Correlation of Retinal Structure and Visual Function Assessments in Mouse Diabetes Models

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Purpose. Diabetic retinopathy results in vision loss with changes to both retinal blood vessels and neural retina. Recent studies have revealed that animal models of diabetes demonstrate early loss of visual function. We explored the time course of retinal change in three different mouse models of diabetes in a longitudinal study using in vivo measures of retinal structure (optical coherence tomography [OCT]) and visual function (optomotor and pupillary responses).

Methods. OCT analysis of retinal microstructure, optokinetic response as a measure of visual acuity, and pupillary response to light stimulation were compared among the db/db, Ins2^{Akita}, and streptozotocin (STZ)-induced mouse models of diabetes at 1.5, 3, 6, and 9 months of diabetes.

Results. The db/db, Ins2^{Akita}, and STZ-induced models of diabetes all exhibited vision loss and retinal thinning as disease progressed. Both structural changes and functional measures were significantly correlated with the blood glucose levels. Despite this, vision loss and retinal thinning were not consistently correlated, except for the inner retinal layer thickness at 6 months of diabetes.

Conclusions. This longitudinal study compiled structural measures and functional outcome data for type 1 and 2 diabetes mouse models commonly used for diabetes studies and demonstrated an overall decline in retinal-related health in conjunction with weight change and blood glucose alterations. The relationship between the structural change and functional outcome could be correlative but is not necessarily causative, as retinal thinning was not sufficient to explain visual acuity decline.

Keywords: diabetic retinopathy, mouse, retina, structure, function

Current estimates suggest that approximately 10% of all Americans have diabetes,[1] leading to an increase in diabetic retinopathy (DR), as the majority of patients with type 1 diabetes and nearly 60% of patients with type 2 diabetes are diagnosed with retinopathy during the first 20 years of having diabetes.[2] The disease manifests with leaky vessels, edema, and neovascularization, all of which contribute to vision loss,[3] and current therapies for DR target vascular abnormalities. Studies also suggest that increased apoptosis of neural tissue and retinal thinning may precede vascular abnormalities,[4] termed retinal diabetic neuropathy (RDN) by Sohn et al.[5] To determine the temporal relationship between DR and RDN in humans, a longitudinal study in people with diabetes with no or minimal DR revealed that RDN may precede signs of characteristic microvascular abnormalities that could be observed in the clinic.[5] However, the relationship between the neural and vascular pathology in DR remains poorly defined.

Multiple models of diabetes have been created using the mouse, through genetic mutation and chemical induction. Three of the most well-known mouse models of diabetes are streptozotocin (STZ)-induction, Ins2^{Akita}, and db/db. Type 1 diabetes is induced by injection of STZ, a glucosamine–nitrosourea compound that damages the insulin-producing pancreatic islet β cells.[6] The Ins2^{Akita} mouse has a spontaneous mutation in the insulin 2 gene which leads to type 1 diabetes through incorrect folding of the insulin protein that induces the unfolded protein response and cell death of the pancreatic β cells, resulting in reduced β-cell mass and reduced insulin secretion.[7] The db/db mouse has a spontaneous mutation that results in leptin deficiency and is used to model aspects of type 2 diabetes.[8]

Previous studies have been conducted to document the course of visual loss in these diabetic mouse models separately.[5,9–14] Barber et al.[15] reported that Ins2^{Akita} mice demonstrated increased vascular permeability and neuronal apoptosis. In contrast, McLenachan et al.[14] reported an absence of classical clinical correlates of human DR in Ins2^{Akita} mice at up to 6 months of age. However, Hombre-bueno et al.[12] observed a progressive thinning of the retina starting at 3 months with the presence of a variety of retinal neuropathic lesions, including the degeneration of cone photoreceptors and severe impairment of synaptic connectivity at the outer plexiform layer detected at 9 months.

By using optomotor responses to assess spatial frequency thresholds and contrast sensitivity, Akimov and Renteria[9] showed that Ins2[Akita] mice have progressive quantifiable vision deficits.

Studies carried out in the db/db mice, a type 2 diabetes model, also revealed significantly lower visual acuity compared with the age-matched control mice as determined by optomotor response.[16] Increased retinal ganglion cell apoptosis, significant alternations of the inner retinal activity measured by pattern electroretinography and altered microglia/macrophage were observed in the db/db mice for over a period of 6 months, and optical coherence tomography (OCT) also revealed obvious retinal thinning.[17]

The STZ-induced diabetic mouse model was used in a longitudinal study[10] that showed that, although visual acuity loss and retinal thinning started early, at 4 weeks after the onset of diabetes, contrast sensitivity was not changed until 18 weeks. The loss of visual function in the STZ-induced diabetic mouse model was escalated by feeding a diet high in fat and carbohydrates.[11] Appropriate animal models and assessment of the deficits in visual function will be critical for determining the underlying mechanisms of retinal neurodegeneration in DR and will be important for developing successful preventative treatments for diabetic complications.[13]

In addition to retinopathy and nephropathy, autonomic neuropathy is a common complication of diabetes. Dynamic pupillometry is a simple and non-invasive technique that has been used to study various disease conditions that affect the autonomic nervous system.[18,19] As the pupil light reflex is under the direct control of the autonomic nervous system, the size of the pupil, controlled by circular (parasympathetic nervous system) and radial (sympathetic nervous system) muscles of the iris, can be evaluated using a simple and non-invasive pupil light reflex technique with a pupillometer. The average baseline pupil size in humans with increasing severity of DR was found to be decreased compared with controls.[18] Decreases in the amplitude and velocity of pupillary constriction were also observed with increasing severity of retinopathy.

