24 weeks of age (Fig. 6A-B). When compared, b-wave reductions of B6.BKS.Lepr mice exceeded those for the a-wave (Fig. 6D), similar to the pattern documented in BKS.Lepr mice (Fig. 4D) and distinct from that noted in the T1D model (Fig. 1G).

The time course of RPE involvement in B6.BKS.Lepr mice was also distinct from the other two models. Assessment of RPE function in these mice demonstrated
insignificant but reproducible reductions to dc-ERG components (Fig. 7A-D). When the amplitudes of each component were normalized to control levels and compared to the normalized a-wave amplitude, most reductions paralleled those of the a-wave (Fig. 7E, the lines superimpose). The sole exception was the reduction of the c-wave noted at 4 weeks of age, the only age at which B6.BKS.Lepr mice are severely hyperglycemic (Fig. 3C). The return to normoglycemia following the initial hyperglycemia observed in this model could account for the recovery of the c-wave amplitude. But this recovery is specific to the RPE as photoreceptor and bipolar cell function progressively decline at later ages where glucose levels are restored to normal (Fig. 6).

In both T2D mouse strains, reductions in the amplitude of either strobe-flash or dc-ERG components cannot be accounted for by cellular damage or degeneration as no gross anatomical abnormalities were observed by SDOCT imaging (Fig. 8A, B). Each retinal layer was distinctly identified and no signs of retinal edema or microaneurysms were found. By SDOCT, an unhealthy retina is identifiable by the loss of specific banding demarking the retinal layers (Kim et al. 2008), which we found to be present in all mouse strains. The RPE also appeared to be normal as evidenced by SLO imaging. Using the AF modality of the SLO, changes to RPE are identifiable by the presence of hyperfluorescent foci and/or lesions (Luhmann et al. 2009), which were absent from both control and diabetic mice. These findings indicated that the electrophysiological changes do not reflect overt structural changes within the RPE or retina. In addition, we
found no evidence of RPE leakage by fluorescein angiography in B6.BKS.Lepr or BKS.Lepr mice (data not shown). We did, however, observe some abnormal patterning on the red free dark reflectance modality of the SLO in B6.BKS.Lepr mice (Fig. 8B arrowheads). The etiology of these spots is unknown; given the advanced age of the mice (50 wks), some abnormal patterning is not unexpected (Bell et al. 2012).

In BKS.Lepr mice, the decrease in amplitude of the dc-ERG components was not completely accounted for by a reduction in photoreceptor function (Fig. 5F). We therefore assessed the relationship between systemic glucose concentration and the amplitude of the a-wave, as well as each significantly affected dc-ERG component. In this model, the a-wave was not reduced until 24 wks (Fig. 4A-B), although significant glucose elevations were seen at earlier ages (Fig. 9A left, arrows denote the ages at which normal a-wave amplitudes are maintained at times of severe hyperglycemia, 8-16 wks). These data suggest that photoreceptor dysfunction occurs after a protracted period of hyperglycemia and the initial hyperglycemic insult does not immediately induce a reduction to the a-wave. In comparison, the amplitude of the c-wave (Fig. 9B), fast oscillation (Fig. 9C) and off response (Fig. 9D) declined at the same ages that correlated with the onset and maintenance of hyperglycemia, and fall below the blue line that demarks relative values equivalent to controls. This is a very different presentation from the B6.BKS.Lepr strain (Fig 9, middle panels) where glucose levels are restored to the normal range by elevated serum insulin and RPE
functional losses match those of photoreceptors (Fig. 7E, 9A-D middle). Note that if the B6.BKS.Lepr graphs in Figure 9 were overlapped, all points would overlap except the c-wave at 4 wks, when B6.BKS.Lepr mice are severely hyperglycemic (Fig. 9B, middle, yellow arrowhead). This suggests that increased insulin delays RPE dysfunction by lowering serum glucose levels. Analysis of the a-wave, c-wave, fast oscillation and off response relative to systemic blood glucose concentration in STZ animals as compared to CNTLS (Fig. 9, right panels) demonstrated that the a-wave is reduced at 2 weeks of hyperglycemia, again illustrating the slight delay in a-wave defects. Each of the dc-ERG components was affected to a similar extent as the a-wave and superimpose with the a-wave symbols, with the exception of the 2 wk c-wave point (yellow arrowhead), which was more severely affected than the a-wave at that time point, and correlated with the rise in blood glucose levels.

