Final Progress Report: AMDCC Pilot and Feasibility Study grant, 9/1/2009 – 8/31/2010

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Title: THE INS2^{AKITA} MOUSE AS A MODEL OF VISION LOSS AND NEURODEGENERATION IN DIABETES

Project Summary

The overall goal of this research is to determine the mechanism of vision loss in diabetic retinopathy. The main objective of the proposed project is to develop functional assays of vision loss in the spontaneously diabetic Ins2^{Akita} mouse, and compare the loss of function with assays of cell death, retinal morphomotry (cell layer thickness and nuclear counts) and synaptic protein content. A new approach that uses the optokinetic reflex will be employed to measure visual acuity and contrast sensitivity in mice. We have also established that a rapid and sensitive cell death ELISA can be used to measure apoptosis in mouse retinas. This assay measures the amount of nucleosomal fragments in the cytoplasm of cells undergoing apoptosis. Furthermore, our published data show that the content of a group of pre-synaptic proteins is depleted in the retinas of diabetic rodents, and that diabetes leads to reductions in the thicknes of the inner retinal layers in mice and rats. These assays can be used to measure loss of function, apoptosis, retinal degeneration and synaptic malfunction in Ins2Akita diabetic mice. Therefore this project will test the general hypothesis that diabetes induces retinal neurodegeneration and compromises visual function in the Ins2Akita mouse. The first specific aim is to measure vision loss, apoptosis and retinal morphomotry in diabetic Ins2Akita mice after different durations of diabetes. The second specific aim is to measure vision loss and synaptic protein content in Ins2Akita mice, again after different durations of diabetes. It is predicted that apoptosis will be detected soon after the onset of diabetes and that visual acuity and contrast sensitivity will be correlated with loss of the retinal cell layers and with reduced synaptic protein content. This project will further establish the Ins2^{Akita} mouse as a model of diabetic retinopathy, and will test the concept that retinal cell apoptosis and synaptic degeneration lead to vision loss.

Furthermore, it will identify the threshold of synaptic protein and retinal cell loss that is required to cause a measurable loss of function in the Ins2^{Akita} mouse.

Final Report

This 12 month project had two Specific Aims: 1) to measure the vision loss and retinal apoptosis in Ins2^{Akita} diabetic mice; and 2) to measure vision loss and depletion of pre-synaptic proteins in retinas of Ins2^{Akita} diabetic mice. The overall goal of the project was to further develop the Ins2^{Akita} mouse as an animal model of diabetic retinopathy, by establishing new endpoints to measure visual function, and to correlate changes in function with measurements of cell death and neurodegeneration in the retina.

Aim 1

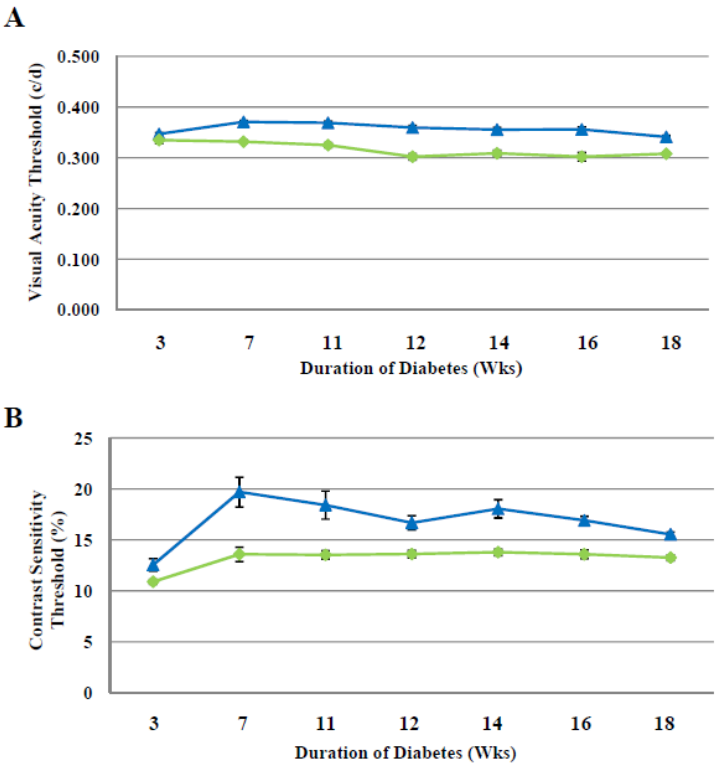
We made good progress on Aim 1 by successfully developing a behavioral methodology to test visual function in diabetic and wild-type Ins2^{Akita} mice. The OptoMotry™ optokinetics testing apparatus can be used to test spatial frequency threshold (sometimes refered to as visual acuity) and contrast sensitivity. Figure 1 shows how these measures were different in a group of Ins2^{Akita} mice over 18 weeks duration of diabetes.