Although many studies have looked at different parameters in diabetes animal models, few have compared the time course of changes across multiple animal models. The current study was designed to assess changes in visual function and compare these longitudinally to changes in OCT and pupillary response with the aim of developing a more comprehensive analysis of progression of retinal pathology. The study revealed the time course of vision loss in each of the three diabetic models. Retinal thinning was observed in all three models and was partially correlated with loss of acuity but did not appear causative, as clear changes in retinal thinning occurred well before changes in visual acuity and acuity decreased without further thinning in the db/db model. Pupillary changes were observed only in the db/db model. These studies suggest that retinal thinning is a common feature of DR in mouse models, but thinning appears unlikely to account for loss of visual acuity.

METHODS

Animal Models

All mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and the guidelines established by the University of Michigan Institutional Animal Care and Use Committee. Male C57BL/6J mice were injected intraperitoneally with STZ (65 mg/kg body weight) for 5 consecutive days at 7 to 8 weeks of age to induce type 1 diabetes. Diabetic mice that displayed glucose levels under 250 mg/dL were excluded from the study. C57BL/6J mice without STZ treatment were used as controls. Spontaneously diabetic heterozygous male C57BL/6-Ins2[Akita]/J mice were used as another type 1 diabetes model. C57BL/6J mice were used as the wild-type (WT) controls. Homozygous male BKS.Cg-*Dock7^m*+/+*Lepr^db*/J mice, otherwise known as db/db mice, were used as a model for type 2 diabetes. Heterozygous littermate db/+ mice were used as controls. For this longitudinal study, the same animals were tested across all time points for all three models.

Body Weight and Blood Glucose Monitoring

The body weight and blood glucose levels of each mouse were taken at intervals to track the progression of the disease in relation to the controls. A small portion (1 to 2 mm) of the tip of the tail was removed with a razorblade; blood from the tail was placed on a blood glucose test strip in a glucometer (OneTouch Ultra; LifeScan, Milpitas, CA, USA), and the blood glucose was recorded. The mice were then weighed. All blood glucose monitoring and weighing were done in the afternoon to avoid blood sugar spikes from overnight eating.

Retinal Microstructure Imaging

A spectral-domain OCT (SD-OCT) system (Bioptigen, Morrisville, NC, USA) was used to measure retinal thickness in each mouse model. Tropicamide (0.5%) drops were used to stimulate eye dilation. The mice were anesthetized with ketamine and xylazine (50 mg/kg and 5 mg/kg bodyweight, respectively). One rectangular scan consisting of 1000 A-scans by 100 B-scans over a 1.4 × 1.4-mm area centered on the optic nerve head (ONH) was done on each eye of each animal. The scans were processed using the Bioptigen Diver analysis software. Retinal thickness was measured at 0.35 mm from the ONH. The inner, outer, and total thicknesses of four points (nasal, temporal, superior, and inferior) on the retina were averaged across the points.

Visual Acuity

A virtual-reality optokinetic tracking system (OptoMotry, CerebralMechanics, Lethbridge, AB, Canada) was used to test visual acuity with an optokinetic head-tracking (OKT) test. Mice were placed on a pedestal inside a chamber consisting of four computer monitors and were allowed to move freely. An alternating rotating sine-wave grating stimulus was presented on the screens in three-dimensional space. The mice tracked the stimulus reflexively until it was no longer visible to them. This tracking was one directional, temporal to nasal, so the acuity of both eyes was tested. The grating was fixed at the eyes of the mouse by constantly recentering the virtual cylinder on the head. The tester recorded the presence and absence of tracking without knowledge of the study group, and a simple staircase method was used to determine the highest level of spatial frequency visible to the mice. The tests were done at maximum contrast with

a drift speed of 12°/s and started at a spatial frequency of 0.042 cyc/deg.

Pupillometer

A pupillometer (A-2000 Pupillometer; Neuroptics, Irvine, CA, USA) was used to measure pupillary responses to light stimuli. This system uses two cameras and two independently controlled light sources that allow for independent stimulation and pupil tracking. The mice were dark adapted for 4 hours prior to testing. They were anesthetized with ketamine and xylazine (50 mg/kg and 5 mg/kg body weight, respectively). The light profile consisted of two red-light stimuli (2.0 log W/m^2) for 1 second; two ultra-low white-light stimuli (−2.5 log W/m^2), two low white-light stimuli (−1.5 log W/m^2), two medium white-light stimuli (−0.5 log W/m^2), and two high white-light stimuli (0.5 log W/m^2), each 0.1 second long; and, finally, a blue-light stimulus (−2.0 log W/m^2) for 1 second. Each stimulus had a weighted delay time to allow the pupil size to recover. After each red-light stimulus there was a 60-second delay; after each ultra-low white-light stimulus, a 19.9-second delay; after each low white-light stimulus, a 29.9-second delay; after each medium white-light stimulus a 39.9-second delay; and after the high white-light stimulus, a 49.9-second delay. The maximum size of the pupil after dark adaptation was quantified at about 2 minutes after anesthesia.

Statistics

Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA, USA). Two-way analysis of variance (ANOVA) was conducted to examine the effect of diabetes status and duration of diabetes on each parameter tested. Figure 1 results are expressed as means, and the error bars represent the standard errors of the means. The results in Figures 2 to 4 and Supplementary Figure S1 are expressed as box-and-whisker plots. Each box represents the 25th to 75th percentile, with the middle line at the median; the whiskers extend to the smallest and largest values ($^*P <$ 0.05, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. db/+, WT, or control at the same time point with the Sidak post hoc test). Correlations among blood glucose levels, visual acuity, and retinal layer thickness (inner, outer, and total) were analyzed with Pearson's correlation coefficient.