Within our testing paradigm, we also recorded light-adapted responses as the cone ERG. A six-step increasing luminance protocol was conducted. Equivalent findings were observed at each intensity and data from the 1.4 log cd s/m² luminance is presented (Fig. 10A-C). While no reductions were observed in the T1D model at any time point (Fig. 10A), significant reductions in amplitude of the cone ERG were found in the B6.BKS.Lepr strain at 12 weeks of age; these persisted and progressed through the 24 week testing period (Fig 10C). In comparison, smaller reductions were identified in the BKS.Lepr mice and were only significant at 24 weeks (Fig. 10B). In STZ-treated rats, changes in cone
morphology have been demonstrated at 12 weeks (Enzsoly et al. 2014). This was noted to precede cone photoreceptor apoptosis, which has typically been found to peak at approximately 6 months (Barber et al. 1998; Park et al. 2003; Santiago et al. 2007) and correlates with our finding in BKS.Lepr mice. The earlier involvement of cone dysfunction that we observed in the B6.BKS.Lepr mice may indicate that cones or the cone pathway are impacted by high insulin.

**DISCUSSION:**

DR is a microvascular disorder and the appearance of vision-impairing microaneurysms in the diabetic retina can be predicted by use of the mfERG (Bearse et al. 2006; Fortune et al. 1999; Harrison et al. 2011; Ng et al. 2008; Tyrberg et al. 2005); however, reductions in the amplitude and changes in the timing of the mfERG cannot be identified prior to development of vascular damage. We have utilized this idea that functional changes may predict development of pathology in the diabetic eye to investigate the role of RPE dysfunction as an early biomarker of DR.

This is the first study to systematically examine the onset of perturbations to RPE and outer retinal neuron function in mouse models of T1D and T2D. By evaluating the function of these cells in STZ and Lepr$^{db/db}$ mice, we demonstrate and establish that hyperglycemia leads to early defects within the outer retina/RPE despite differing etiologies. The functional defects documented here are not attributable to overt structural damage to the RPE or outer retina as evidenced by normal SLO fundus photographs and SDOCT scans (Fig 8).
Furthermore, in each strain, at least one component of the dc-ERG, the c-wave, is reduced by a greater magnitude than that of the a-wave. Because the light-evoked responses of the RPE are dependent upon photoreceptor activity, this initial reduction is significant in demonstrating that a specific and independent insult to the RPE concurrently with hyperglycemia. We further propose that insulin may prevent progression of RPE dysfunction but not outer retinal neuron dysfunction due to the different pattern of results obtained from BKS.Lepr and B6.BKS.Lepr mice.