Group	At 4.5 weeks old		At 23 weeks old	
	n	BG (mg/dl)	n	BG (mg/dl)
Control	8	178 ± 6.09	6	147 ± 3.32
Diabetic	8	416 ± 34.75**	7	539 ± 43.04**

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

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

Blood glucose is significantly elevated in Ins2^{Akita} mice 4-5 weeks after birth. Diabetes duration is measured from 5 weeks of age.



Contrast sensitivity was initially measured at 0.064 c/d (cycles per degree) spatial frequency.
The difference in acuity (A) and contrast sensitivity (B) between diabetic (green line) and wild-type control (blue line)mice was significant at all time points after 3 weeks duration ($p<0.05$).

Note that in all cases the control mice were wild-type littermates of the diabetic Ins2^{Akita} mice, and so were age-matched and genetically similar. Also, only males were used

in these studies, because the female Akita mice develop a less severe diabetic phenotype.

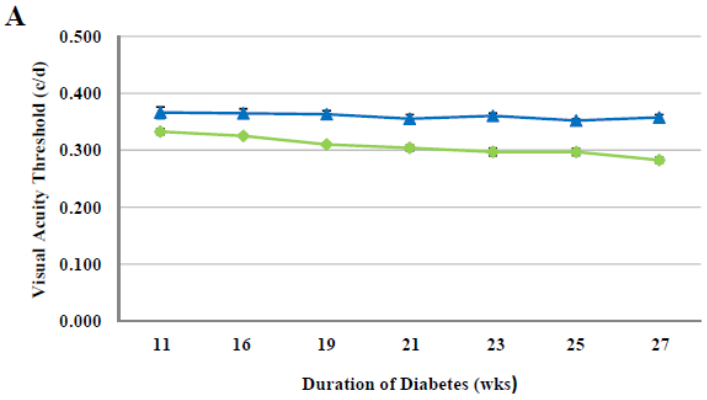
Figure 2 shows results of a second study using older mice.

	At 4.5 weeks old		At 31 weeks old	
Group	**n**	**BG (mg/dl)**	**n**	**BG (mg/dl)**
Control	8	209 ± 6.32	8	147 ± 12.38
Diabetic	8	457 ± 42.40**	6	705 ± 22.69**

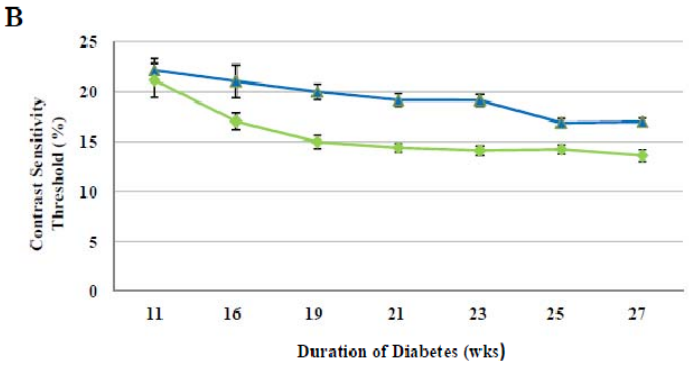
** $p < 0.01$, unpaired t-test relative to corresponding control group

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

Again the blood glucose of Ins2^{Akita} mice was significantly higher than wild-type mice within 5 weeks of age, and remained high for the 31 week duration



The visual acuity (A) and contrast sensitivity (B) were significantly reduced in the diabetic mice (green line) compared to the wild-type control mice (blue line) ($p<0.05$).