RESULTS

Comparison of Weight and Blood Glucose in Diabetes Models

To track the progression of diabetes, body weight and blood glucose measurements were taken for the db/db, Ins2[Akita], and STZ diabetes models during the course of the study (Fig. 1). As expected, the type 2 db/db model had rapid weight gain compared with the db/+ counterparts, reaching almost twice the weight of the littermate group (db/db, 48.4 ± 4.7 g; db/+, 28.1 ± 0.5 g; $P < 0.0001$). Opposite of the db/db, the Ins2[Akita] and STZ groups had similar body-weight trends; both models exhibited a failure to gain weight compared with the nondiabetic controls at the end of the study: Ins2[Akita] (24.3 ± 0.6 g) versus WT (31.1 ± 0.4 g) ($P < 0.0001$) and STZ (26.1 ± 1.2 g) versus control (33.1 ± 0.6 g) ($P < 0.0001$). The Ins2[Akita] model was noted to be at a lower weight from the beginning of testing (Ins2[Akita], 20.0 ± 0.2 g; WT, 24.5 ± 0.4 g; $P < 0.0001$), and the diabetic animals gained less weight over the course of study (Ins2[Akita], 4.3 g; WT, 6.6 g). The STZ group also failed to gain weight after diabetes induction and had much less weight gain than the control group (STZ, 3.2 g; control, 8.7 g) at the end of study.

Blood glucose levels of all three diabetic models were elevated. Blood glucose values higher than 250 mg/dL were considered diabetic, as in previous studies.[20] The db/db model had blood glucose levels that were twice those of the db/+ controls (db/db, 354 ± 43 mg/dL; db/+, 158 ± 5 mg/dL; $P < 0.0001$) at the onset of testing. At 40 weeks, blood glucose was four times higher than the db/+ controls
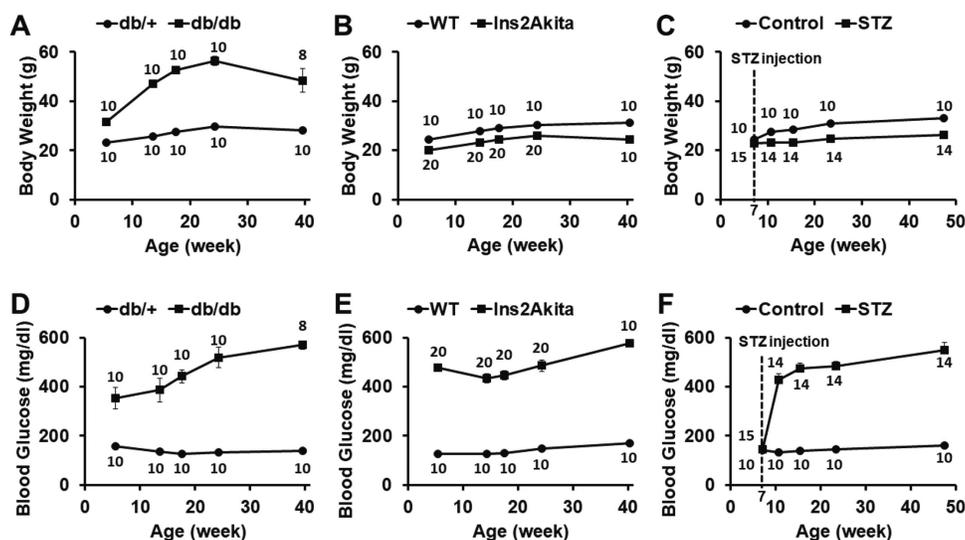
FIGURE 1. Body weight and blood glucose levels of three mouse diabetes models. Body weight (**A–C**) and non-fast blood glucose levels (**D–F**) were obtained from each mouse in the afternoon at different time points near assessments during the course of study. Results are expressed as the mean and standard error of the mean (SEM). The number of animals at each time point is indicated. Except at the time of STZ injection in the STZ model, significant differences were observed at all time points for both body weight and blood glucose levels in all three models.
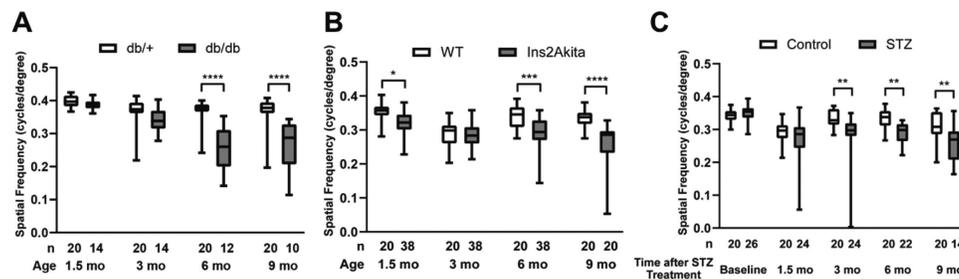
FIGURE 2. Changes in visual acuity for the three mouse diabetes models. The visual acuity of the mouse was assessed using a CerebralMechanics OptoMotry virtual-reality optokinetic tracking system. A simple staircase method was used to determine the highest level of spatial frequency visible to the mouse. The tests were performed at maximum contrast with a drift speed of 12°/s, starting at a spatial frequency of 0.042 cyc/deg. Results are expressed as box-and-whisker plots. The box represents the 25th to the 75th percentile, with the middle line at the median; the whiskers extend to the smallest and largest values. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus db/+, WT, or control mice at the same time point (Sidak post hoc test). The number of animals at each time point is indicated.