**Type 1 diabetic mice**

STZ-injected T1D mice displayed reductions in RPE function (Fig 2) and a-wave and b-wave amplitudes (Fig 1D-F) beginning at 2 weeks post onset of hyperglycemia. We have previously shown in the *Prph*Rd2/− mouse model of photoreceptor degeneration (Samuels et al. 2010) that RPE function was preserved despite a significant loss in rod outer segment and outer nuclear layer thickness and the development of vacuoles and hypertrophy. This result indicates that the RPE function measured by the dc-ERG, while dependent upon rod photoreceptor activity for its initiation, is not simply a surrogate measure of photoreceptor function. Here, dc-ERG changes exceed photoreceptor dysfunction, confirming a functional insult independent of the photoreceptor response.
Rodent models of diabetes have been widely used to understand the pathophysiological mechanisms of DR. For example, STZ-injected rats demonstrated early defects in psychophysical correlates of vision (visual acuity at 3 weeks and contrast sensitivity at 9 weeks post STZ treatment) by the optokinetic tracking response (Aung et al. 2014) (OKR) and ERG OP changes at 4 weeks (Hernandez et al. 2013; Layton et al. 2007; Shinoda et al. 2007; Zhang et al. 2011) as well as a- and b- wave changes (Aizu et al. 2002). Additional studies demonstrated decreases in visual acuity and OPs in STZ-injected mice at 3-4 weeks (Aung et al. 2013) and in b-wave amplitude of alloxan-induced diabetic mice at similar time points (Johnsen-Soriano et al. 2008; Miranda et al. 2007). Initial evidence of neurodegeneration in the outer retina (photoreceptor inner segment swelling and vacuole presence, outer segment disorganization) and abnormal morphology of the RPE (decreased RPE thickness, reduced RPE65 staining) was identified at 12 weeks following diabetes in two albino rat strains (Enzsoly et al. 2014). Akita mice, which mimic T1D by reduced insulin signaling as a result of a missense mutation in Ins2, display similar reductions in a- and b-wave amplitudes although these deficits do not appear until 8 months following onset of hyperglycemia (Barber et al. 2005; Gastinger et al. 2008; Han et al. 2013). While it is not ideal to compare findings between rats and mice as phenotypes vary between strain, species and protocol for diabetes induction (Lai and Lo 2013), it is noteworthy that in all models of T1D, dysfunction and pathology attributable to the neural retina occur at times that follow the functional RPE defects identified in the present study. Notably, in contrast to our findings,
the majority of the aforementioned studies do not report reductions in a-wave amplitude. While the nature of this discrepancy is unknown, inconsistent reductions in a- and b- wave amplitude at early time points following hyperglycemia have been found (Aung et al. 2013). We hypothesize that the difference may reflect differences in anesthetic protocols for ERG testing. Most ERG recording paradigms utilize ketamine/xylazine mixtures for anesthesia, which is known to induce sustained hyperglycemia in mice (Brown et al. 2005). We utilized sodium pentobarbital which does not impact blood glucose levels (Brown et al. 2005). Therefore, it is possible that ketamine/xylazine-induced increases in blood glucose concentrations of control animals masked differences in ERG component amplitudes between control and diabetic groups.

**Type 2 diabetic mice**

 Investigations of DR and neurodegeneration in Lepr<sup>db/db</sup> mice have been performed for nearly 25 years (Cheung et al. 2005; Lai and Lo 2013; Midena et al. 1989; Robinson et al. 2012). Although structural abnormalities, including reductions in thickness of the retina and RPE basement membrane have been identified (Clements et al. 1998; Tang et al. 2011), only one study conducted a functional assessment of the BKS.Lepr model (Bogdanov et al. 2014). These mice demonstrated reductions in a- and b-wave amplitudes, as well as other abnormalities beginning at 16 weeks of age (Bogdanov et al. 2014). Our studies of the BKS.Lepr strain, which has a similar time course of severe and sustained hyperglycemia, replicate these observations (Fig 4). In the second T2D model studied here, B6.BKS.Lepr mice, a-wave reductions were noted at the same time
point (Fig. 6A), while b-wave reductions were present earlier (Fig 6B).
Importantly, each of these defects occurs concurrently with or at time points
which follow the identification of RPE defects and therefore may result as a
secondary effect of the RPE dysfunction.

