The Pennsylvania State University
The Graduate School
College of Medicine

VISUAL ACUITY AND CONTRAST SENSITIVITY IN DIABETIC INS2AKITA MICE

A Thesis in
Anatomy
by
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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

May 2010
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Abstract

Purpose: Diabetes causes a loss of retinal neurons in the Ins2\(^{\text{Akita}}\) mouse. The purpose of this study was to determine the affect diabetes has on photopic (daylight) visual function in mice. The hypothesis tested was that diabetes progressively reduces visual acuity and contrast sensitivity in Ins2\(^{\text{Akita}}\) mice.

Methods: Ins2\(^{\text{Akita}/+}\) diabetic and Ins2\(^{+/+}\) wild-type (non-diabetic) littermates were bred in the Penn State Hershey Comparative Medicine barrier facility. Only male mice were used because the females develop only mild diabetes. Visual acuity and contrast sensitivity were measured using an optokinetic testing apparatus (Cerebral Mechanics, Inc) by recording the tracking response (optokinetic reflex) to a rotating visual stimulus displayed on LCD panels surrounding the mouse. Visual acuity was measured at 100% contrast, while contrast sensitivity was measured at spatial frequencies of either 0.064 c/d or 0.092 c/d. Thresholds were obtained once a day for three days and averaged. Statistical comparisons were made by ANOVA or unpaired t-test.

Results: Visual acuity and contrast sensitivity were decreased by 10% - 16% and 15% - 31%, respectively, after 5 - 18 weeks of diabetes in Ins2\(^{\text{Akita}/+}\) diabetic mice compared to wild-type litter-mates (p < 0.01). In a second study visual acuity was decreased 9% - 21% in Ins2\(^{\text{Akita}/+}\) diabetic mice after 10 to 26 weeks of diabetes compared to controls (p < 0.01). Contrast sensitivity was also reduced; however, a significant loss of 16% - 26% was found after 18 weeks of diabetes and continued to decrease up to 26 weeks.

The significant deficit in visual acuity and contrast sensitivity in Ins2\(^{\text{Akita}/+}\) mice after 2 weeks of diabetes was reversed by treatment with subcutaneous insulin implants. When the implants were exhausted (5 weeks post implantation), blood glucose returned to levels
significantly higher than the wild-type controls, but acuity and contrast sensitivity were significantly better than $\text{Ins}^2_{\text{Akita}^+/+}$ diabetic animals.

**Conclusions:** Visual acuity and contrast sensitivity in diabetic $\text{Ins}^2_{\text{Akita}^+/+}$ mice are decreased soon after the onset of diabetes. This reduction lasts for the entire duration of diabetes. Treatment with insulin restored visual function, which was maintained even when insulin was no longer being delivered.
Table of Contents

List of Tables ................................................................................................................... vii
List of Figures ................................................................................................................. viii
Abbreviations .................................................................................................................... ix
Acknowledgements ........................................................................................................... xi

Chapter 1: Introduction .......................................................................................................1
  Epidemiology of Diabetes ............................................................................................2
    Prevalence of Diabetes ..............................................................................................1
    Cost Associated with Diabetes ..............................................................................2
  Diabetic Retinopathy .................................................................................................3
    Characteristics of Diabetic Retinopathy .............................................................3
    Associated Complications of Diabetes / Risk Factors / Treatment for Diabetes ........5
  Retinal Structure and Function ...............................................................................6
    Cellular Organization and Function ....................................................................6
    Phototransduction Cascade ..................................................................................9
  Morphological and Molecular Changes of Retina ..................................................10
    Cellular and Lamellar Changes ...........................................................................11
    Synaptic Transmission .........................................................................................13
    Vascular Permeability ............................................................................................14
  Functional Changes of Retina .................................................................................15
    Electroretinography ..............................................................................................15
    Visual Acuity and Contrast Sensitivity ...............................................................17
  Optokinetics Used To Measure Vision in Diabetic Ins2Akita Mice .........................20
    Optokinetics Apparatus .......................................................................................20
    Visual Acuity .........................................................................................................21
    Contrast Sensitivity ..............................................................................................22
    Generation of Optokinetic Reflex Response .....................................................23
    Monocular Visual Threshold Values ...................................................................24
    Optomotry Program Used to Obtain Threshold Values ......................................25
  Diabetic Ins2Akita Mouse-Model ...........................................................................26
  Aim of Optokinetics Research ..................................................................................29

Chapter 2: Materials and Methods ..................................................................................30
  Animals .......................................................................................................................30
  Blood Glucose Monitoring .......................................................................................31
  Threshold Value Determination ...............................................................................31
  Cell Death ELISA ......................................................................................................32
  Insulin Pellet Implantation .......................................................................................33
  Data and Statistical Analysis ....................................................................................34

Chapter 3: Results .............................................................................................................35
  Loss of Visual Function in Diabetic Ins2Akita Mice ...............................................35
  Reversing Diabetes with Insulin Corrects Visual Function ......................................41
List of Tables

Table 1. Summary of Loss in Contrast Sensitivity in Diabetic Patients ..............................9
Table 2. Average Blood Glucose Levels ..........................................................................35
Table 3. Average Blood Glucose Levels ..........................................................................37
Table 4. Average Blood Glucose Levels ..........................................................................39
Table 5. Average Blood Glucose Levels ..........................................................................40
Table 6. Average Blood Glucose Levels Pre- and Post- Insulin Implant .........................42
Table 7. Significant Difference Between Groups .............................................................45
List of Figures

Figure 1. Structure of the Retina .........................................................................................9
Figure 2. Optokinetics Apparatus ..........................................................................................21
Figure 3. Virtual Rotating Cylinder .....................................................................................21
Figure 4. Visual function diminished in Ins2\textsuperscript{Akita} ..............................................................36
Figure 5. Visual function diminished in Ins2\textsuperscript{Akita} ..............................................................38
Figure 6. Visual function diminished at late stage of diabetes .............................................39
Figure 7. Visual function diminished at late stage of diabetes and increased apoptosis
        in diabetic retina ..................................................................................................................41
Figure 8. Contrast sensitivity curve of Ins2\textsuperscript{Akita} mice at various spatial frequencies ......42
Figure 9. Visual function rescued in diabetic Ins2\textsuperscript{Akita/} treated with insulin .............45
Figure 10. Visual function diminished in Ins2\textsuperscript{Akita} ...........................................................46
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABTS</td>
<td>2,2’-Azino-di[3-ethylbenzthiazoline-sulfonate]</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOS</td>
<td>Accessory optic system</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BG</td>
<td>Blood glucose</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>c/d</td>
<td>Cycles per degree</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>deg/sec</td>
<td>Degree per second</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IGM</td>
<td>Impaired glucose metabolism</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KDM</td>
<td>Known diabetic individuals</td>
</tr>
<tr>
<td>LCD</td>
<td>Liquid crystal display</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mg/dl</td>
<td>Milligram per deciliter</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>n</td>
<td>Number of animals used in study</td>
</tr>
<tr>
<td>NDM</td>
<td>Newly diagnosed diabetes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NGM</td>
<td>Normal glucose metabolism</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>°</td>
<td>degree</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
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</table>
Acknowledgements

I gratefully acknowledge the guidance of my thesis research advisor Dr. Alistair J. Barber and the support I was given by the Barber Lab. I would also like to thank my committee members Dr. Patricia McLaughlin and Dr. Patricia Grigson for their feedback and assistance with my research project.
Chapter 1: Introduction

1.1 Epidemiology of Diabetes

1.1.1 Prevalence of Diabetes

Diabetes is characterized by high levels of blood glucose (hyperglycemia), resulting from defects in the production of, or the resistance to, the hormone insulin, which is responsible for the uptake of glucose by cells in the body. Clinically, diabetes is diagnosed as either Type I or Type II. Type I is a consequence of the destruction of insulin producing pancreatic beta cells by the immune system. This form of diabetes accounts for 5%-10% of the diagnosed population and usually occurs in children and young adults. Type II diabetes is characterized by cells that fail to respond to insulin and ultimately become insulin resistant. It accounts for 90-95% of the diagnosed population and develops with age and obesity, and occurs in families that have a history of diabetes (CDC, 2007).

High blood glucose levels do not immediately classify an individual as diabetic; some individuals have blood glucose levels above normal but not high enough to be considered diabetic. This condition is known as pre-diabetes. Individuals with pre-diabetes have fasting glucose and/or glucose tolerance levels above normal. This increases the risk of developing Type I or Type II diabetes if it is left unmanaged. An estimated 57 million Americans in the United State that were 20 years of age or older in 2007 were deemed pre-diabetic (CDC, 2007).

Diabetes, aside from its biological definition, is of significant importance because of its endemic footprint in the United States. In the National Diabetes Fact Sheet: General information and national estimates on diabetes in the United States, 2007, from the Centers
of Disease Control and Prevention, it was estimated that 23.5 million people have diagnosed and undiagnosed diabetes and are 20 years of age or older. Furthermore, survey data from 2003 – 2006 projected that in 2007, 2.6% of the population that fell between the ages of 20 – 39, 10.8% of the population that were in the 40 – 59 years old group, and 23.1% of the population that are 60 years of age or above have diabetes. Lastly, approximately 186,300 people under the age of 20 have either Type I or Type II diabetes (CDC, 2007).

In 2006 diabetes was reported as the 7th leading cause of death. This report was based on 72,507 death certificates issued that declared that the main cause of death was due to diabetes. However, this is not completely accurate because diabetes is usually not listed as the main cause of death but rather a contributor. In 2005 there were 233,619 cases in which diabetes was listed as a contributor to death. Studies have indicated that approximately 35% - 40% of the deceased had listed that they were diabetic on their death certificate, but only 10% -15% had it listed as the cause of death (CDC, 2007). These data illustrate that diabetes is underreported, that its prevalence continues to grow among the American population and that there is an increasing importance for continued research in this field of science to remedy these concerns.

1.1.2 Cost Associated with Diabetes

The American Diabetes Association estimated that the total national cost due to diabetes in 2007 in the United States was approximately $174 billion. Of this $174 billion, $116 billion was attributable to medical expenditures. More than half of the cost associated with healthcare expenditures was due to an increase in hospital admission and longer average length of stay per admission due to diabetes and associated complications. The other $58
billion was a result of a loss in productivity or money associated with work-related absenteeism, unemployment, disability, premature mortality and decreased work performance. The annual per capita healthcare expenditure for a diabetic individual was projected to be $11,744. Additionally, the average cost per medical visit, such as inpatient hospital stay and emergency visit in 2007 for diabetes and associated complications, which include cardiovascular, renal, and ophthalmic conditions was estimated as well. Inpatient hospital stays for diabetics was roughly $1,853, and diabetics with cardiovascular, renal, and ophthalmic conditions was approximately $3,225, $1,872, and $2,408 respectively. The average cost per emergency visit for an individual with diabetes was $696. Lastly, emergency expenses for those with cardiovascular, renal and ophthalmic complications corresponded to nearly $680, $576, and $1,623 per visit (American Diabetes Association, 2008).