(db/db, 569 ± 14 mg/dL; db/+, 139 ± 2 mg/dL; $P < 0.0001$). The Ins2[Akita] model had blood glucose levels that stayed consistently high throughout the testing period (576 ± 16 mg/dL at the beginning of the study) and never dropped below three times the blood glucose levels of their littermate WT control group (169 ± 11 mg/dL). STZ injection induced blood glucose levels to increase to three times the control for the duration of testing (STZ, 550 ± 30 mg/dL; control, 161 ± 8 mg/dL; $P < 0.0001$).

The above physical data indicate that each of the models displayed diabetic phenotypes. The db/db group exhibited rapid weight gain and sustained the high blood glucose levels indicative of type 2 diabetes. Both type 1 diabetes models (Ins2[Akita] mice and mice that underwent STZ treatment) had similar trends of high blood glucose levels and failed to gain weight.

Visual Acuity Changes Over Course of Diabetes

The visual acuity of each diabetic model was measured using a simple staircase method to determine the highest level of spatial frequency visible to each mouse in an OKT test. The averaged results of the three models can be found in Figure 2. Consistent differences were noted between the db/db and db/+ groups. At 3 months, the db/db group showed a slight decrease in visual acuity compared with the db/+ group (9.2%, $P > 0.05$). At 6 and 9 months, the difference became very significant (44.0% at 6 months, $P < 0.0001$; 41.4% at 9 months, $P < 0.0001$). These consistent differences resulted in a trend of declining acuity in the diabetic mice, which was demonstrated by the two-way ANOVA. There was a statistically significant difference in visual acuity by both diabetes status ($F_{1,122} = 69.84$, $P < 0.0001$) and by duration of diabetes ($F_{3,122} = 24.77$, $P < 0.0001$), and the interaction between the diabetes status and duration of diabetes was also significant ($F_{3,122} = 11.46$, $P < 0.0001$). For the Ins2[Akita] diabetes model, a decline in visual acuity could be seen as early as 1.5 months of age (9.7%, $P < 0.05$). However, this difference was not seen at 3 months, as the average for the WT mice demonstrated a transient decrease. The difference in visual acuity was observed again at the 6-month (13.8%, $P < 0.001$) and 9-month time points (29.9%, $P < 0.0001$). As with the db/db mice, acuity declined over the course of the testing period in control animals. Despite this, there was a significant difference in all three factors: diabetes status ($F_{1,206} = 47.45$, $P < 0.0001$), duration of diabetes ($F_{3,206} = 16.84$, $P < 0.0001$), and the interaction between them ($F_{3,206}$

$= 6.279$, $P < 0.001$). The STZ group had an initial decrease in visual acuity at 1.5 months after STZ treatment and demonstrated a consistent decrease in visual acuity compared with the controls beginning at 3 months (12.1%; $P < 0.01$) after STZ treatment. The difference continued to be observed at 6 months (15%; $P < 0.01$) and at 9 months (18.7%; $P < 0.01$). These consistent differences are mirrored by the two-way ANOVA test, which showed that, like the db/db and Ins2[Akita] models, the diabetes status ($F_{1,200} = 25.83$, $P < 0.0001$), duration of diabetes ($F_{4,200} = 14.66$, $P < 0.0001$), and the interaction between the diabetes status and duration of diabetes ($F_{4,200} = 3.334$, $P < 0.05$) were also significant.

Retinal Microstructure Imaging Reveals Most Changes Occur in the Inner Retina

Retinal thickness was measured at four time points of disease. The inner, outer, and total thicknesses were defined and quantified for each diabetes model (Fig. 3A). An average was obtained for each eye from four points 0.35 mm from the ONH. The averages for each retinal layer of each model can be found in Figures 3B to 3J. The db/db diabetes model displayed significant thinning across all four time points for the inner retinal thickness measurements, specifically by diabetes status ($F_{1,121} = 72.61$, $P < 0.0001$) and by duration of diabetes ($F_{3,121} = 9.219$, $P < 0.0001$). Significant differences in layer thickness compared with the nondiabetic db/+ controls were observed at all four time points (1.5 months, 0.005 mm, $P < 0.0001$; 3 months, 0.0049 mm, $P < 0.0001$; 6 months, 0.0036 mm, $P < 0.01$; 9 months, 0.0033 mm, $P < 0.01$). Although no significant difference was observed in the outer retinal thickness by direct comparison, there was a significant difference in thickness by duration of diabetes ($F_{3,121} = 19.94$, $P < 0.0001$).