Our findings demonstrate that both strains of Lepr\textsuperscript{db/db} mice exhibit altered RPE
function evidenced by reduced dc-ERG waveform components. Significant
reductions in the c-wave, fast oscillation and off response precede those of the a-
wave, most notably in BKS.Lepr mice (Fig. 5, 7). In this model, the fast
oscillation was affected first (Fig. 5B, 7B, 9C). The fast oscillation is generated
by a restoration of subretinal [K\textsuperscript{+}] and a [Cl\textsuperscript{-}] dependent hyperpolarization of the
basal RPE membrane, so investigation of the channels underlying these currents
and how they are affected by hyperglycemia will be important to examine in
future studies. RPE dysfunction appears to be reversible, as the c-wave, fast
oscillation and off response in B6.BKS.Lepr mice return to control amplitudes
(Fig 7A-D) when mice become normoglycemic (Fig. 3C). This return to
normoglycemia in B6.BKS.Lepr mice is correlated with significantly elevated
insulin levels (Fig. 3D). While BKS.Lepr also display increased insulin levels, the
elevation is only moderate. The Lepr\textsuperscript{db/db} mutation is identical in BKS.Lepr and
B6.BKS.Lepr mice; however, these strains differ in H2 haplotype and it has been
proposed that differences in metabolism of endogenous androgens and estrogen
on the two backgrounds may account for their apparent differences in
susceptibility and severity of diabetes (Coleman and Hummel 1973; Santiago et
These differences make it difficult to conclude whether hyperinsulinemia is causative of RPE function stabilization. Importantly, no defects in RPE function are found prior to onset of hyperglycemia though and, in B6.BKS.Lepr mice, when glucose concentrations are lowered due to hyperinsulinemia, RPE defects normalize to the reduction in the a-wave. Future studies to test the hypothesis that insulin reduces RPE defects could be performed by treating BKS.Lepr mice with exogenous insulin so that serum levels match those of B6.BKS.Lepr mice, then determining if the amplitude of dc-ERG waveform components are normalized to the a-wave. In support of the possibility that insulin may protect the RPE is the recent finding by Enzsoys et al (2014), which demonstrated that BKS.Lepr mice fed a restrictive diet to lower blood glucose levels did not display some of the ERG abnormalities found in their hyperglycemic counterparts. Additional support that elevations in glucose affect RPE function is provided by EOG data that demonstrated a reduction in fast oscillation amplitudes with acute elevations of D-glucose (Schneck et al. 2000) and data from mfERG studies that demonstrated an association of increased abnormal neuroretinal function with poor long-term glucose control (Lakhani et al. 2010; Laron et al. 2012).

**b-wave reductions**

Reductions in b-wave amplitude of diabetic mice and rats have been routinely reported by other groups (Barber et al. 1998; Hammes et al. 1995; Harrison et al. 2011; Lai and Lo 2013; Ng et al. 2008; Robinson et al. 2012; Zhang et al. 2008).
and we demonstrate similar reductions here. In juxtaposition to our findings for dc-ERG components generated by the RPE, it appears that once a hyperglycemic insult has occurred, the reduction of the b-wave is immutable. Despite a return of normal glucose concentrations as observed in B6.BKS.Lepr mice, the b-wave continued to decline with age in both Lepr strains (Fig 6B). It is important to bear in mind that the corneal-positive b- and c-waves are influenced by a corneal-negative ERG component, slow PIII, which is generated by a Kir4.1 conductance in Müller glia cells (Kofuji et al. 2000; Wu et al. 2004a). As a consequence, the b- and/or c-wave reductions could reflect an increase in slow PIII amplitude. We are using the Nyx<sup>nob</sup> mouse (Pardue et al. 1998), which lacks the ERG b-wave to genetically isolate slow PIII and thus determine how the response properties of this component are impacted by diabetes.

**Controlling hyperglycemia**

It is well established that chronic hyperglycemia is a major cause of DR and other microvascular complications associated with diabetes (1993; King et al. 1999). Our findings support the notion that the RPE, responsible for transport of glucose across the blood-retinal-barrier, is significantly impacted immediately following onset of hyperglycemia. Campochiaro and colleagues (Lu et al. 2013) have shown that modulation of the glucose transporter, GLUT1 in the eye as a whole can modulate glucose concentrations within the retina and prevent diabetes-associated hallmarks of pathology. It is an intriguing possibility that specific reduction of GLUT1 within the RPE of diabetic mice could protect the retina from
elevations in retinal glucose concentration. In addition, evaluation of RPE function in animal models of diabetes with the dc-ERG or in diabetic patients by the EOG following pharmacotherapy may be useful in measuring efficacy of treatments to slow and prevent diabetes-associated damage to the neural retina.

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**Figure Legends**

**FIG. 1:** T1D STZ mice display reductions in strobe flash ERGs beginning two weeks post onset of hyperglycemia. *A:* schematic illustrating the STZ injection and ERG testing paradigms *B:* systemic non-fasting glucose concentration *C:* serum insulin levels at each time point of ERG testing *D:*
averaged ERG waveform tracings from 3 representative flash luminances. CNTL mice are in black, STZ-injected diabetic mice are in gray. 