1.2 Diabetic Retinopathy

1.2.1 Characteristics of Diabetic Retinopathy

There are an assortment of complications associated with diabetes; one of them being blindness caused by diabetic retinopathy, a retinal degenerative disease. Each year it causes approximately 12,000 - 24,000 new cases of blindness (CDC, 2007) and is more prevalent in the working-class population (Ciulla et al., 2003). Diabetic retinopathy is classified as being non-proliferative or proliferative which are determined by a variety of clinically diagnostic criterion. The non-proliferative form defines the initial stages of diabetic retinopathy. A hallmark pathophysiological change which occurs in the microvasculature is the presence of retinal microaneurysms. They arise in regions where capillaries close and increase in number.
as the disease progresses. In later stages of non-proliferative diabetic retinopathy, microaneurysms may erupt in the inner nuclear layer of the retina which is known as a blot hemorrhage. Hard and soft exudates may also be observed within the retina. Leakage of blood vessels in the posterior regions of the retina along with lipid accumulation leads to the presence of hard exudates. Soft exudates, in contrast, develop when infarction of the closed capillaries occurs in the nerve fiber layer resulting in fluid filled lesions containing cellular debris (Silvio et al., 2005). These microvascular abnormalities are indicative of the non-proliferative stages of diabetic retinopathy.

Proliferative diabetic retinopathy, which represents the later stages of the disease, is characterized by the growth of blood vessels in an attempt to supply oxygenated blood to the hypoxic retina which resulted from capillary loss (Ciulla et al., 2003). They tend to expand into the vitreous and can hemorrhage, leading to vision problems (Silvio et al., 2005). Additionally, it is common to see an increase in vascular permeability due to the breakdown of the blood-retinal-barrier (Barber et al., 2003b). This can lead to macular edema, which has been found to correlate with increased vision loss in diabetes (Moss et al., 1988). However, the mechanisms in which abnormal blood flow, closure of retinal vasculature and increased retinal permeability occurs are not well understood.

The abovementioned changes in the structure of the retinal microvasculature are used clinically to determine the severity of diabetic retinopathy via fundus photographs. The International Clinical Diabetic Retinopathy Disease Severity Scale is a set of guidelines that clinicians use to identify the stage of diabetic retinopathy. These guidelines however are not used to detect the onset of vision impairment. Diabetic retinopathy is a progressive disease with minimal symptoms and at the point which microvascular abnormalities are recognized,
vision loss is irreversible. Laser photocoagulation and vitrectomy are the current treatment methods to improve the quality of life of the patients by reducing the progression of diabetic retinopathy and delaying vision loss. Unfortunately these techniques do not rescue the loss in vision. Furthermore, these treatments run the risk of creating an even larger visual deficit in the patients (Cuilla et al., 2003).

Thus far visual loss associated with diabetic retinopathy has been explained as a consequence of retinal vasculature changes. However, it is the communication between the retinal neuronal cells that provides individuals with the ability to see. Therefore, research investigations have turned towards explaining the loss of vision to retinal neurodegeneration as well as vascular changes. For the reasons mentioned above, it is of the upmost importance to determine the specific mechanisms involved in triggering a loss in vision so a more sensitive diagnostic test can be developed to detect this deficit in the early stages of diabetic retinopathy.

1.2.2 Associated Complications of Diabetes / Risk Factors / Treatment for Diabetes

There are a variety of complications associated with the diabetes. In 2002, a study investigated possible associations of blood pressure, cholesterol, body mass index (BMI), and triglyceride count, with glucose metabolism in 626 individuals. The individuals were categorized into groups based on their blood glucose metabolism: known diabetic individuals (KDM), participants with normal glucose metabolism (NGM), others with impaired glucose metabolism (IGM), and newly diagnosed diabetics (NDM). Fundoscopic exams were used to determine who had retinopathy. Results indicated that amongst all groups, a strong correlation exists between increased blood pressure and occurrence of retinopathy,
particularly in the NGM and KDM groups. Similarly, an association between total cholesterol levels and frequency of retinopathy was found, specifically in the IGM and NDM groups. However, there is no strong trend between retinopathy and BMI or triglycerides count within the groups. Overall, there was an increase in risk of developing retinopathy in the individuals with diabetes who presented with high blood pressure and/or cholesterol (Van Leiden et al, 2002). Other complications related to diabetes are amputation, blindness, heart disease, kidney disease and stroke. Treatments for diabetes, which do not eradicate the disease but rather help maintain normal blood glucose levels to circumvent the abovementioned complications involve the use of oral medications, insulin injections, increase in physical activity, strict diet plans and monitoring of blood glucose levels (CDC, 2007).

1.3 Retinal Structure and Function

The retina is a transparent vascularized neural tissue that is positioned between the retinal pigment epithelium and the vitreous body. The primary function of the retina is to convert photons into electrochemical energy or action potentials that propagate from cell-to-cell to facilitate transmission of the electrochemical signal to the occipital lobe for translation. These events are stimulated by light environments ranging from bright to dark.

1.3.1 Cellular Organization and Function

The retina has a lamellar oriented structure (Fig. 1) that is comprised of microvascular networks, non-neural and neural cells which play a part in transducing a light stimulus into a neural signal. The non-neural cells present within the neurosensory retina are Muller cells,
astrocytes and microglial. Muller cells span the length of the lamellar structure with their nuclei residing in the inner nuclear layer while astrocytes do not. Both however, contact blood vessels allowing lactic acid, glucose and amino acids to exit the circulation and regulate the blood-retinal barrier, and provide nutritional and structural support to the retina (Cibis, 2001; Antonetti et al., 2006). The Muller cells play a role in neurotransmission and the phototransduction process by producing ATP which is vital in maintaining ion gradients open at synaptic connections and conducting the neural signal through the retinal layers. Unlike the other non-neural cells, microglia have immunological functions. They regulate the retinal environment via interactions with neurons, non-neural cells and the vasculature. They secrete proinflammatory cytokines and phagocytose apoptotic cells (Antonetti et al., 2006).

Neural cells are found spread throughout the retinal layers as well. The outer most layer of the retina is known as the retinal pigmented epithelium. Its outer surface lies adjacent to the choroidal vascular network of the eye and takes part in the blood-retinal barrier. Meanwhile, its inner surface neighbors the photoreceptor layer providing nourishment and plays an important part in the process of converting photon energy into a neural response which is then transmitted through the neurosensory retina. The apical surfaces of the photoreceptors are in contact with the retinal pigmented epithelium layer and contain photopigments arranged in membranous disks. The photopigment molecules transduce light into neural signals which are synaptically transmitted throughout the rest of the layers. The retinal pigmented epithelium functions to remove used disks via phagocytosis and recycles photopigment molecules after they have converted light into a neural stimulus (Alloway et al., 2007).
There are two different types of photoreceptors: rods and cones, which are depolarized to a resting membrane potential of -40 mV and hyperpolarized in the presence of light to -70 mV. The rods have the ability to detect low levels of illumination (wavelength of 496 nm) and thus take part in scotopic or night vision. Meanwhile, the cones have a low sensitivity to light and need higher levels of illumination (wavelengths of 531 nm and 559 nm) and therefore participate in photopic or day vision. The rods and cones have an inner and outer segment. The inner segment contains the nucleus of the photoreceptor, which lies in the outer nuclear layer and contains synaptic terminals which project into the outer plexiform layer. The outer segment comes in contact with the retinal pigmented epithelial layer, as previously stated, and contains the membranous disks and photopigments, one in particular known as opsins (Cibis, 2001).

The photoreceptors are responsible for neurotransmission of light through the retina via synaptic connections with the horizontal and bipolar cells. The processes of the horizontal cells extended horizontally throughout the outer plexiform layer interconnecting adjacent photoreceptors mediating lateral inhibition at their synapses. The bipolar cells are vertically oriented with their dendritic processes synapsing with the rods and cones and their axonal processes transmitting the neural stimulus to the inner plexiform layer to synapse with ganglion cells. The cell bodies of the bipolar, horizontal and amacrine cells lay in the inner nuclear layer which is sandwiched between the outer and inner plexiform layers. The processes of the amacrine cells which have a similar function as the horizontal cells lie in the inner plexiform layer and regulate the output of the bipolar cells onto the dendritic processes of the ganglion cells. The cell bodies of the ganglion cells lie in the ganglion cell layer and their axonal processes extend through the nerve fiber layer, exiting the eye through the optic
disk forming the optic nerve. Axons of the optic nerve project to the lateral geniculate nucleus in the thalamus where information about edges and contrasts in color are processed. Additionally, some of these cells project to other areas in the brain to coordinate eye movements in response to light (Cibis, 2001).

**1.3.2 Phototransduction Cascade**

Phototransduction is a cascade of molecular events that begins at the photoreceptor layer. Opsin, a G-protein coupled receptor that absorbs light is the key phototransduction molecule which is present in both rod and cone photoreceptors. There are different types of
Opsin molecules. Rod photoreceptors contain rhodopsin while cones contain photopsin. Both types are sensitive to light but at different intensities. These molecules transduce light into a neural stimulus which is then processed in the retina and transmitted to the optic nerve. The visual information is then identified and interpreted in the visual cortex. This process is what enables us to have day and night vision.

The cascade begins when light is absorb by the photoreceptor which contains the G-protein coupled receptor Opsin which is associated to a 11-cis retinal molecule. When light hits 11-cis retinal it causes the molecule to undergo a conformational change to an all-trans configuration which has an agonistic affect on Opsin leading to its activation. Transducin is a G-protein that interacts with the receptor which in the presence of light stimulates cyclic guanosine monophosphate phosphodiesterase to hydrolyze cGMP. This results in a reduction of cGMP in the cytosol, which normally maintains Na\(^+\) ion channels open that are located on the outer segment. Meanwhile, a Na\(^+\)/K\(^+\) ATPase pump located on the inner segment continues to operate, further decreasing the total amount of Na\(^+\) in the receptor. The combination of these is what leads to hyperpolarization of the photoreceptor to -70 mV. This results in a decrease in the release of the neurotransmitter glutamate at the synaptic terminals of the photoreceptors triggering “OFF” and “ON” bipolar cells to hyperpolarize and depolarize, respectively. The depolarized bipolar cells carry on the phototransduction cascade by synapsing with ganglion cells in the inner plexiform layer (Fisherman et al, 1990).

1.4 Morphological and Molecular Changes of Retina

Diabetic retinopathy is typically classified as a vascular disease in which the cells undergo apoptosis. However, it has been brought to our attention that neural degeneration is
also occurring and is a critical contributing factor to the development and progression of diabetic retinopathy. Therefore, a variety of histological, microscopic and molecular methods have been employed in animal models and human subjects to determine which cells in conjunction with vascular cells are degenerating due to diabetes.

1.4.1 Cellular and Lamellar Changes

Five postmortem human retinas were investigated by Barber et al, (1998) via TUNEL-HRP assays. Three of the five subjects did not have a history of diabetes while the other two subjects had a history of 6 and 30 years of insulin-dependent diabetes. The diabetic individuals expressed nearly a 104% increase in apoptotic cells compared to the individuals without diabetes (Barber et al, 1998). Proapoptotic mediators were analyzed in a different study via immunohistochemistry in human donor eyes from 5 diabetic and 4 nondiabetic subjects (Abu-El-Asrar et al, 2004). An upregulation of apoptosis-promoting factors caspase-3, Fas, and Bax were found in the ganglion cells of the diabetic retinas compared to the controls (Abu-El-Asrar et al, 2004).