Thinning in the inner layer was translated to significant changes in the first two time points for the total retinal thickness measurements (1.5 months, 0.0057 mm; $P < 0.001$; 3 months, 0.0055 mm; $P < 0.001$). However, there were no significant differences in the total thickness at the end of the testing period between the db/+ and db/db groups, as the age-related thinning in retinal layers was observed in the db/+ mice. Despite this, thickness differences caused by diabetes status ($F_{1,121} = 38.81$, $P < 0.0001$) and by duration of diabetes ($F_{3,121} = 23.33$, $P < 0.0001$) were significant. The Ins2[Akita] model of diabetes resulted in varying retinal thickness differences while maintaining a trend of
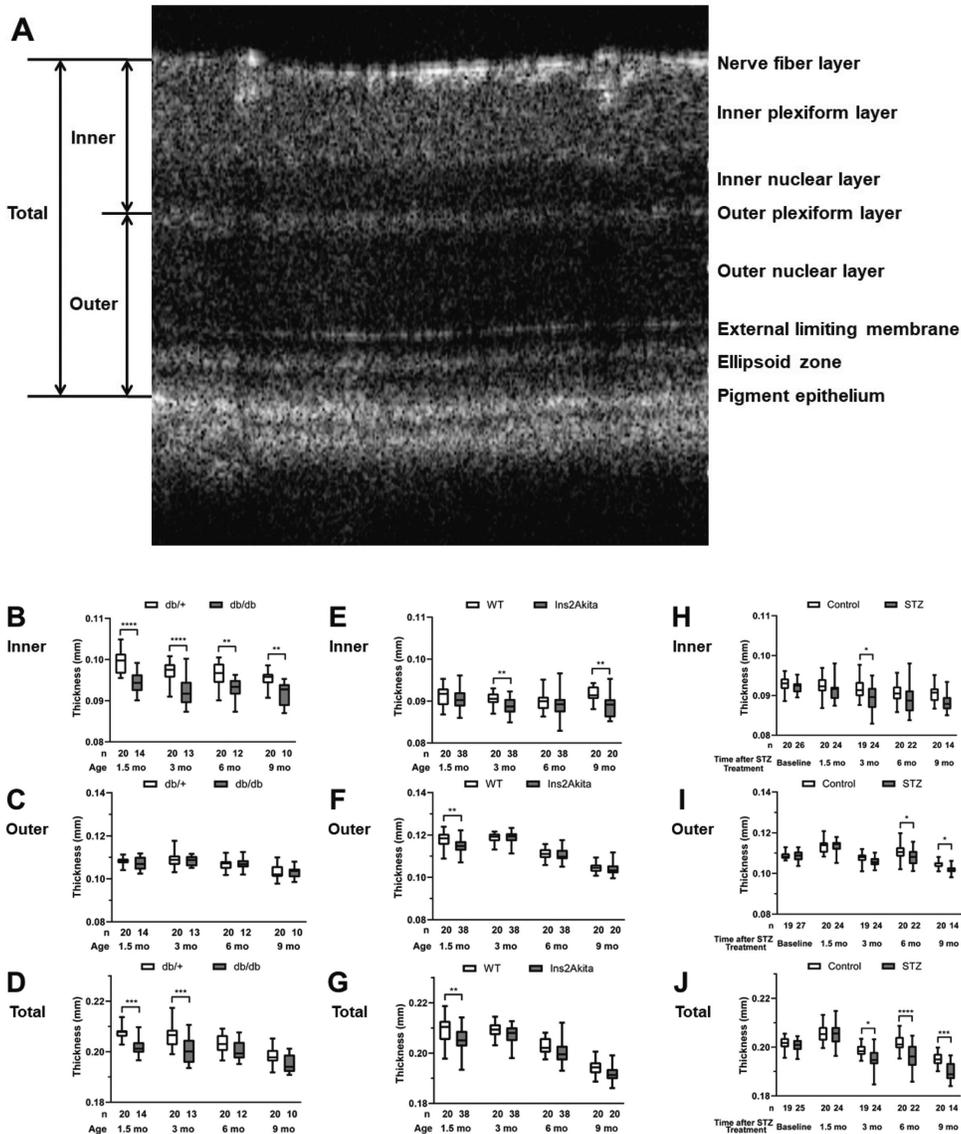
FIGURE 3. Changes in retinal thickness for the three mouse diabetes models. Retinal images were obtained at different time points with a Bioptigen Envisu R2200 SD-OCT imaging system. The rectangular scan of each eye was processed to obtain the thicknesses of the inner, outer, and total retinal layers with Bioptigen Diver software (**A**). Measurements of total retinal thickness from the top of the retinal ganglion cell layer (included) to the top of the retinal pigment epithelium (excluded) were obtained. Rectangular volumes consisting of 1000 A-scans by 100 B-scans over a 1.4 × 1.4-mm area centered on the ONH were obtained. Retinal thicknesses at 0.35 mm relative to the ONH of the nasal, temporal, superior, and inferior regions were quantified and averaged for each eye. Retinal images were acquired at different time points. The inner (**B**, **E**, **H**), outer (**C**, **F**, **I**), and total (**D**, **G**, **J**) layers were compared to the db/+, WT, or control at the same time point. Results are expressed as box-and-whisker plots. The box represents the 25th to the 75th percentile, with the middle line at the median; the whiskers extend to the smallest and largest values. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ versus db/+, WT, or control at the same time point (Sidak post hoc test). The number of animals at each time point is indicated.

overall thinning compared with the WT control. The inner layer only had significant differences at 3 months (0.003 mm; $P < 0.01$) and at 9 months (0.0027 mm; $P < 0.01$). Thus, differences caused by diabetes status ($F_{1,206} = 23.79$, $P < 0.0001$) and by duration of diabetes ($F_{3,206} = 4.870$, $P < 0.01$) were both significant, although the interaction between them was not. The outer and total thicknesses of the Ins2^{Akita} group were significantly thinner than those for the WT group at the 1.5-month time point (outer, 0.0029 mm, $P < 0.01$; total, 0.0038 mm, $P < 0.01$). However, this initial difference was not permanent, as the rest of the time points showed no significant changes between the

Ins2^{Akita} and the WT control. Note that thickness in the WT controls decreased as the mice aged.