E: a-wave amplitudes obtained in response to a 1.4 log cd s/m² flash. F: b-wave amplitudes obtained in response to a 1.4 log cd s/m² flash. G: Relative changes in a- and b-wave amplitudes observed in STZ mice. Data are plotted relative to the average of the age-matched CNTL mice. The diagonal line indicates an equivalent reduction in a- and b-wave amplitudes. CNTL, control; data points indicate the average ± sem. At each time point, n≥3 for each group. * indicates p<0.05 between CNTL and STZ by Two-way ANOVA with Tukey post-hoc analysis.

FIG. 2: T1D STZ mice display reductions in RPE function by the dc-ERG which coincides with decreased photoreceptor function. A: c-wave. B: fast oscillation. C: light peak. D: off response amplitude at the three time points tested. E: representative dc-ERG waveform traces at each time point. CNTL traces are black, STZ traces are in gray. The major dc-ERG components are labeled in the upper trace. F: Relative amplitude of the c-wave plotted as a function of the relative a-wave amplitude. G: Relative amplitude of the fast oscillation plotted as a function of the relative a-wave amplitude. H: Relative amplitude of the off response plotted as a function of the relative a-wave amplitude. Black diamonds, 1 week; gray, 2 weeks; white, 4 weeks post onset of hyperglycemia. Statistical analysis was performed separately at each time point between groups. n≥3 for each group at each time point. *p<0.05.
FIG. 3: Lepr\textsuperscript{db/db} mice are obese, and display different extents of hyperglycemia and hyperinsulinemia based on background strain. A: schematic illustrating the experimental timeline of ERG testing for both strains of Lepr mice. B: Body weight of B6.BKS.Lepr (left) and BKS.Lepr (right) mice over the time course of testing. C: systemic non-fasting blood glucose concentration over the time course of testing. Arrows indicate times of hyperglycemia. D: serum insulin concentration of the time course of testing. For all graphs, Lepr\textsuperscript{+/+},db/\textsuperscript{+} mice are in black, Lepr\textsuperscript{db/db} mice are in gray. Data points indicate average ±sem. n≥3 for each group; Student’s t-test was performed between genotypes at each time point. *p<0.05.

FIG. 4: T2D BKS.Lepr\textsuperscript{db/db} mice display reductions in a- and b-wave amplitude. A: averaged ERG waveform tracings from 3 representative flash luminance intensities at the ages examined. B-C: average a-wave and b-wave amplitudes at each time point in response to a 1.4 log cd s/m\textsuperscript{2} flash. Lepr\textsuperscript{+/+},db/\textsuperscript{+} mice are in black, Lepr\textsuperscript{db/db} mice are in gray. Boxes surrounding ages indicate times of hyperglycemia. D: Amplitude of the b-wave plotted relative to that of the a-wave. All data are normalized by the response amplitude of control littermates. The diagonal line indicates an equivalent reduction in a- and b-wave amplitude. The WT point represents the average relative amplitude ±sem calculated for Lepr\textsuperscript{+/+},db/\textsuperscript{+} mice that was utilized to determine statistical significance of Lepr\textsuperscript{db/db} data. Data points indicate average ±sem of at least 3 mice for each group.
*p<0.05 by Two-way ANOVA with Tukey post-hoc analysis (B-C) and Student’s t-test (D).

FIG. 5: T2D BKS.Lepr\textsuperscript{db/db} mice display reductions in RPE function by the dc-ERG which precede reductions in photoreceptor function. A: c-wave B: fast oscillation C: light peak D: off response amplitude of BKS.Lepr\textsuperscript{+/-,db/+} and BKS.Lepr\textsuperscript{db/db} mice at the ages tested. Data points indicate average ±sem of at least 3 mice for each group. Boxes surrounding ages indicate times of hyperglycemia. E: representative dc-ERG waveform traces at each time point. BKS.Lepr\textsuperscript{+/-,db/+} traces are black, BKS.Lepr\textsuperscript{db/db} traces are in gray. F: Relative amplitude of the a-wave, c-wave, fast oscillation and off response as a function of age. Data are normalized to responses obtained from control BKS.Lepr\textsuperscript{+/-,db/+} littermates. Student’s t-test was performed between genotypes at each time point. *p<0.05, **p<0.001, ***p<0.0001.