Barber et al, (1998) also used TUNEL-HRP assays on whole-mounted retinas after 1, 3, 6, and 12 months of STZ-induced diabetes in male Sprague-Dawley rats. Results revealed 9 - 10 fold increase of apoptotic cells at each time point in the diabetic retinas compared to age-matched controls. To determine if these apoptotic cells were solely vascular, cryostat sections of the retinas were simultaneously labeled with TUNEL-FITC and vWf, an immunohistochemical marker for vascular endothelial cells. TUNEL-FITC labeling was found in the ganglion cell layer and was not colocalized with the vWf marker, indicating that ganglion and amacrine cells, as well as astrocytes, became apoptotic with diabetes (Barber et
Barber et al. (1998) found a 10% reduction in ganglion cell density in the diabetic retinas of Sprague-Dawley rats compared to the controls. Similarly, Gastinger et al. (2006) used a TUNEL assay which revealed significantly increased apoptotic activity in the inner nuclear and ganglion cell layers in diabetic Ins2^{Akita/+} mice and Sprague-Dawley rats compared to controls. These apoptotic results that were established in diabetic animal models are comparable to those found in humans. It can be concluded that an apoptotic milieu is introduced by diabetes in the human and animal retina that results in the loss of a variety of neurons, specifically targeting amacrine and ganglion cells.

The thickness of the nerve fiber layer, which contains the axons of the ganglion cells, was analyzed via scanning laser polarimetry, in the human diabetic retina of 128 patients and compared to 50 age-matched controls. Results showed a significant decrease in the thickness of the nerve fiber layer. Additionally, thinning of the layer increased with the severity of diabetic retinopathy (Takahashi et al., 2006). OCT images of the retina of 57 diabetic patients with no or little diabetic retinopathy showed that the ganglion cell/inner plexiform layer and inner nuclear layer were significantly thinner than age matched controls by 2.7 um and 1.1 um, respectively. Other retinal layers remained unchanged (Van Dijk et al., 2009).

Barber et al. (1998) and Barber et al. (2005) measured the thickness of retinal layers in diabetic animal models and found comparable results to those reported in the diabetic human retina. Sprague-Dawley rats that had STZ-induced diabetes for 7.5 months experienced a 22% and 14% reduction in thickness of the inner plexiform layer and inner nuclear layer, respectively (Barber et al., 1998). Barber et al. (2005) observed a significant loss of cells in the inner retinal layer of diabetic Ins2^{Akita/+} mice compared to Ins2^{+/+} litter-
mates. These studies conclude that diabetes causes structural changes which may be a contributing factor to a variety of the retinal complications, specifically vision loss.

Experimental observations in both diabetic humans and diabetic animal-models have detected a consistent increase in apoptotic activity in the diabetic retina as well as a decrease in retinal layer thickness. The changes in structure may be a result of the loss of retinal cells such as amacrine and ganglion cells or may be due to another mechanism. Whichever it may be, these changes could be a factor in the loss of vision due to diabetes.

1.4.2 Synaptic Transmission

Other reports have found that not only is there a loss in retinal cell bodies but there is also a change in protein content at the molecular level. VanGuilder et al, (2008) investigated the synaptic function of retinal neural cells by measuring the expression of presynaptic protein and mRNA content in STZ-induced diabetic Sprague-Dawley rats. Immunohistochemistry and RT-qPCR were used to analyze the following presynaptic proteins: synapsin I, synaptophysin, vesicle-associated membrane protein 2, synaptosomal associated protein of 25 kDa and postsynaptic density protein of 95 kDa. Immunoreactivity revealed that the proteins were concentrated mainly in the inner and outer plexiform layers. Overall, these proteins were reduced in rats with 3 months of diabetes compared to controls. In addition, RT-qPCR showed that all mRNA levels of all proteins were significantly decreased after 3 months of diabetes. To determine if the levels of the retinal presynaptic proteins were also diminished within the synapse, synaptosomes from rats with a duration of diabetes for 1 month were isolated and immunoblotted. Results showed a reduction of the proteins in the STZ-induced diabetic rat retinas compared to the age-
matched controls (VanGuilder et al., 2008). A different study explored another aspect of synaptic transmission. The metabolism of the neurotransmitter, glutamate, into glutamine by glial cells by way of ion exchange chromatography of 9 month diabetic and control Sprague-Dawley rats was investigated. Findings showed that the metabolism of glutamate was significantly impaired in the retina of the diabetic rat (Lieth et al., 1998). These results entertain the possibility that a reduction in synaptic protein and mRNA content in the diabetic retina may alter the transduction of neural stimuli. Furthermore, changes in the metabolic glutamate/glutamine cycle which controls neurotransmission between neurons and prevents excitotoxicity (Antonetti et al., 2006) may be a result of a failure in Muller cell function which provides neurotransmission support. It is possible that dysfunction in these processes due to diabetes may alter the phototransduction cascade and/or cause neural degeneration which leads to a deficit in visual function.

1.4.3 Vascular Permeability

Upon the onset of diabetes, there is an early breakdown of the blood-retinal barrier, which can be detected before all of the aforementioned lesions are clinically noticeable. The early breakdown of the blood-retinal-barrier increases the vascular permeability resulting in macular edema, which was shown via vitreous fluorophotometry in diabetic patients (Cunha-Vaz et al., 1975) and diabetic rats (Carmo et al., 1998). The diabetic rats displayed a significant increase in permeability by 75% which was localized to the photoreceptor and inner nuclear layer (Carmo et al, 1998). The mechanisms in which an increase in permeability occurs is unclear; however, studies have established that in the diabetic retina there is a loss in the protein occludin, which is involved in tight junctions (Barber et al,
2003a; Barber et al, 2005) which may play a part in the occurrence of retinal edema. A side effect associated with these findings is that the rate of vision loss increased in diabetic patients with macular edema; consequently resulting in a decrease in visual acuity (Moss et al., 1998). These reports have confirmed that diabetes increases permeability which appears to target the photoreceptor layer and cause vision loss. However the mechanism in which this visual deficit occurs is not clearly defined and may be associated with photoreceptor function.

In summary, the above reported findings have shown that diabetic retinopathy is not only a vascular disease but also a neural degenerative disease. These data suggest that vision impairment is a result of a combination rather than a distinct event of retinal changes due to diabetes. However, the understanding of which of these events is the primary contributor to the onset of diabetic retinopathy remains a gap in science that needs further investigation.

1.5 Functional Changes of Retina

1.5.1 Electroretinography

Electroretinography is a technique that is used experimentally to quantify neurotransmission within the retina. It measures electrical potential changes generated in neural and non-neural cells when exposed to a stimulus of light which is displayed on an electroretinogram. The electroretinogram that is produced is comprised of several wave forms: the a-wave is a product of the hyperpolarization of the photoreceptors, the b-wave is depolarization of the bipolar cells, the c-wave is a result of the hyperpolarization of the retinal pigment epithelial cells, and the oscillatory potentials which are found on the rising slope of the b-wave, represent the depolarization of the Amacrine cells. Electroretinograms
can be scotopic (dark-adapted eye) which a response from the rod photoreceptors or photopic (light-adapted eye) which reflects the activity of cone photoreceptors (Fisherman et al., 1990).

Experimental studies have analyzed electroretinograms of diabetic patients and diabetic-animal models. After dark adaptation results of diabetic patients showed that the cone-b-wave not rod-b-wave amplitude was reduced at advanced stages of diabetic retinopathy. Similarly, dark adapted STZ-induced diabetic Sprague-Dawley rats models showed no change in rod-b-wave amplitudes compared to controls (Fisherman et al., 1990). A different study investigated STZ-induced diabetic Long-Evans rats and found the curve of the cone-b-wave was shifted to the right in diabetes (Hancock et al., 2004). These results imply that in diabetes cone photoreceptors are malfunctioning under photopic environments and photoreceptor kinetics is delayed implicating a desensitization of their triggering mechanisms.

Investigations of diabetic patients showed no change in a-wave amplitude but found a decrease in the sensitivity parameter of the a-wave, which represents the transduction function of the photoreceptors at the receptoral level (Holopigian et al., 1997). In contrast, Phipps et al., (2006) found a reduction in the a-wave amplitude with normal sensitivity in STZ-induced diabetic Sprague-Dawley male rats when compared controls. Interestingly, it seems that photoreceptor responses are to some extent inconsistent between diabetic humans and animals when analyzed via electroretinography.

There appears to be a discrepancy in photoreceptor function in diabetes when using electroretinography. Nevertheless, electroretinography lends itself to using waveform components as a way to detect retinal changes attributable to diabetes early on, prior to any
clinically visible abnormalities that lead to retinopathy. However, to add some clarity, visual function measurements using a virtual optomotor system are also being investigated.

1.5.2 Visual Acuity and Contrast Sensitivity

Clinically the visual acuity and contrast sensitivity thresholds are a routine part of an eye examination. These techniques are used to determine if there are any problems with vision in young children, adolescents and adults. Abnormal results indicate that the patient may need glasses or contacts or depending on the severity of the loss in vision it may be a sign of ocular disease. For that reason, these routine eye exams are common in diabetic patients because as aforesaid visual loss is an adverse side affect of diabetes. These tests have been used in clinical experimentations to establish the degree of visual deficit in diabetics as well as to determine if these techniques could provide additional information such as the onset of visual loss related to the duration of the disease.

One study examined four different groups: one control and three non-insulin dependent diabetic groups which had diabetes for duration of 6, 10 or 16 years, each with more advanced diabetic retinopathy. Contrast sensitivities were determined using high and low contrast charts that measured threshold values at 0.5 c/d and 2 c/d. Thresholds revealed that contrast sensitivities were significantly less in the diabetic groups than that of controls ranging from 6%, 9% and 12% which correspond to increasing severity of diabetic retinopathy (Ismail et al., 1998). Contrast sensitivities of insulin-dependent diabetes mellitus and non-insulin-dependent diabetes mellitus subjects with no or minimal diabetic retinopathy were compared to age-matched controls. Individual contrast sensitivity threshold values were obtained by means of a series of sinusoidal black and white stationary gratings at six different
spatial frequencies ranging between 0.5 to 22.8 c/d. Additionally, all subjects had normal acuity threshold values with little or no retinopathy. Results revealed that insulin-dependent diabetes mellitus individuals without retinopathy had normal contrast sensitivities compared to the controls at all spatial frequencies. In contrast, non-insulin-dependent diabetes mellitus subjects without retinopathy exhibited significantly less than normal contrast sensitivity at a spatial frequency of 22.8 c/d, while those with retinopathy experienced significantly lower contrast sensitivity values at all spatial frequencies compared to controls (Sokol et al., 1985). A different study showed similar results to Sokol et al, (1985). Contrast sensitivity threshold values were evaluated in 24 individuals with short duration diabetes, without any signs of retinopathy. Dynamic and static contrast sensitivity modes were analyzed at eight different spatial frequencies between 0.6-12.2 c/d. Results obtained via dynamic testing showed a significant loss in threshold values of 33.3% in diabetic subjects compared to age-matched controls at all but the highest spatial frequency of 12.2 c/d. Static results also demonstrated a loss of 72.9% in contrast sensitivity at 1.0, 1.4, 2.2, 7.1, and 9.6 c/d (Di Leo et al., 1992). Arend et al, (1997) also found a significant reduction in static analyses of contrast sensitivity thresholds; however, the loss in visual function was not as dramatic as that reported by Di Leo et al, (1992). Contrast sensitivity thresholds obtained at 6 and 12 c/d in diabetic individuals which had diabetes for a duration of 13 years were significantly reduced by 6% and 13% respectively, compared to healthy subjects. An assessment of visual acuity and contrast sensitivity threshold values was done on pediatric and adolescent insulin-dependent diabetes mellitus patients who had diabetes for duration of 6 years. No significant difference was found in visual acuity values between insulin-dependent diabetes mellitus patients and
age-matched control subjects. Static contrast sensitivity values were significantly decreased at spatial frequencies of 3 and 6 by 20% and 33%, respectively (North et al., 1997).