Despite the lack of significance in direct comparison, the two-way ANOVAs of both the outer and total thickness displayed significant differences: for outer thickness, diabetes status ($F_{1,206} = 5.097$, $P < 0.05$), diabetes duration ($F_{3,206} = 216.1$; $P < 0.0001$), and interaction ($F_{3,206} = 2.742$, $P < 0.05$); for total thickness, diabetes status ($F_{1,206} = 22.09$, $P < 0.0001$) and diabetes duration ($F_{3,206} = 138.8$, $P < 0.0001$). The STZ group showed a significant difference in the inner layer at 3 months (0.0021 mm; $P < 0.05$), and differences in the outer layer were observed at 6 months
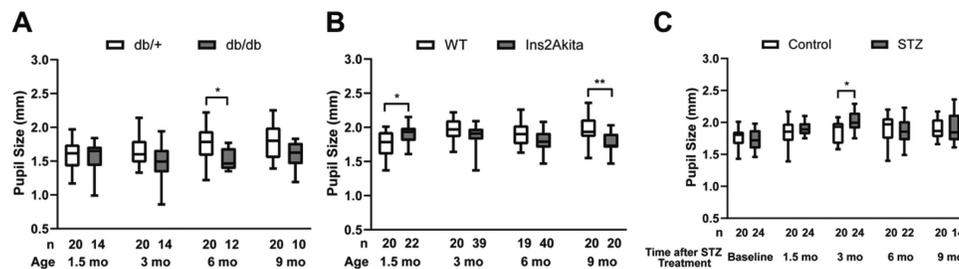
FIGURE 4. Changes in pupil size for the three mouse diabetes models. The maximum pupil size of the mouse was assessed using a Neuroptics A-2000 pupillometer after dark adaptation for 4 hours. Results are expressed as box-and-whisker plots. The box represents the 25th to the 75th percentile, with the middle line at the median; the whiskers extend to the smallest and largest values. *$P < 0.05$ and **$P < 0.01$ versus db/+, WT, or control at the same time point (Sidak post hoc test). The number of animals at each time point is indicated.

(0.0026 mm; $P < 0.05$) and 9 months (0.003 mm; $P < 0.05$). These trends resulted in significant values in the two-way ANOVA calculations for both the inner layer (diabetes status, $F_{1,199} = 19.17$, $P < 0.0001$; diabetes duration, $F_{4,199} = 10.82$, $P < 0.0001$) and the outer layer (diabetes status, $F_{1,199} = 13.10$, $P < 0.001$; diabetes duration, $F_{4,199} = 64.07$, $P < 0.0001$; interaction, $F_{4,199} = 2.543$, $P < 0.05$).

The total retinal thickness was significantly different at the last three time points (3 months, 0.004 mm, $P < 0.05$; 6 months, 0.0053 mm, $P < 0.0001$; 9 months, 0.0051 mm, $P < 0.001$). Due to this direct significance, the differences in diabetes status ($F_{1,197} = 34.86$, $P < 0.0001$), duration of diabetes ($F_{4,197} = 64.20$, $P < 0.0001$), and the interaction between diabetes status and duration of diabetes ($F_{4,197} = 3.825$, $P < 0.01$) were also significant.

Pupillary Response in Diabetes

The maximum pupil size was measured after dark adaptation and prior to stimulus exposure (Fig. 4). Maximum pupil size, latency, and constriction velocity were tested, as differences in dark-adapted pupil size, constriction velocity, and latency have been documented in humans with diabetes.[18,21–24] We saw no consistent differences in pupillary response to light in the diabetes models for constriction velocity or latency, although some specific time points revealed differences identified by two-way ANOVA (Supplementary Fig. S1). However, the db/db model of diabetes displayed a significant difference in maximum pupil size by diabetes status ($F_{1,122} = 16.43$, $P < 0.0001$), and significance

was observed at the 6-month time point (11.8%, $P < 0.05$). The Ins2[Akita] model had a similar trend, although this model started with a higher maximum pupil size (8.9%, $P < 0.05$) and steadily decreased below the control group to become significantly lower at the 9-month time point (9.8%, $P < 0.01$). Similarly, this decline yielded a significant difference in maximum pupil size with regard to diabetes status ($F_{1,192} = 5.002$, $P < 0.05$) and the interaction between diabetes status and duration of diabetes ($F_{3,192} = 8.348$, $P < 0.0001$). The STZ group was significantly dilated compared with the control group at the 3-month time point (8%, $P < 0.05$), and the two-way ANOVA revealed a significant difference in maximum pupil size by duration of diabetes ($F_{4,198} = 6.809$, $P < 0.0001$).

Correlation of Blood Glucose with Retinal Thickness or Visual Acuity

Correlation coefficients were calculated for OKT visual acuity against their corresponding blood glucose levels for each time point. Unfortunately, the 9-month data may be skewed as some glucose readings indicated the maximum levels for the reader, thus the true value was not able to be used. Additionally, the group sizes for each diabetic group declined around 9 months due to the loss of several animals. Therefore, the correlation was determined only through 6 months. As expected, correlation between blood glucose levels and visual acuity was observed in all models (Table 1). Correlation was observed in the db/db model at 6 months;

TABLE 1. Correlation Between Blood Glucose Levels and Visual Acuity or Retinal Layer Thickness in Mouse Diabetes Models