FIG. 6: T2D B6.BKS.Lepr\textsuperscript{db/db} mice display reductions in a- and b- wave amplitude. A: averaged ERG waveform tracings from 3 representative flash luminances over the time course of testing. B-C: average a-wave and b-wave amplitude at each age in response to a 1.4 log cd s/m\textsuperscript{2} flash. Boxes surrounding ages indicate times of hyperglycemia. B6.BKS.Lepr\textsuperscript{+/-,db/+} mice are in black, Lepr\textsuperscript{db/db} mice are in gray D: Amplitude of the b-wave plotted relative to a-wave. Data are normalized to the responses obtained from control B6.BKS.Lepr\textsuperscript{+/-,db/+} mice at the same time point. The diagonal line indicates an equivalent reduction
in a- and b-wave amplitudes. The WT point represents the average relative amplitude ±sem calculated for \( Lepr^{+/+},db/+ \) mice. \( n \geq 8 \) for each group. Two way ANOVA with post-hoc Tukey test (B-C) and Student’s t-test (D) was performed.*\( p<0.05 \), **\( p<0.001 \), ***\( p<0.0001 \).

**FIG. 7:** T2D B6.BKS.\( Lepr^{db/db} \) mice display reductions in RPE function by the dc-ERG which are largely attributable to decreased photoreceptor activity. A: c-wave B: fast oscillation C: light peak D: off response amplitude of B6.BKS.\( Lepr^{+/+},db/+ \) and B6.BKS.\( Lepr^{db/db} \) mice at the ages tested. Boxes surrounding ages indicate times of hyperglycemia. E: Relative amplitude of the a-wave, c-wave, fast oscillation and off response in B6.BKS.\( Lepr^{db/db} \) mice as a function of age. Data are normalized to responses obtained from B6.BKS.\( Lepr^{+/+},db/+ \) mice. \( n \geq 8 \) for each group. Student’s t-test was performed between genotypes at each time point. *\( p<0.05 \), **\( p<0.001 \).

**FIG. 8:** \( Lepr^{db/db} \) mice do not show signs of RPE permeability or overt structural damage even at late ages. A: Representative photomicrographs from SLO imaging and SDOCT of BKS.\( Lepr^{+/+},db/+ \) (top) and BKS.\( Lepr^{db/db} \) (lower) mice at 16 weeks of age. B: Representative photomicrographs from SLO imaging and SDOCT of B6.BKS.\( Lepr^{+/+},db/+ \) (top) and B6.BKS.\( Lepr^{db/db} \) (lower) mice at 50 weeks of age. No overt structural damage to the retina or RPE is observed in either strain. IRDF, infrared darkfield; RFDF, red free dark field; AF, autofluorescence. Arrowheads indicate abnormal patterning observed.
**FIG. 9:** Reductions in RPE function of BKS.**Lepr**<sup>db/db</sup> mice are concomitant with increased glucose concentration. Averaged relative A: a-wave B: c-wave C: fast oscillation and D: off response amplitudes (±sem) of BKS.**Lepr**<sup>db/db</sup> (left), B6.BKS.**Lepr**<sup>db/db</sup> (middle) and STZ (right) mice plotted against average (±sem) blood glucose concentration. Amplitude measures are normalized to average control (either **Lepr**<sup>+/+;db/+</sup> or CNTL) responses. Vertical blue lines highlight 250mg/dl glucose, which is the cutoff for hyperglycemia. Horizontal blue lines highlight the average normalized control response. In BKS.**Lepr**<sup>db/db</sup> mice, arrows indicate a decrease in the amplitude of dc-ERG components at ages where a-wave amplitudes remain unaffected. A different pattern is seen in B6.BKS.**Lepr**<sup>db/db</sup> mice, however, where only the c-wave is selectively reduced at 4 weeks, when mice are hyperglycemic (yellow arrowhead). In STZ mice, amplitude reductions are comparable for all ERG measures except the 2 week c-wave (yellow arrowhead). Black diamonds, 4 weeks; gray, 8 weeks; white, 12 weeks; red, 16 weeks; blue, 24 weeks.