Studies on contrast sensitivity threshold values have shown that a visual deficit is present with diabetes; yet, the spatial frequency at which the deficit occurs and degree of visual loss is rather variable (Table 1.1). However there appears to be a discrepancy between reports of visual acuity thresholds in diabetic patients that endured diabetes for a short period of time compared to controls. Some studies have found no statistical significance difference in visual acuity threshold values between diabetic and control groups (Olafsdottir et al., 2007; Lee et al., 1995; North et al., 1997) while others have (Ismail et al., 1998). Also, it has been reported that a loss in vision significantly diminishes after long durations of diabetes (Moss et al., 1988). Nevertheless, evaluation of contrast sensitivity threshold values may serve as an adjunct to identifying individuals with diabetes early on. In addition, contrast sensitivity assessment may act as an early index of anatomical changes associated with diabetic retinopathy.

<table>
<thead>
<tr>
<th>Duration of Diabetes (yrs)</th>
<th>% Loss</th>
<th>Spatial Frequency (c/d)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>6; 13</td>
<td>6; 12</td>
<td>Arend et al. 1997</td>
</tr>
<tr>
<td>Short duration</td>
<td>73; 33</td>
<td>1.0 - 9.6; 0.6 - 12.2</td>
<td>Di Leo et al, 1992</td>
</tr>
<tr>
<td>6,10, &amp; 16</td>
<td>6; 9; 12</td>
<td>0.5 &amp; 2</td>
<td>Ismail et al, 1998</td>
</tr>
<tr>
<td>6</td>
<td>20; 30</td>
<td>3, 6</td>
<td>North et al, 1997</td>
</tr>
</tbody>
</table>

There have not been any reports on diabetic-animal model studies that evaluated these different visual function parameters. For that reason, we have chosen to investigate visual acuity and contrast sensitivity threshold values in diabetic Ins2^Akita/+ and control Ins2^+/+ mice.
via the use of an optokinetics apparatus. We hope to provide some valuable information on when visual loss can be detected and to what degree vision is impaired in diabetes.

1.6 Optokinetics Used To Measure Vision in Diabetic Ins2\textsuperscript{Akita} Mice

1.6.1 Optokinetics Apparatus

A method developed by Prusky et al., (2004) known as a virtual-reality optomotor system can measure visual acuity and contrast sensitivity in rodents (Fig. 2). The apparatus consists of four computer monitors arranged in a square-like fashion, surrounding an elevated platform centered within the chamber in which the rodents are allowed to move freely. Vertical gratings of black and white are projected onto the screens of the computer monitors via a computer program, OptoMotry, creating the illusion of a virtual cylinder. The cylinder is rotated around the rodent at different drift speeds, spatial frequencies, and contrast ratios between the vertical bars (Fig. 3). A video camera is mounted to the lid of the apparatus which is used by the investigator to observe the optokinetic reflex response of the rodents head in the direction of the rotating vertical bars. Once a response is no longer elicited the visual acuity or contrast sensitivity threshold is ascertained. The video image is essential for generating accurate visual acuity or contrast sensitivity threshold responses because it allows the investigator to keep the cursor centered between the eyes of the freely moving rodent in order to maintain a constant visual angle regardless of how close the rodent moves towards the rotating cylinder. As a result, the spatial frequency between the gratings remains constant, which is advantageous for reducing variation in the results. For this reason, this system can be used to provide an efficient way to determine visual acuity and contrast sensitivity thresholds of rodents and thus be useful in evaluating treatments of retinal diseases.
1.6.2 Visual Acuity

Visual acuity is the ability to resolve fine detail and is mainly derived from the synaptic convergence of the cone system in the retina. In humans, rod photoreceptors are more abundant in the peripheral retina while cone photoreceptors are more highly concentrated at the macula, the center of focus. The fovea which is in the center of the macula contains only cones. In this region the layers except for the photoreceptor layer, are displaced laterally. This allows for light to directly interact with the photoreceptors and
transmit neural stimuli to the bipolar and ganglion cells. This one-to-one communication between the bipolar and ganglion cells allows the retina to detect the exact location of a stimulus in the visual field, resulting in a high degree of spatial resolution or visual acuity. Rods in the periphery do not have this one-to-one communication; rather they merge onto many bipolar cells and in turn project to ganglion cells resulting in a lower spatial resolution (Alloway et al., 2007; Cibis, 2001).

1.6.3 Contrast Sensitivity

Contrast sensitivity, another measure of visual function, is the degree in which one is able to distinguish between different light intensities which is a function of the retinal ganglion cells. Interpreting contrast is essential for distinguishing an object from its background. The retinal ganglion cells have a circular receptive field with light evoking a response at the center or the circumference of the field. These ganglion cells are described as either “on-center” or “off-center.” If light is shined in the circumference of an on-center ganglion cell the cells ability to discharge is inhibited. The opposite is true for the off-center ganglion cells (Kuffler et al., 1951). These antagonistic characteristics allow the on-center and off-center retinal ganglion cells to differentiate between light and dark edges of an object. Furthermore, the retinal ganglion cells produce different discharge patterns and rates under different illumination stimulus conditions which allows for distinguishing objects in the visual field (Alloway et al., 2007).
1.6.4 Generation of Optokinetic Reflex Response

To determine if the response to the virtually rotating cylinder was in fact a reflex, unilateral cortical lesions in the primary visual cortex were made surgically in both mice and rats. Visual threshold values were obtained post surgery. Results showed no change in threshold values compared to those established prior to surgery for either the ipsilateral or contralateral eye; meaning the temporonal directions of the rotating stimulus to invoke a response in individual eyes was unaffected by the cortical lesions. Subsequently, bilateral cortical lesions were surgically created and similar results were obtained (Douglas et al., 2005). These results suggest that the response is a reflex. However it is not the retinal afferent cortical projections to the primary visual cortex that invoke the behavioral response to a moving stimulus which generates the visual acuity and contrast sensitivity threshold values.

The optokinetic reflex response is evoked by the movement of images within the visual field. This behavioral response is a result of the Accessory Optic System (AOS), which is comprised of fasciculi projecting from the optic tract and several neurons. It was found that the AOS in rabbits receive input from retinal ganglion cells (Simpson et al., 1979); in particular the on-direction-selective-ganglion cells which respond to moving stimuli direction and speed. As a result, the AOS is responsible for processing direction selectivity and speed of a moving visual pattern (Grasse et al., 1982 and Simpson et al., 1979). More specifically, it is the dorsal terminal nucleus of the AOS that processes horizontal temporonal movement occurring in the visual field, which is recognized by the retinal ganglion cells (Simpson et al., 1979). Lesions studies of the AOS showed that the horizontal optokinetic nystagmus is eliminated (Montgomery et al., 1982).
A study using retrograde axonal transport of HRP showed that projections of the medial terminal accessory optic nucleus in rat and rabbit led to the superior and lateral vestibular nuclei (Giolli et al., 1985). They focused on the activity of the vestibular nuclei in an alert mouse subjected to an optokinetic reflex stimulus. Different combinations of a rotating turn table (vestibular stimulation) and visual drum (full field visual stimulation) were used in conjunction with electrophysiology to determine which of the vestibular nuclei evoked dissociated and combined head and eye movements. Analyses of action potentials obtained via electrophysiology to the stimuli were used to group the vestibular nuclei into two categories: vestibular only neurons and eye-movement-sensitive neurons. Results showed that the nuclei categorized as vestibular only were not provoked in the presence of the full field visual stimulation, unlike the eye-movement-sensitive neurons. (Beraneck et al., 2007).

These findings indicate that the optokineti
cs response is in fact a reflex that is a result of a collaboration of higher order neuron function. Specially, it is the Accessory Optic and Vestibular nuclei that work together to elicit the optokinetic reflex response caused by a moving visual stimulus. These neural inputs are what allow us to obtain visual acuity and contrast sensitivity threshold values and enables us to use animal models to investigate vision loss associated with diabetes via an optokineti
cs apparatus.

1.6.5 Monocular Visual Threshold Values

A recent study using the optokinetic testing apparatus analyzed responses in the individual eyes of normal pigmented rats and retinal degenerate transgenic rats who received a monocular retinal transplant. The virtual cylinder rotated at a constant speed
counterclockwise and clockwise per eye for 60 seconds each direction. Monocular temporonasal and nasotemporal head tracking responses were monitored and recorded for each animal. Asymmetrical responses were detected, with the temporonasal direction eliciting a significantly stronger response (Thomas et al., 2004).

A study by Douglas et al., (2005) concluded that visual acuity determined via optokinetics for either monocular or binocular visual conditions are equivalent and are guided by temporonasal stimulation in both rats and mice. It was discerned that the left eye would respond to gratings moving in a clockwise direction but not in a counterclockwise direction. The opposite was true for the right eye. It would respond to the stimulus moving in a counterclockwise direction, but not a clockwise direction. Consequently, the individual eyes would respond only to stimuli in a temporonasal direction, which are similar to previous findings by Thomas et al., (2004), but no response would arise to a stimulus moving in a nasotemporal direction. Therefore, visual acuities for each eye can be determined solely by changing the direction of rotation of the virtual cylinder and can be ascertained under binocular conditions (Douglas et al., 2005).

1.6.6 OptoMotry Program Used To Obtain Threshold Values

The animals are free to move on a raised platform surrounded by vertical bars rotating around them in a cylindrical fashion while being video monitored in order to track an optokinetic reflex response or slow small head movement in the direction of the stimulus. Visual acuity thresholds are obtained by changing the spatial frequency between the vertical bars while the contrast sensitivity thresholds are acquired by adjusting the contrast between the bars. Thresholds are reached when a response is no longer elicited by the rat.
The investigator scores the optokinetic reflex response on a “yes or no” system using the image generated by the video camera. Reflex responses are scored by keeping the cursor centered between the eyes of the freely moving rodent in order to maintain a constant distance between the virtual cylinder and the rodent’s head/eyes. The “yes or no” system is based on if a response is elicited or not when different constant stimuli values of either spatial frequency or contrast sensitivity are presented via a staircase method to the animal while the stimulus is rotating in either a clockwise or counterclockwise fashion. The staircase method uses a binary search algorithm to identify a threshold value, continually halving stimuli based on 7 direction reversals (yes or no) for each stimulus direction until a threshold is identified. Reporting “yes” indicated that the animal can see the stimulus which causes the spatial frequency or contrast sensitivity to advance to a higher value; whereas reporting “no” implied that the stimulus is not visible by the animal and would thus be decreased to something visible (Prusky et al., 2004).