In a separate experiment we again measured the visual function of a group of aging Ins2^{Akita} and wild-type mice, and used the retinas to measure apoptosis (Cell Death ELISA™). The results in figure 3 show that visual acuity (A) was significantly reduced in a group of mice assessed after 27 weeks and 32 weeks of diabetes ($p<0.01$). The contrast sensitivity was also compromised in the Ins2^{Akita} diabetic mice after 27 weeks of diabetes ($p<0.05$), although the controls also appeared to have reduced contrast sensitivity at the 32 weeks time point, probably due to their advancing age. The amount of apoptosis (C), as measured by the amount of cytoplasmic histone-associated DNA fragments (Cell Death ELISA™), and was found to be significantly greater in the Ins2^{Akita} diabetic

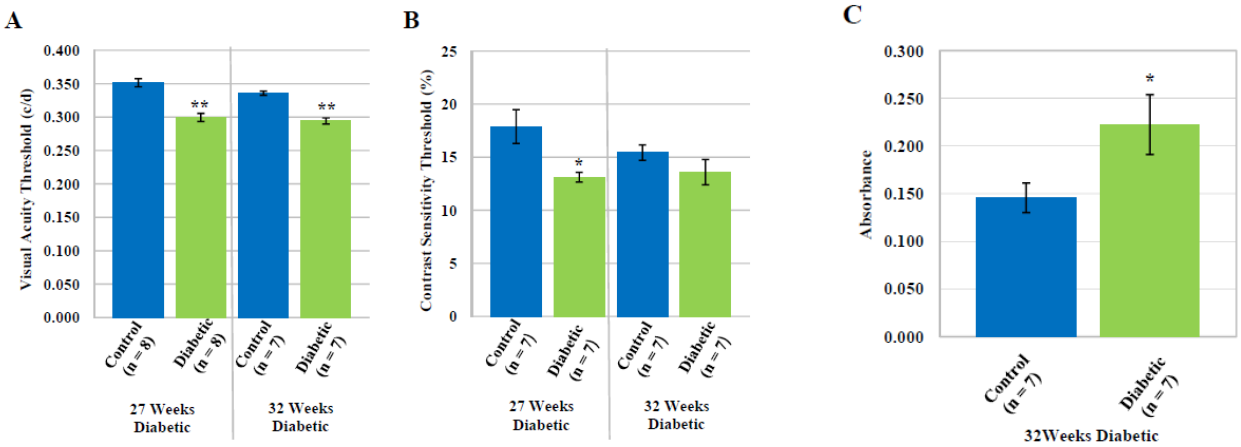
mice compared to controls. These results are similar to data obtained previously from STZ-diabetic rats.

Attempts to correlate the cell death ELISA with visual function were ambiguous, given the small sample size in this study; however there was no statistically significant correlation between cell death and visual function.

Group	At 4.5 weeks old		At 36 weeks old	
	n	BG (mg/dl)	n	BG (mg/dl)
Control	8	203 ± 11.09	7	207 ± 7.27
Diabetic	8	477 ± 29.65*	7	1065*