**FIG. 10:** Cone ERGs are reduced in T2D but not T1D mice. A: Amplitude (right) and representative waveform tracings (left) of the cone ERG in CNTL and STZ mice in response to a 1.4 log cd/m<sup>2</sup> flash at each time point examined B: representative waveform tracings (top) and amplitude (bottom) of the cone ERG in BKS.**Lepr**<sup>+/+,db/+</sup> and BKS.**Lepr**<sup>db/db</sup> mice in response to a 1.4 cd/m<sup>2</sup> flash at each age C: representative waveform tracings (top) and amplitude (bottom) of
the cone ERG in B6.BKS.\textit{Lepr}^{+/-,db/+} and B6.BKS.\textit{Lepr}^{db/db} mice in response to a 1.4 cd/m$^2$ flash at each age. Note that B6.BKS.\textit{Lepr}^{db/db} mice develop reductions in the light-adapted response earlier than the BKS strain and no changes are observed in the STZ mice at any of the time points analyzed. Bars indicate average ±sem, Boxes surrounding ages indicate times of hyperglycemia. n≥3 for each group. Student’s t-test was performed between control and diabetic groups at each separate time point. *p<0.05, **p<0.001, ***p<0.0001.

REFERENCES:


Gallemore RP, and Steinberg RH. Light-evoked modulation of basolateral membrane


Pardue MT, McCall MA, LaVail MM, Gregg RG, and Peachey NS. A naturally occurring mouse model of X-linked congenital stationary night blindness. *Investigative ophthalmology & visual science* 39: 2443-2449, 1998.


Treatment/Testing Paradigm:

Age: 6wks 7wks 8wks 9wks 11wks
Diabetes: baseline 0wks 1wk 2wks 4wks

ERG testing: Standard strobe flash & dc-ERG at 1, 2 and 4 weeks diabetes
Streptozotocin (STZ) injection (25mg/kg on three consecutive days between 6 and 7 weeks of age)

B. Non-fasting blood glucose (mg/dl) vs. Duration of diabetes (wks)

C. Serum Insulin (ng/ml) vs. Duration of diabetes (wks)

D. Graph showing waveforms for different log cd s/m^2 levels:

-2.4
-0.6
1.4

E. Graph showing a-wave amplitude for 1wk, 2wk, 4wk

F. Graph showing b-wave amplitude for 1wk, 2wk, 4wk

G. Graph showing relative b-wave vs. relative a-wave for 1wk, 2wk, 4wk
A. Treatment/Testing Paradigm:

Age: 4wks 8wks 12wks 16wks 24wks
Diabetes: B6.BKS BKS
Times of ERG testing (Standard & dc- ERG, weighing, blood glucose and insulin measurements)

B. B6.BKS

Body Weight (g)

0 10 20 30 40 50 60 70 80
0 4 8 12 16 20 24
Age (weeks)

Lepr +/+;+/-db
Lepr db/db

BKS

Body weight (g)

0 10 20 30 40 50 60 70 80
0 4 8 12 16 20 24
Age (weeks)

C. non-fasting blood glucose (mg/dl)

0 100 200 300 400 500 600
0 4 8 12 16 20 24
Age (weeks)

D. Insulin (ng/ml)

1 10 100
0 4 8 12 16 20 24
Age (weeks)
**A**

- 4wks
- 8wks
- 12wks
- 16wks
- 24wks

- 100μV
- 100 msec

- Log cd s/m^2

**B**

**A-wave**

- Bar graph showing amplitude (μV) vs. age (weeks) for different genotypes.

**C**

**B-wave**

- Bar graph showing amplitude (μV) vs. age (weeks) for different genotypes.

**D**

- Graph showing relative b-wave vs. relative a-wave for different genotypes (4wk, 8wk, 12wk, 16wk, 24wk, WT), with significance markers (*, **, ***).