1.6 Diabetic Ins2\textsuperscript{Akita} Mouse-Model

The Ins2\textsuperscript{Akita} strain is of a C57BL/6 (B6) mouse background and is a model for Type I diabetes. An autosomal dominant trait that is a result of a point mutation on the MODY4 locus of the Insulin 2 gene on chromosome 7 is what causes diabetes (Yoshioka et al., 1997). A cysteine residue is substituted for a tyrosine on the A chain of the Insulin 2 gene which disrupts disulfide bond formation between the A and B chains. This causes misfolding of the Insulin 2 gene product which leads to its accumulation in the endoplasmic reticulum of the pancreatic β-cells and thus leads to their dysfunction (Wang et al., 1999). Mice heterozygous for this mutation are not accompanied with obesity or insulitis but develop hyperglycemia.
which is detectable 4 weeks after birth (Yoshioka et al., 1997) which is considered the onset of diabetes. Hyperglycemia is irreversible in these animals and the phenotype is 100% penetrant (Gastinger et al., 2008). Mice that do not have this mutation are referred to as \( \text{Ins}_2^{+/+} \) while those that have the mutation are identified as diabetic \( \text{Ins}_2^{\text{Akita/}} \).

Studies have been done to determine if this model can be used to investigate diabetes and if comparable results to those of STZ diabetes induced animal models can be obtained. Barber et al, (2005) labeled cell bodies in the retinal ganglion layer with Hoechst in diabetic \( \text{Ins}_2^{\text{Akita/}} \) mice and compared them to \( \text{Ins}_2^{+/+} \) litter-mates. Results showed that the number of ganglion cell bodies was significantly reduced by 23.4% in the diabetic retina. Gastinger et al, (2006) further confirmed the loss of ganglion cells. An increase in TUNEL-positive nuclei in 12 week diabetic \( \text{Ins}_2^{\text{Akita/}} \) of 72\% in the inner nuclear and ganglion cell layers was found. Later on Gastinger et al., (2008) explored \( \text{Ins}_2^{\text{Akita/}} \) diabetic mice crossed with transgenic mice that expressed cyan fluorescent protein (CFP), which is under the regulation of the Thy1 promoter and is found on the cell bodies of retinal ganglions. The peripheral retina of 3-month old diabetic \( \text{Ins}_2^{\text{Akita/}} \) mice showed a 16.4\% reduction in CFP-positive cells when compared to wild type mice. In contrast, there was no quantitative difference found in the number of CFP-positive cells between the mice in the central retina. This implies that peripheral ganglion cells are being adversely affected by diabetes.

A variety of other results were found using the \( \text{Ins}_2^{\text{Akita}} \) mouse which are similar to those previously mentioned in other diabetic animal-models. Gastinger et al, (2006) investigated whole retinas of the diabetic \( \text{Ins}_2^{\text{Akita/}} \) mice which were immunofluorescently labeled with antibodies specific for cholinergic and dopaminergic amacrine cells. There was a significant decrease of 20\% and 16\% of cholinergic and dopaminergic amacrine cells,
respectively, in the diabetic \textit{Ins}2^{\text{Akita/+}} mice compared to their \textit{Ins}2^{+/+} litter-mates. Morphological changes of retinal ganglion cells of diabetic \textit{Ins}2^{\text{Akita/+}} mice were also reported. The soma size of 3 month old diabetic \textit{Ins}2^{\text{Akita/+}} mice were 20%-25% larger than \textit{Ins}2^{+/+} litter-mates. Furthermore, there was an increase in dendritic length and number of terminal dendrites by 18.6% and 32.4%, respectively, in the 3 month old diabetic \textit{Ins}2^{\text{Akita/+}} compared to age-matched controls Gastinger et al, (2008).

Other studies by Barber et al, (2005) examined vascular permeability in the retina by systemically injected of bovine serum albumin conjugated with FITC. They established that the vascular permeability in diabetic \textit{Ins}2^{\text{Akita/+}} was increased by 67% after 12 weeks of diabetes compared to \textit{Ins}2^{+/+} litter-mates. The thickness of the retinal layers was also observed in diabetic \textit{Ins}2^{\text{Akita/+}} mice after they that endured diabetes for 22 weeks. In the peripheral retina the inner plexiform layer thickness and inner nuclear layer thickness were significantly less in the diabetic \textit{Ins}2^{\text{Akita/+}} mice by 27% and 15.7%, respectively, compared to control \textit{Ins}2^{+/+} mice. In the central retina the inner plexiform layer thickness was 16.7% less in the diabetic \textit{Ins}2^{\text{Akita/+}} than in the \textit{Ins}2^{+/+} litter-mates.

These studies have confirmed that genetically modified \textit{Ins}2^{\text{Akita}} mice provide an alternative yet comparable model to streptozotocin induced diabetic rats for studying the effects of diabetes on the retina. Furthermore, the results obtained from these studies are similar to those that were found in humans with diabetes. For these reasons we have chosen to use the diabetic \textit{Ins}2^{\text{Akita}} mouse model to investigate visual function via the use of an optokinetics apparatus.
1.7 Aim of Optokinetics Research

Diabetes is known to cause a variety of retinal changes, specifically a loss in retinal neurons in humans. Therefore, the purpose of this study was to determine the affect diabetes has on photopic visual function in Ins2<sup>Akita</sup> mice using the optokinetics apparatus. The hypothesis tested was that diabetes progressively reduces visual acuity and contrast sensitivity in diabetic Ins2<sup>Akita/+</sup> mice which can be rescued by insulin treatment. Additionally, the hypothesis that vision loss is correlated to cell death was also investigated.

The optokinetics technique allows us to establish a timeline in which a loss in vision can or cannot be reversed in managed and unmanaged diabetes. We are also able to identify the degree of vision loss related to diabetes and determine the point at which a visual deficit can be detected after the onset of diabetes. This study may add some clarity to the gap between molecular and functional changes in vision due to diabetes. Furthermore, this method of analyzing visual function may have possible clinical application in evaluating at which time point vision loss will have a debilitating affect on the daily activities of diabetic individuals.
Chapter 2: Materials and Methods

2.1 Animals

The Ins2$^{Akita}$ mice are bred by the Bronson Laboratory (Hershey, PA) and housed in the Penn State University College of Medicine animal facility. Institutional Animal Care and Use Committee guidelines and the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals are followed. The Bronson Laboratory breeds C57/B6J female mice acquired from the Jackson Laboratory (Bar Harbor, ME) to inbred Ins2$^{Akita/+}$ male mice which are obtained from an in-house inbred Ins2$^{Akita}$ colony. Males exhibiting a blood glucose level over 300mg/dl (Lifescan meter) were chosen to be a breeder. One C57/B6J female is housed in a cage with one Ins2$^{Akita/+}$ male for 2 weeks. The mice are then separated and the male is set up for another breeding. The gestation period for a litter containing a mix of control Ins2$^{+/+}$ and diabetic Ins2$^{Akita/+}$ is 19-21 days. Thereafter the pups are weaned from their mother at 4.5 weeks of age, upon which they are ear tagged and blood glucose levels are taken by the Bronson Laboratory. These blood glucose levels are used to assign the mice to control (Ins2$^{+/+}$) or diabetic (Ins2$^{Akita/+}$) groups which are determined by blood glucose levels of $< 250$ mg/dl or $> 250$ mg/dl, respectively. For this study, only males are used because the development of diabetes in females is slower and more variable (Yoshioka et al., 1997). Subsequently, the mice are kept in a plastic cage which encompasses an air filtration system to provide a housing environment that is specific pathogen free. Mice were maintained on a 12/12 hour light/dark schedule, with lights on at 6 am. They are fed a 2019 Teklad Global 19% Protein Extruded Diet obtained from Harlan Laboratories, Inc, which consists of 19% protein, 55% carbohydrates and 9% fat. At the end of the experiment the mice were weighed and blood glucose was measured. Mice were
sacrificed by a lethal injection of a final dose of 0.136 mg ketamine + 0.0096 mg xylazine / g body weight (which is a 14:1 Ketamine/Xylazine mix). A volume of 0.12 ml per 30 g mouse was injected followed by decapitation. Retinas were dissected rapidly for cell-death analysis.

2.2 Blood Glucose Monitoring

The AlphaTRAK blood glucose meter and blood glucose strips are used to determine blood glucose levels in the Ins2\textsuperscript{Akita} mice after they have been received from the Bronson Laboratory. This meter and the blood glucose strips are used for subsequent testing throughout the trials. For mice the glucose meter is calibrated to Code 29 and values are multiplied by a factor of 1.42 to determine the final blood glucose (mg/dl) value. When blood glucose levels are $> 750$ mg/dl the meter display screen will report “High” (HI). To include these mice in the statistical comparison between control Ins2\textsuperscript{+/+} and diabetic Ins2\textsuperscript{Akita/+} we record these blood glucose levels as 1065 mg/dl. Unless noted, mice did not receive insulin therapy.

2.3 Threshold Value Determination

The visual acuity and contrast sensitivity threshold tests were obtained using the virtual optokinetic testing equipment developed by CerebralMechanics, Inc. Individual tests were administered using the OptoMotry program associated with the optokinetic apparatus. In this study, both the right and left eye visual acuity and contrast sensitivity values were determined by rotating the virtual cylinder in a clockwise and then counterclockwise direction. The average threshold values represent the average between the left and right eyes in each animal in this study.
For each visual acuity threshold test, the virtual cylinder was rotated at a drift speed of 12 deg/sec and the contrast between the vertical black and white bars was maintained at 100% (Prusky et al., 2004). The same drift speed was used for the contrast sensitivity threshold tests; however, the spatial frequency (visual acuity) between the vertical gratings was varied. Douglas et al. (2005), showed that the most sensitive spatial frequency value that achieved a maximum contrast sensitivity threshold in C57/B6J mice was at 0.064 c/d. However, we generated a contrast sensitivity curve for the Ins2^Akita mice which illustrated that this mouse strain is most sensitive to a spatial frequency of 0.092 c/d when testing contrast sensitivity thresholds. Contrast sensitivity thresholds measured during the early part of this study were recorded at 0.064 c/d spatial frequency, while the later studies were recorded using 0.092 c/d.

Each trial began when the animal was placed on the platform and allowed to settle down and adjust to the environment. Repositioning and grooming movements were ignored during the test and the trial was resumed. If the animal fell or jumped off of the platform they were gently replaced and the trial was continued. Each trial lasted a maximum of one hour per animal. The tests ended when the visual acuity and contrast sensitivity threshold values were both obtained or the maximum one hour test time was reached.

2.4 Cell Death ELISA

A cell-death ELISA (Roche) was used to quantify DNA-histone fragments (nucleosomes) present in the cytoplasm of apoptotic retinal cells. Extracted retinal samples were placed in 100 µl of lysis buffer followed by hand homogenization for 10 seconds and placed on ice. Samples were incubated by rocking at room temperature for 30 minutes. After
incubation, each sample was centrifuged at 12,500 rpm for 10 minutes at 4°C. The pellet was discarded and 20 µl of the supernatant was added per well to a streptavidin-coated microtiter plate. Each well received 80 µl of the immunoreagent, a mixture containing biotinylated anti-histone mouse monoclonal antibody, peroxidase-labeled anti-DNA mouse monoclonal antibody and incubation buffer, and incubated for 2 hours at room temperature. The solution was aspirated and each well washed 3X with 200 µl of incubation buffer. Sample wells received 100 µl of ABTS solution (2,2’-Azino-di[3-ethylbenzthiazoline-sulfonate]) and the covered microtiter plate incubated on a plate shaker for 15-20 minutes until color developed. Color development was stopped with 100 µl per well of ABTS stop solution and quantified by spectrophotometry at a wavelength of 405 nm (reference wavelength 490 nm).