	1.5 Months			3 Months			6 Months		
				Diabetes Model					
	db/db ($n = 34$)	**Ins2[Akita]** ($n = 58$)	**STZ** ($n = 44$)	**db/db** ($n = 34$)	**Ins2[Akita]** ($n = 58$)	**STZ** ($n = 44$)	**db/db** ($n = 32$)	**Ins2[Akita]** ($n = 58$)	**STZ** ($n = 42$)
Blood glucose vs. visual acuity									
Correlation	−0.2790	−0.4296	−0.2280	−0.2368	−0.0377	−0.4251	−0.7304	−0.4213	−0.5304
P	0.1100	**0.0008**	0.1366	0.1774	0.7787	**0.0045**	**<0.0001**	**0.0010**	**0.0003**
Blood glucose vs. inner retinal thickness									
Correlation	−0.7034	−0.2059	−0.3487	−0.6369	0.0946	−0.4733	−0.4905	−0.4155	−0.3491
P	**<0.0001**	0.1209	**0.0204**	**0.0001**	0.4800	**0.0014**	**0.0044**	**0.0012**	**0.0235**
Blood glucose vs. outer retinal thickness									
Correlation	−0.1973	−0.3631	−0.0084	−0.0598	0.1075	−0.3981	0.0230	0.6071	−0.3213
P	0.2633	**0.0051**	0.9569	0.7408	0.4219	**0.0082**	0.9008	**<0.0001**	**0.0380**
Blood glucose vs. total retinal thickness									
Correlation	−0.7096	−0.3483	−0.2014	−0.4752	0.1037	−0.5479	−0.3407	0.4640	−0.5445
P	**<0.0001**	**0.0074**	0.1899	**0.0052**	0.4385	**0.0001**	0.0564	**0.0002**	**0.0002**

Bold entries indicate a statistically significant correlation, with a P value less than 0.05.

TABLE 2. Correlation Between Visual Acuity and Retinal Layer Thickness in Mouse Diabetes Models

	1.5 Months			3 Months			6 Months		
				Diabetes Model					
	db/db ($n = 34$)	Ins2^{Akita} ($n = 58$)	STZ ($n = 44$)	db/db ($n = 34$)	Ins2^{Akita} ($n = 58$)	STZ ($n = 44$)	db/db ($n = 32$)	Ins2^{Akita} ($n = 58$)	STZ ($n = 42$)
Visual acuity vs. inner retinal thickness									
Correlation	0.1887	0.1456	0.2129	0.2077	−0.1866	0.1776	0.5255	0.4266	0.3312
P	0.2852	0.2755	0.1653	0.2461	0.1607	0.2606	**0.0020**	**0.0008**	**0.0321**
Visual acuity vs. outer retinal thickness									
Correlation	0.0723	0.0483	0.0204	0.1968	−0.0739	0.3619	0.1627	−0.3035	0.0883
P	0.6844	0.7186	0.8954	0.2723	0.5815	**0.0185**	0.3737	**0.0206**	0.5781
Visual acuity vs. total retinal thickness									
Correlation	0.2141	0.1006	0.1290	0.2478	−0.0701	0.3283	0.5042	−0.1392	0.3343
P	0.2241	0.4522	0.4039	0.1644	0.6012	**0.0338**	**0.0033**	0.2972	**0.0305**

Bold entries indicate a statistically significant correlation, with a P value less than 0.05.
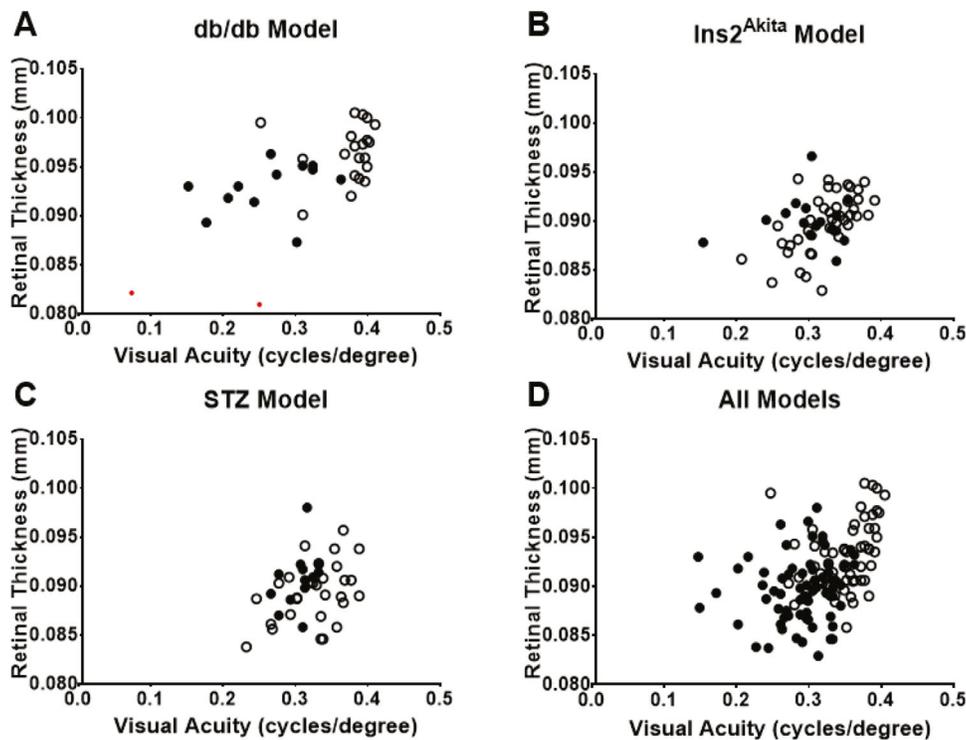
FIGURE 5. Correlations between visual acuity and inner retinal thickness in the three mouse diabetes models. Correlations between visual acuity and inner retinal thickness were analyzed for all three mouse diabetes models at 6 months: (**A**) db/db ($n = 32$), (**B**) Ins2^{Akita} ($n = 58$), (**C**) STZ ($n = 42$), and (**D**) all three models combined ($n = 132$). Results for the diabetic mice (db/db. Ins2^{Akita}, or STZ) are indicated by *solid circles*, and results for the controls (db/+, WT, or control) are indicated by *open circles*.

in the Ins2^{Akita} model, at 1.5 and 6 months; and in the STZ model, at 3 and 6 months. Correlation coefficients were also calculated for retinal thicknesses against their corresponding blood glucose levels for each time point (Table 1). In the db/db model, correlation between blood glucose levels and retinal layer thickness was observed at early time points due to inner retinal thinning. In the STZ model, the correlations were observed in inner, outer, and total retinal thicknesses at 3 and 6 months, but it was only observed in the Ins2^{Akita} model at 6 months.