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

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



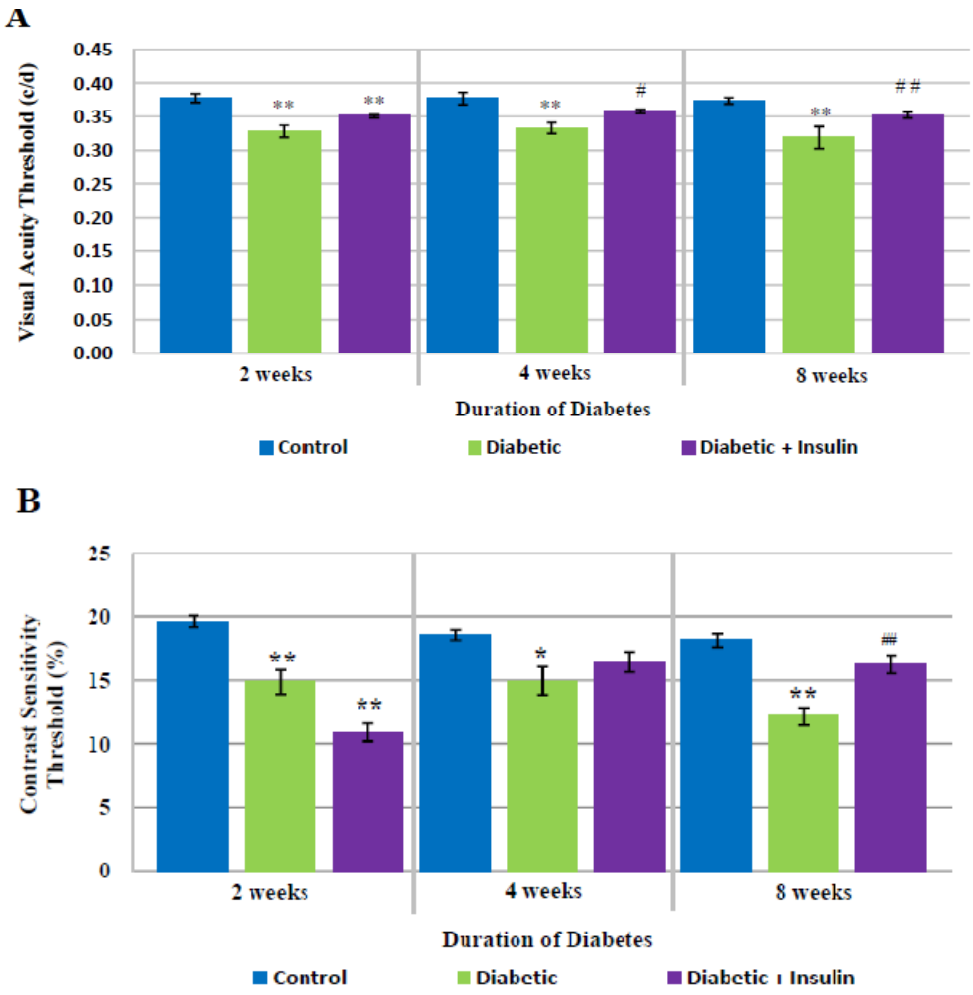
In further studies we used subcutaneous insulin implants (Linshin™,Canada) to reverse the diabetic state in $Ins2^{Akita}$ mice. Figure 4 shows results of a study in which visual function was measured in young mice with continuous subcutaneous insulin delivery (2 units/24 hours). Visual function was measured before, and during the insulin implant, and also measured 5 weeks after the implant, when most of the insulin would have been depleted. The table shows that blood glucose was initially high in both diabetic groups (4.5 weeks of age) and was normalized 1-week after the insulin implant. Five weeks after the implant, blood glucose had returned to high levels, indicating that the insulin was depleted. Figure 4A shows that an initial deficit in acuity in both diabetic groups (left panel) was reversed by the insulin implant (middle panel). The visual acuity was significantly higher in the insulin treated diabetic group compared to the untreated group, both during (middle panel) and after (right panel) insulin treatment (##p<0.01).

Group	At 4.5 Weeks Old		1 Week Post-Insulin Implant (4 weeks of Diabetes)		5 Weeks Post-Insulin Implant (8 weeks of Diabetes)	
	n	BG (mg/dl)	n	BG (mg/dl)	n	BG (mg/dl)
Control	5	193 ± 3.91	5	172 ± 12.97	5	219 ± 15.32
Diabetic	5	380 ± 14.32**	5	613 ± 95.92**	5	740 ± 137.09**
Diabetic + Insulin	6	340 + 12.85**	6	109 ± 15.13##	5	462 + 65.90*