2.5 Insulin Pellet Implantation

Insulin pellets (LinBits) were purchased from LinShin, Inc (Toronto, Ontario, Canada). The pellets were composed of insulin and micro-recrystallized palmitic acid. The insulin release rate is approximately 0.1 Units/24 hr/ implant for >30 days, subcutaneously. The dose used per mouse is based on their individual body weight. Two LinBit pellets are implanted subcutaneously for the first 20 grams of body weight, using a 12 gauge trocar. For each additional 5 grams of body weight another pellet is added.

The Ins2Akita mice were weighed and appropriate insulin doses were calculated. Individual blood glucose values were also obtained. Each mouse was anesthetized, via an intraperitoneal injection, with a final dose of 0.136 mg ketamine + 0.0096 mg xylazine / g body weight (which is a 14:1 Ketamine/Xylazine mix). A volume of 0.03 ml per 30 gram mouse was administered prior to implantation of insulin pellets. During this time, the insulin
pellets were immersed in a dilute (~2%) Betadine solution. Additionally, the 12-gauge trocar is bathed in the Betadine solution before each implantation. Once the mouse was fully anesthetized, which was determined by observing loss of hind limb retraction reflex, the trocar was loaded with the proper number of insulin pellets using forceps and a stylet. Then the loaded trocar was inserted subcutaneously with the bevel side facing upward. After the trocar was inside, it was rotated so the bevel side faced downward and the stylet was used to push the individual insulin pellets out of the distal end of the trocar. As soon as the pellets were implanted, the trocar was removed and a drop of Betadine solution was placed on the skin puncture, which was then pinched closed.

Aftercare consisted of keeping the mice warm until they woke up and ensuring that food and water were available at all times. Wounds were examined daily and kept clean until fully healed. Blood glucose levels were obtained and recorded 1 week post implantation.

2.6 Data and Statistical Analysis

Contrast sensitivity threshold values expressed in the figures were expressed as the inverse of the averaged threshold for each animal, and then multiplied by 100 to obtain an average percent value. As a result, the larger the percent, the greater the ability the animal has to distinguish between shades of gray and white. Similarly, larger visual acuity thresholds signify that the animal has a better ability to resolve fine detail. Statistical comparisons between the control Ins2^{++} and diabetic Ins2^{Akita/+} groups were made using unpaired t-test and ANOVA.
Chapter 3: Results

3.1. Loss of Visual Function in Diabetic Ins2\(^{Akita}\) Mice

Binocular visual acuity and contrast sensitivity thresholds of Ins2\(^{+/+}\) litter-mates and diabetic Ins2\(^{Akita/+}\) mice of different ages were obtained using a virtual optomotor system. Contrast sensitivity values for the following experimental studies were determined at a spatial frequency of 0.064 c/d while visual acuity values were obtained at 100% contrast. The ages of the diabetic Ins2\(^{Akita/+}\) mice compared to control Ins2\(^{+/+}\) ranged from 7 to 36 weeks old and experienced diabetes for a duration of 3 to 32 weeks, respectively.

In Study 1, diabetic Ins2\(^{Akita/+}\) mice endured diabetes from 3 to 18 weeks. Table 2 shows that the blood glucose (BG) levels of diabetic Ins2\(^{Akita/+}\) were significantly increased and decreased, respectively, at 4.5 weeks of age and time of harvest at 23 weeks of age when compared to control Ins2\(^{+/+}\) (p<0.01). Diabetic Ins2\(^{Akita/+}\) (n = 7) visual acuity thresholds were significantly less than that compared to Ins2\(^{+/+}\) litter-mates (n = 6) by 9.78% - 15.98% after 5 to 18 weeks of hyperglycemia (p<0.01, Fig. 4A). Similarly contrast sensitivity threshold values for diabetic Ins2\(^{Akita/+}\) were significantly less than that of the control Ins2\(^{+/+}\) by 13.22% - 31.03% after 3 to 18 weeks of diabetes (p<0.05, Fig. 4B).

<table>
<thead>
<tr>
<th>Table 2. Average Blood Glucose Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Diabetic</td>
</tr>
</tbody>
</table>

**p < 0.01, unpaired t-test relative to corresponding control group

Group determination by blood glucose (BG) testing at 4.5 weeks old
Figure 4. Visual function diminished in Ins2\textsuperscript{Akita}. Visual function measurements of control Ins2\textsuperscript{+/+} (n = 6, ▲) and diabetic Ins2\textsuperscript{Akita/+} (n = 7, ◇) were compared from 7 to 22 weeks of age. (A) Visual acuity threshold values at 100% contrast were significantly less at all data points in which Ins2\textsuperscript{Akita/+} had diabetes for a duration of 7 to 18 weeks by 9.78% - 15.98% compared to control Ins2\textsuperscript{+/+} (p < 0.01). (B) Contrast sensitivity threshold values at 0.064 c/d for diabetic Ins2\textsuperscript{Akita/+} mice which endured diabetes for 3 to 18 weeks were significantly less than control Ins2\textsuperscript{+/+} by 13.22% - 31.03% at all time points (p < 0.05). Error bars at each time point represent standard error values.

In Study 2 visual function measurements of diabetic Ins2\textsuperscript{Akita/+} that were hyperglycemic for 11 to 27 weeks were analyzed. Table 3 shows that the blood glucose
levels of diabetic Ins2^{Akita/+} were significantly higher and lower, respectively, at 4.5 weeks of age and time of harvest at 31 weeks of age compared to control Ins2^{+/+} (p<0.01). Visual acuity threshold values are significantly less in diabetic Ins2^{Akita/+} (n = 6) by 9.18% - 20.87% after a duration of diabetes of 11 to 27 weeks compared to Ins2^{+/+} litter-mates (n = 8, p<0.01, Fig. 5A). Contrast sensitivity was also lower in diabetic Ins2^{Akita/+} compared to controls Ins2^{+/+}; a significant loss of 15.83% - 26.36% was found after 19 weeks of diabetes and persisted for the rest of the duration of the disease (p < 0.01, Fig. 5B).

![Graph A](image)

**Table 3. Average Blood Glucose Levels**

<table>
<thead>
<tr>
<th>Group</th>
<th>At 4.5 weeks old</th>
<th>At 31 weeks old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>BG (mg/dl)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>209 ± 6.32</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8</td>
<td>457 ± 42.40**</td>
</tr>
</tbody>
</table>

** p < 0.01, unpaired t-test relative to corresponding control group

Group determination by blood glucose (BG) testing at 4.5 weeks old.
In Study 3 diabetic Ins2^Akita/+ mice that had diabetes for 23 to 30 weeks were compared to control Ins2^+++. Table 4 shows that the blood glucose levels of diabetic Ins2^Akita/+ were significantly higher and lower, respectively, compared to control Ins2^+++ at 4.5 weeks of age and time of harvest at 34 weeks of age (p < 0.01). There was a significant loss in visual acuity of 10.58% and 16.33% in diabetic Ins2^Akita/+ mice that had diabetes for a duration of 23 to 30 weeks, respectively, compared to control Ins2^+++ (p < 0.01, Fig. 6A). Additionally, contrast sensitivity thresholds of diabetic Ins2^Akita/+ were significantly less than those of the control Ins2^+++/ by 26.18% after 30 weeks of diabetes (p < 0.01, Fig. 6B).
Table 4. Average Blood Glucose Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>At 4.5 weeks old</th>
<th></th>
<th>At 34 weeks old</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>BG (mg/dl)</td>
<td>n</td>
<td>BG (mg/dl)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>171 ± 5.79</td>
<td>7</td>
<td>214.6 ± 6.72</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8</td>
<td>429 ± 26.42**</td>
<td>6</td>
<td>1065**</td>
</tr>
</tbody>
</table>

** p < 0.01, unpaired t-test relative to corresponding control group

Group determination by blood glucose (BG) testing at 4.5 weeks old

Figure 6. Visual function diminished at late stage of diabetes. Visual function measurements of control Ins2+/+ and diabetic Ins2^Akita/+ were compared at 27 then 34 weeks of age. (A) Visual acuity threshold values at 100% contrast in diabetic Ins2^Akita/+ that endured diabetes for 23 and 30 weeks were significantly less than that of control Ins2+/+ mice by 10.58% and 16.33%, respectively (p < 0.01). (B) Contrast sensitivity threshold values at 0.064 c/d of diabetic Ins2^Akita/+ that had diabetes for 30 weeks were significantly less than that of control Ins2+/+ by 26.18% (p < 0.01). Error bars at each time point represent standard error values.

Study 4 analyzed diabetic Ins2^Akita/+ that were hyperglycemic for 29 to 32 weeks. Table 5 shows that the blood glucose levels of the diabetic Ins2^Akita/+ were significantly increased and decreased, respectively, at 4.5 weeks of age and time of harvest at 36 weeks of age compared to Ins2^+/+ litter-mates (p < 0.01). Visual acuity was significantly less in diabetic Ins2^Akita/+ after 29 and 32 weeks of diabetes by 14.76% and 12.41%, respectively,
when compared to control Ins2\(^{+/+}\) (p<0.01, Fig. 7A). Additionally, contrast sensitivity threshold values of diabetic Ins2\(^{Akita/+}\) that endured diabetes for 29 weeks were significantly less than the control Ins2\(^{+/+}\) by 26.68% (p<0.05, Fig. 7B). At the end of this study the retinas of the 36 week old mice were analyzed by cell death ELISA. Retinas of the Ins2\(^{Akita/+}\) diabetic mice had 52.73% more nucleosome fragments compared to Ins2\(^{+/+}\) litter-mate controls (p<0.05; Fig 7C).

**Table 5. Average Blood Glucose Levels**

<table>
<thead>
<tr>
<th>Group</th>
<th>At 4.5 weeks old</th>
<th>At 36 weeks old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>BG (mg/dl)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>203 ± 11.09</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8</td>
<td>477 ± 29.65*</td>
</tr>
</tbody>
</table>

* p < 0.01, unpaired t-test relative to corresponding control group

Group determination by blood glucose (BG) testing at 4.5 weeks old.
Figure 7. Visual function diminished at late stage of diabetes and increased apoptosis in diabetic retina. Visual function measurements of control Ins2+/+ and diabetic Ins2^{Akita/+} were compared at 33 then 36 weeks of age, followed by retinal apoptosis analysis. (A) Visual acuity threshold values at 100% contrast of diabetic Ins2^{Akita/+} were significantly less than control Ins2+/+ after 29 and 32 weeks of diabetes by 14.76% and 12.41%, respectively (p < 0.01). (B) Contrast sensitivity threshold values at 0.064 c/d were significantly less in diabetic Ins2^{Akita/+} compared to control Ins2+/+ after 29 weeks of diabetes by 26.68% (p < 0.05). (C) Cell death ELISA revealed that diabetic Ins2^{Akita/+} had 52.73% significantly more nucleosome fragments than control Ins2^{+/+} at time of harvest (p < 0.05). Error bars represent standard error values.