Correlation Between Visual Acuity and Retinal Thickness

The data were also analyzed to determine if there was a correlation between OKT visual acuity and retinal thickness. The correlation plots between visual acuity and inner retinal thickness for all three models are shown in Table 2. A correlation between visual acuity and inner retinal thickness was observed in all three diabetes models at 6 months (Fig. 5). However, despite changes in inner retinal thickness at

1.5 months in the db/db model, no correlation with visual acuity was observed until 6 months. A significant correlation in the outer retinal thickness was only observed in the Ins2[Akita] model and not in the other two models. A correlation with total retinal thickness was observed in the db/db and STZ models at 6 months.

DISCUSSION

In this study, we characterized visual functional changes and morphological alterations to the retina induced by diabetes in three animal models over time. All three models revealed changes in retinal thickness as observed by OCT. Retinal thinning was observed at the earliest time point measured in the db/db mice and exclusively in the inner retina. Thinning of the inner retina occurred in the Ins2[Akita] mice and STZ mice, as well, but less dramatically than in the db/db mice, with some time points failing to reach significance. The STZ model revealed thinning of the outer retina in the later time points. This retinal thinning correlated with the increased apoptosis observed in each of the retinal models.[15,25,26]

In addition, visual acuity and pupillary responses were measured in all three diabetes models. All models revealed a robust change in visual acuity over time due to diabetes. The Ins2[Akita] mice revealed a decrease in acuity at the earliest time measured, whereas the STZ model required 3 months of diabetes and the db/db model was not statistically different until 6 months. The change in STZ-induced diabetic mice in visual deficits was delayed compared with recently published studies[10,11] and with slower retinal thinning.[5,10] Further, the db/db and Ins2[Akita] models revealed a decrease in the baseline pupil diameter at late time points, similar to findings reported in humans with diabetes.[18] However, no differences in response to light were observed in any of the models.

Recent studies have suggested that a number of neural retinal changes occur in both humans and animal models that precede observable vascular changes. In a longitudinal study, humans with diabetes with no or minimal vascular abnormalities demonstrated RDN, defined as thinning of the retinal nerve fiber and ganglion cell layer, before signs of characteristic microvascular abnormalities could be observed in the clinic.[5] A number of retinal alterations occur before clinically observable vascular pathology, including reduced electrical response of the retina and diminished contrast sensitivity.[27,28] Therefore, it was of interest to determine if there was a correlation between the loss of visual acuity and retinal thinning observed in the current longitudinal study. The studies revealed that inner retinal thinning and loss of visual acuity were well correlated at 6 months of diabetes in all models. In the STZ model, there was inner retinal thinning at 3 months that was statistically different, and at the same time point the STZ animals demonstrated a significant loss of visual acuity. However, the db/db animals revealed dramatic inner retinal thinning at 1.5 months without significant loss of visual acuity until 6 months. Further, the Ins2[Akita] mice demonstrated loss of visual acuity at the earliest point tested, 1.5 months; however, they had no inner retinal thinning but did have outer retinal thinning at this time point. Collectively, the data suggest that inner retinal thinning may correlate with OKT-measured acuity loss in rodents but inner retinal thinning may not directly induce this vision loss or at least may not be sufficient.

Changes in vascular permeability have previously been reported for all three models[15,29,30] and may contribute to vision loss. The time points for changes in permeability vary dramatically based on the laboratory performing the study, the method used, and the marker under investigation. Studies in STZ diabetic animals using vitreous fluorophotometry have revealed changes as early as 8 days.[31] Using concanavalin A crosslinking as a marker of leak, changes as early as 2 weeks of diabetes were observed.[32] Other studies have reported changes over 3 to 6 months.[33] Ultimately, genetic intervention targeting the vascular or neural degeneration may provide insight into causation. Expression of a mutant form of occludin-preventing, site-specific phosphorylation of Ser490 specifically in endothelial cells was shown to prevent loss of visual function at 4 months of diabetes.[34] The link between structural changes in the retina and functional outcome will require more direct interventional evidence in future research.

In this study, two type 1 models of diabetes, STZ and Ins2[Akita], targeted β-cell destruction, and a type 2 pre-diabetes obesity model, db/db mice, was used to complete a longitudinal analysis of retinal thinning and visual response changes including OKT-measured acuity and pupillary response. All models demonstrated a loss of visual acuity and retinal thinning, with the db/db mouse revealing the most dramatic changes in both. Further, the db/db and Ins2[Akita] mouse models demonstrated basal pupillary constriction in diabetes. These data demonstrate an overall decline in retinal-related health in conjunction with blood glucose abnormalities. However, changes in visual acuity measured by OKT were not well correlated with OCT-measured morphological changes. As these mouse models are widely used for DR study, variation in the timing of outcome measures should be carefully considered for experimental design and interpreting results.

Disclosure: **S.R. Sheskey**, None; **D.A. Antonetti**, None; **R.C. Rentería**, None; **C.-M. Lin**, None

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