3.2 Reversing Diabetes with Insulin Corrects Visual Function

The effect of correcting diabetes with insulin on visual function was investigated in control Ins2^{+/+} and diabetic Ins2^{Akita/+} mice. Table 6 shows blood glucose levels of the Ins2^{Akita} groups at 4.5 weeks of age, after 4 weeks of diabetes (1 week post-insulin implant) and after 8 weeks of diabetes (5 weeks post-insulin implant) compared to Ins2^{+/+} litter-mates. At 4.5 weeks of age diabetic Ins2^{Akita/+} and the diabetic Ins2^{Akita/+} designated to receive insulin treatment (Diabetic + Insulin) had significantly higher blood glucose levels of 96.89% and 76.17%, respectively, compared to control Ins2^{+/+} (p<0.01). After 2 weeks of diabetes a
contrast sensitivity curve was established to determine which spatial frequency produced the greatest difference in contrast sensitivity (Fig. 8). Results revealed that a spatial frequency of 0.092 c/d generated the largest difference in contrast sensitivity threshold values between the control Ins2$^{+/+}$ (n = 6) and diabetic Ins2$^{Akita/+}$ (n = 11) group.

Table 6. Average Blood Glucose Levels Pre & Post-Insulin Implant

<table>
<thead>
<tr>
<th>Group</th>
<th>At 4.5 Weeks Old</th>
<th>1 Week Post-Insulin Implant (4 weeks of Diabetes)</th>
<th>5 Weeks Post-Insulin Implant (8 weeks of Diabetes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>BG (mg/dl)</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>193 ± 3.91</td>
<td>5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>5</td>
<td>380 ± 14.32**</td>
<td>5</td>
</tr>
<tr>
<td>Diabetic + Insulin</td>
<td>6</td>
<td>340 ± 12.85**</td>
<td>6</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, unpaired t-test relative to corresponding control group
## p<0.01, unpaired t-test relative to corresponding diabetic group

Group determination by blood glucose (BG) testing at 4.5 weeks old

Figure 8. Contrast sensitivity curve of Ins2$^{Akita}$ mice at various spatial frequencies. After 2 weeks of diabetes the greatest difference in contrast sensitivity occurred at a spatial frequency of 0.092 c/d between control Ins2$^{+/+}$ (▲) and diabetic Ins2$^{Akita/+}$ (♦) mice. Error bars represent standard error values.
A significant loss in visual acuity of 9.77% was found in the Ins2^{Akita/+} diabetic group compared to the control Ins2^{+/+} after 2 weeks of diabetes (p < 0.01, Fig. 9A). Similarly, a significant loss of 24.31% and 44.48% in contrast sensitivity was found in the diabetic Ins2^{Akita/+} mice and the diabetic Ins2^{Akita/+} designated for insulin treatment (Diabetic + Insulin group), respectively, after 2 weeks of diabetes compared to the Ins2^{+/+} litter-mates at 0.092 c/d (p<0.01, Fig. 9B).

After 3 weeks of diabetes, 6 diabetic Ins2^{Akita/+} mice underwent insulin pellet implantation. One week post implantation (4 weeks of diabetes) blood glucose levels and visual function were examined. Table 5 depicts the average blood glucose levels for control Ins2^{+/+} (n = 5), diabetic Ins2^{Akita/+} (n = 5) and diabetic Ins2^{Akita/+} with insulin (n = 6). The average blood glucose for diabetic Ins2^{Akita/+} was 256% significantly higher than that of the control Ins2^{+/+} (p<0.01). Additionally, there was a significant rise of 461% in average blood glucose in the diabetic Ins2^{Akita/+} compared to the diabetic Ins2^{Akita/+} with insulin (p<0.01). Visual acuity thresholds were significantly less in the diabetic Ins2^{Akita/+} group compared to the control Ins2^{+/+} and diabetic Ins2^{Akita/+} with insulin groups by 11.62% and 6.94%, respectively (p<0.05). The threshold values between the control Ins2^{+/+} and diabetic Ins2^{Akita/+} with insulin were not significantly different (Fig. 9A). The diabetic Ins2^{Akita/+} experienced a significant loss of 19.45% in contrast sensitivity threshold when compared to control Ins2^{+/+} (p < 0.05). The contrast sensitivity threshold of the diabetic Ins2^{Akita/+} with insulin were not significantly different from the control Ins2^{+/+} and diabetic Ins2^{Akita/+} groups (Fig. 9B).

The blood glucose levels and visual function were compared again once the insulin was exhausted, which occurred 5 weeks post insulin implantation (8 weeks of diabetes).
Diabetic Ins2^{Akita/+} (n = 5) and diabetic Ins2^{Akita/+} with insulin (n = 6) groups had significantly greater blood glucose levels compared to the control Ins2^{+/+} (n = 5) by 237.77% (p<0.01) and 110.58% (p<0.05), respectively (Table 5). Visual acuity threshold values 5 weeks post insulin implantation revealed that the diabetic Ins2^{Akita/+} visual acuity was significantly less than the control Ins2^{+/+} and diabetic Ins2^{Akita/+} with insulin by 14.61% and 9.51%, respectively (p < 0.01, Fig. 9A). Similar results were found when contrast sensitivity thresholds were examined. The diabetic Ins2^{Akita/+} contrast sensitivity was significantly less than the control Ins2^{+/+} and diabetic Ins2^{Akita/+} with insulin by 33.05% and 25.31%, respectively (p < 0.01, Fig. 9B). Table 7 summarizes the significant differences in visual function between the groups after 2, 4, and 8 weeks of diabetes.
Figure 9. Visual function rescued in diabetic Ins2\(^{Akita/+}\) treated with insulin. Visual acuity and contrast sensitivity measurements of control Ins2\(^{+/+}\), diabetic Ins2\(^{Akita/+}\), and diabetic Ins2\(^{Akita/+}\) treated with insulin were compared after a duration of 2, 4, and 8 weeks of diabetes. At a duration of diabetes of 2 weeks, the diabetic + insulin group did not receive insulin treatment at this point. Measurements obtained at 4 weeks were 1 week post insulin treatment for the diabetic + insulin group while values obtained at 8 weeks were 5 weeks post insulin treatment in which the insulin was exhausted. *p<0.05, **, p<0.01 unpaired t-test are relative to corresponding control group. *p<0.05, **p<0.01 unpaired t-test are relative to corresponding diabetic group. Error bars at each time point represent standard error values. See Table 6 for significant differences in visual function between groups at 2, 4, and 8 weeks of diabetes.

### Table 7. Significant Difference Between Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Visual Acuity Threshold Differences (%)</th>
<th>Contrast Sensitivity Threshold Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks of Diabetes</td>
<td>4 weeks of Diabetes</td>
</tr>
<tr>
<td>Diabetic</td>
<td>** &gt; 13.02</td>
<td>** &gt; 11.62</td>
</tr>
<tr>
<td>Diabetic + Insulin</td>
<td>** &gt; 7.06</td>
<td># &lt; 6.94</td>
</tr>
<tr>
<td></td>
<td>** &gt; 24.31</td>
<td>* &gt; 19.45</td>
</tr>
<tr>
<td>Diabetic + Insulin</td>
<td>** &gt; 44.48</td>
<td>-</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01, unpaired t-test relative to corresponding control group
* p<0.05, ** p<0.01, unpaired t-test relative to corresponding diabetic group

** > means less than corresponding control group
##< means greater than corresponding diabetic group
The study investigating the effect of insulin on visual function is still underway. Additional visual acuity and contrast sensitivity threshold values will be compared between the diabetic $\text{Ins}2^{\text{Akita/+}}$ and control $\text{Ins}2^{+/+}$ at a later age. These comparisons will be done to determine if the restoration or reversal of vision loss in the group of diabetic $\text{Ins}2^{\text{Akita/+}}$ that had received a month’s treatment with insulin is still present. Lastly, a cell death ELISA will be performed at the end of the study to analyze the degree of retinal apoptosis in each of the groups.

A similar study, which is still in progress, examined visual acuity thresholds at 100% contrast and contrast sensitivity thresholds at 0.092 c/d on a population of diabetic $\text{Ins}2^{\text{Akita/+}}$ that experienced diabetes for 22 weeks, followed by insulin treatment. The insulin data is still being generated. Blood glucose levels at 4.5 weeks of age in diabetic $\text{Ins}2^{\text{Akita/+}}$ ($n = 8$) were significantly elevated by 126.76% compared to $\text{Ins}2^{+/+}$ litter-mates ($n = 6$, $p<0.01$, Fig. 10A). Figure 7B illustrates a significant deficit of 16.43% in visual acuity threshold levels in the diabetic $\text{Ins}2^{\text{Akita/+}}$ mice compared to $\text{Ins}2^{+/+}$ litter-mates ($p < 0.01$). Contrast sensitivity thresholds were significantly reduced as well. Compared to control $\text{Ins}2^{+/+}$, diabetic $\text{Ins}2^{\text{Akita/+}}$ experienced a loss of 29.99% in threshold ($p < 0.01$, Fig. 10C).
Figure 10. Visual function diminished in Ins2<sup>Akita</sup>. Blood glucose levels at 4.5 weeks of age and visual function of control Ins2<sup>++</sup> (n = 6) and diabetic Ins2<sup>Akita/</sup> (n = 8) were compared at 26 weeks of age. (A) The average blood glucose levels of diabetic Ins2<sup>Akita/</sup> (n = 8) were 126.76% significantly higher compared to the control Ins2<sup>++</sup> (p < 0.01). (B) Visual acuity threshold values at 100% were significantly less in the diabetic Ins2<sup>Akita/</sup> compared to the control Ins2<sup>++</sup> by 16.43% (p< 0.01). (C) The diabetic Ins2<sup>Akita/</sup> contrast sensitivity was significantly less compared to that of the control Ins2<sup>++</sup> by 29.99% (p < 0.01). Error bars represent standard error values.
Blood glucose levels and visual acuity and contrast sensitivity threshold values will be obtained 1 week post insulin implant and compared between the control Ins2<sup>+/+</sup>, diabetic Ins2<sup>Akita/+</sup> and diabetic Ins2<sup>Akita/+</sup> with insulin. Like the previous study, the same abovementioned factors will be measured and evaluated among the three groups once the insulin is exhausted, which occurs 5 weeks post insulin implant. This will be followed by another comparison of blood glucose levels and visual acuity and contrast sensitivity threshold values at a later time point without further insulin treatment between the same three groups. Lastly, at time of harvest retinas a cell-death ELISA will be performed to examine the degree of apoptosis present within the three groups.
Chapter 4: Discussion

This project used the optokinetics reflex to detect deficits in vision in diabetic mice. The hypothesis tested was that diabetes progressively reduces visual acuity and contrast sensitivity in diabetic $\text{Ins}^2\text{Akita}^+/+$ mice which can be rescued by insulin treatment. Additionally, the hypothesis that vision loss is correlated to cell death was also investigated. Results showed that visual acuity and contrast sensitivity threshold values in diabetic $\text{Ins}^2\text{Akita}^+/+$ mice were less than that of $\text{Ins}^2^{+/+}$ litter-mates. This loss persisted with the duration of diabetes and was detectible two weeks after the onset of hyperglycemia. Furthermore, the degree of apoptosis in diabetic $\text{Ins}^2\text{Akita}^+/+$ mice was significantly greater than in control $\text{Ins}^2^{+/+}$ mice. No correlation, however, was found between apoptosis and diabetes. Lastly, it was found that the treatment of diabetes with insulin restored visual function in young mice, which had been diabetic for less than one month.

The results indicate that a loss in visual function in $\text{Ins}^2\text{Akita}$ mice begins soon after the onset of diabetes, but is reversible. Initially, we hypothesized that a loss in vision was due to retinal apoptosis since it has been confirmed through many studies that there is an increase in apoptotic activity in the presence of diabetes (Barber et al., 1998; Abu-El-Asrar et al., 2004). Our findings, however, report that a visual deficit as a result of diabetes can be rescued by insulin treatment. This suggests that, at least during the early stages of the disease since the neurons cannot be regenerated, vision loss is not due to the depletion of neurons by apoptosis. From this we can infer that another mechanism other than apoptosis must alter the function of the retinal cells which causes a loss in vision in diabetes.
Conclusions drawn from other studies identified that in diabetes the function of the cone-photoreceptors, specifically their activity and sensitivity, is altered (Fisherman et al., 1990; Hancock et al., 2004; Holopigian et al., 1997). Other reports indicated that there is a decrease in synaptic proteins (VanGuilder et al., 2008) and the metabolism of glutamate, the major neurotransmitter of the retina is impaired (Lieth et al., 1998). These reports in conjunction with our findings allow us to speculate that a loss in vision may be a result of altered photoreceptor function at the synaptic level and not a product of apoptosis. It is possible that persistent high blood glucose levels caused a decrease in the sensitivity of the photoreceptors (in particular the opsin molecules) to light, resulting in a reduction in synaptic transmission and thus diminished vision. Another mechanistic explanation for a loss in vision may be a product of reduced neurotransmitter vesicle binding at the pre-synaptic level due to the loss in synaptic proteins. This may also lead to a change in synaptic transmission. Lastly, visual deficits in diabetes could be related to changes in Muller cells and/or glutamine synthetase regulatory roles of the glutamine/glutamate metabolic cycle that regulates neurotransmission.

As previously stated, this study is still underway. We will be investigating the effect of insulin on a much older diabetic Ins2^{Akita/+} group. Since we believe that apoptosis is not the mechanism that is causing a loss in vision, given that visual function was reversed, we predict that insulin will partially rescue vision in these mice similar to what was seen in 1 month diabetic Ins2^{Akita/+}. 
5.1 Obtaining Objective Results

In behavioral studies such as this as this one, it is possible that the investigator can influence or bias the outcome of the study. In this study, several approaches were taken to prevent results from being subjectively influenced. First, an average value of three trials obtained for visual acuity and contrast sensitivity threshold was recorded to eliminate individual trial variation that may have occurred during testing. Variations could be attributed to the attentiveness of either the operator or mouse as well as the time of day that the trial was performed. Secondly, initial studies were performed by two different operators testing the same group of animals, to test the reproducibility of threshold values. It was found the scores for each group of mice was highly reproducible between investigators. Lastly, the OptoMotry interface was moved to the bottom of the computer screen, which hides the range of values being tested. The target stimulus could not be seen by the investigator, helping keep the investigator unaware of the threshold scores until completion of data collection for each trial.

The apparatus enables the virtual cylinder to provide a constant spatial frequency regardless of the distance between the mouse’s head and the screen. At the beginning of a trial the investigator must calibrate the location of the platform using a crosshair pointer which is recognized by the Optomotry software as the center of the cylinder rotation. As the mouse moves freely on the platform the crosshair is used to follow the mouse’s head. It is important that the investigator follows the mouse because the positional coordinates of the crosshair are used to center the virtual cylinder rotation on the mouse’s head as they move closer to and farther away from the computer monitors that are displaying the virtual gratings. This positional feedback is used to maintain the desired visual angle at which the
trial is being carried out. Furthermore, the Optomotry program controls the speed of the cylinder as well as the contrast of the stimulus (Prusky et al., 2004). These built-in features help ensure objective threshold results between users and between trials with the same animal.

5.2 Adaptation to Stimulus

A concern using the optokinetics apparatus is the possibility that the mouse will become adapted to the rotating stimulus if they are in there for prolonged periods or are tested multiple times in a day. As a result the mouse will become inactive to the point where it appears that they are asleep so an optokinetics reflex response will not be clearly detected. To remedy this issue the investigator carries out short testing periods of a maximum of 1 hour per trial once a day. Additionally, the investigator can make noises and tap the apparatus to startle the mouse when they appear dormant, thereby decrease the time in which they are exposed to a specific threshold value. Furthermore, the programs stair-case method for determining the threshold limits the likelihood of adaptation as well. It does so by changing the spatial frequency or contrast if visual acuity or contrast sensitivity is being tested, respectively, in a transient manner, so the mouse does not become accustomed to a specific threshold value. Additionally, the direction of the grating stimulus is alternated to stimulate individual eyes and introduces a variety of stimuli the mouse experiences in a single trial (Prusky et al., 2004). A combination of these methods limits the possibility of the mouse adapting to the virtual stimulus.
5.3 Limitations of Optokinetics Apparatus

The apparatus measures the optokinetic reflex response which is indicated by the movement of the mouse’s head in the direction of the rotating stimulus at a given threshold (Thomas et al., 2004; Douglas et al., 2005). As the threshold is approached, the movement of the head becomes less evident until it reaches a point at which a response is no longer detectible; indicating that the maximum threshold value has been achieved. One possible limitation with this approach is that the investigator is unable to directly visualize the movement of the eyes of the mouse. Therefore, at the point where head movements are no longer occurring, it is possible that there is still a residual reflex response in which only the eyes move in the direction of the stimulus. Thus, the threshold values obtained may be slightly lower than the actual threshold.

Another limitation is that since the animals are light-adapted and the visual stimulus is bright, the test only measures photopic, (or cone photoreceptor) responses. This restricts the analysis of threshold responses to a fairly small subpopulation of photoreceptors and retinal neurons that participate in daylight vision only. Therefore, the task measures only a small part of the total functional capacity of the mouse retina.

5.4 Visual Acuity and Contrast Sensitivity Comparisons Between Humans & Mice

It is difficult to make a direct comparison of visual function measurements between humans and mice. The standard Snellen Eye chart, which is comprised of lines of block letters of diminishing size, is used to determine a patient’s visual acuity threshold to a static image. The patients must stand 20 feet away from the chart in order to maintain a constant visual angle. Monocular measurements are performed by having the patient read the
individual lines of letters out loud. Final thresholds are obtained by recording which of the lines the patient was able to read out loud correctly. One important point to take note of is that visual acuity measurements are not typically used for diagnostic purposes or a primary clinical endpoint because this technique is considered relatively insensitive. A loss in visual acuity, especially in the diabetic population, is not reduced until advanced stages of diabetic retinopathy and long duration of diabetes (Moss et al., 1988). It is used more as part of a routine check up to make sure drastic visual changes have not occurred in a short period of time. Secondly, it is important to realize that the acuity measured in this study is based determined by a moving target; while in humans acuity is determined from a stationary measurement that assesses the fovea’s ability to detect detail, which a mouse lacks.

Similarly, to test contrast sensitivity a patient will be asked to look at a chart that displays bars or dots with different contrasting backgrounds to their threshold. These stationary clinical techniques are different from the dynamic stimulus the Ins2\textsuperscript{Akita} mice experience when using the optokinetics apparatus. Therefore, it is difficult to directly compare the numerical values of contrast sensitivity between humans and mice. Optokinetics testing however, allows us to identify a loss in visual function and to what degree this is occurring in the diabetic Ins2\textsuperscript{Akita} mouse models. The percent loss could be indirectly compared between diabetic humans and mice to determine the effect diabetes has on visual function.

5.5 Human vs. Rodent Cone Photoreceptor Population

As previously stated visual acuity is the ability to resolve fine detail and is mainly derived from the synaptic convergence of the cone system. The average human retina
contains 4.6 million cones, which is 5% of the photoreceptor population. They are highly localized at the fovea of the macula located in the central retina. In contrast, there are on average 96 million rods which are more concentrated in the peripheral retina (Curcio et al., 1990).

Mice lack a macula and only 3% of the photoreceptor population is cones which are dispersed throughout the central and peripheral retina (Carter-Dawson et al., 1979). There are 437,000 cells/mm\(^2\) or 6.4 million rods per mouse retina compared to 12,400 cells/mm\(^2\) or 180,000 cones per mouse retina (Jeon et al., 1998). Even though the cone population in mice is far less than that in humans, it was still possible to obtain visual acuity measurements. For that reason, the optokinetics apparatus is a promising technique to analyze visual function in diabetic-animal models.

5.6 Significance of Optokinetic Research

Clinically, if functional measurements such as visual acuity and contrast sensitivity can be used to detect initial stages of diabetic retinopathy it may be possibly to restore or decelerate the loss of vision. Furthermore, visual deficit treatments due to altered synaptic transmission or retinal degeneration could be investigated in animal-models and analyzed via optokinetics for clinical use. Lastly, mechanisms causing a decrease in neural transmission can be isolated and possibly give rise to therapies that remedy specific cellular functions.

Research with diabetic animal models, such as the Ins2\(^{Akita}\) mouse, lends itself to developing new ways to assess vision and the functional aspects in the clinic. The outcomes of these studies provide a rationale for clinical changes observed in the retina such as cell death, differences in vascular permeability, and changes in neural transmission. Additionally,
using animal models can improve current diagnostic tests in the clinic. Overall animal models are imperative in gaining knowledge of the retina and remedying any malfunctions caused by diabetes.

5.7 Conclusion

Diabetic retinopathy has been a topic of investigation for a long period of time. The use of optokinetics provides more insight into the pathological changes associated with diabetes. Utilizing this technique we were able to show that vision is impaired early on and progresses with the disease. To further bridge the gap between histological and functional changes that occur in diabetes it may be worthwhile doing electroretinography studies with diabetic Ins2Akita/+ mice. This technique can provided more detail on which cell population is being targeted and what part of the phototransduction cascade the loss of vision is occurring. Moreover, this method has the capability of testing photopic and scotopic vision. Therefore, a larger group of retinal cells can be investigated.

A variety of future experiments which could test our mechanistic hypotheses about vision loss and reversibility with insulin could be to use the optokinetics technique in conjunction with transgenic mice. The transgenic mice could over express synaptic proteins, have a protective mechanism in which opsins molecules do not become desensitized to light, or down-regulate the expression of glutamate dehydrogenase so there is an increase of glutamate in the synapse such that phototransduction neurotransmission is not diminished. If these transgenic models are used in combination with the optokinetics apparatus we may be able to establish which mechanism is the cause of the loss in visual function in diabetes and determine the role of insulin in the rescue of vision. These experiments may provide us with
a little more insight on which region of the retina is being affected and if it is related to synaptic transmission, cellular function, or metabolism.
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