* 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

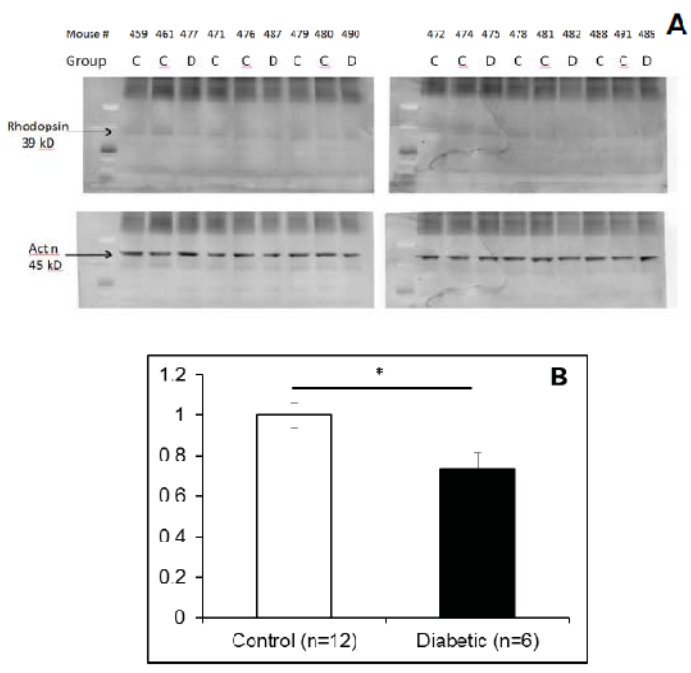


For contrast sensitivity results (Figure 4B), there was a significant deficit in both diabetic groups after 2 weeks of diabetes (left panel). The deficit as not significantly reversed by insulin (middle panel), however there was a significant increase in contrast sensitivity after 8 weeks, when the insulin was depleted.

These data show that the spatial frequency threshold (visual acuity) and contrast sensitivity are compromised in Ins2^{Akita} diabetic mice soon after the onset of diabetes, and persist throughout the duration of diabetes. Insulin therapy can partially reverse the visual function deficits, although the affect may not be immediate. Furthermore, the protective effect of insulin appears to be prolonged, after the insulin has worn off, and the loss of function does not correlate with blood glucose. The mechanisms of this delayed rescue with insulin is unclear.

Aim 2

Retinas from the mice in figures 1 and 2 were used in an attempt to measure synaptic protein content. Our previous work showed that presynaptic neurotransmitter vesicle-associated proteins, synaptophysin, synapsin1, VAMP2 and SNAP25, are depleted in the retinas of STZ-diabetic rats, within the first 1-2 months of diabetes. While this finding has been replicated several times we had greater difficulty confirming these measures in Ins2^{Akita} mice. New primary antibodies were purchased because the ones used in rats were found not to work on the mouse tissue. The results of western blots on mouse tissue were highly variable between animals and difficult to quantify, leading to ambiguous results. While blots showed a trend for reduction in all four synaptic proteins of interest, the differences were not large enough to be statistically significant, leading to doubt about the statistical power in these studies. As an alternative, we quantified photoreceptor-associated proteins rhodopsin, transducin and phosphodiesterase, in these samples, and found that the relative content of rhodopsin was reduced in three separate groups of diabetic Ins2^{Akita} mice (figure 5), compared to controls, but transducing and phosphodiestrase were not altered in the same samples (data not shown). These data suggest that rhodopsin is depleted from rod photoreceptors in the diabetic Ins2^{Akita} mice, rather than an overall reduction in the rod photoreceptor cell population.



Regulation of rhodopsin protein content has not previously been considered as a component of diabetic retinal dysfunction. These data suggest that there may be an

early dysregulation of the photoreceptor pigment in Ins2^{Akita} mice.

In further studies we established protocols to measure the scotopic (dark adapted) electrophysiological response (ERG) of the Ins2^{Akita} mouse retina, using new recording equipment (Diagnosys™, Espion2). Several components of the ERG were measured, including the amplitude and implicit time of the a- and b-waves, the summated oscillatory potentials, and the scotopic threshold response, which is a low intensity response thought to originate from the inner retinal ganglion and amacrine cells. All electrophysiological studies were performed in mice that were 11 weeks of age, so they had diabetes for about 1.5 months.

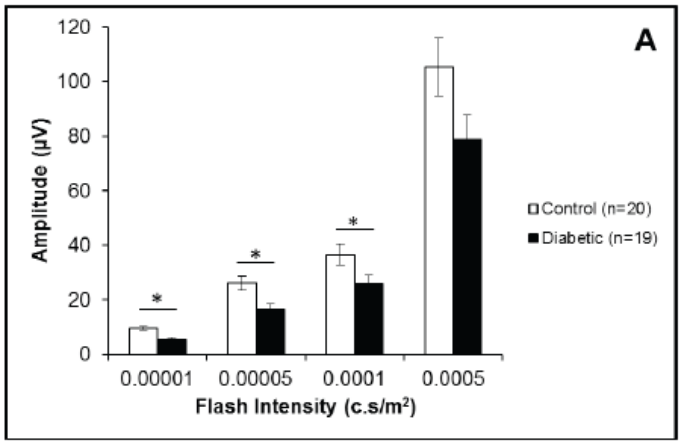
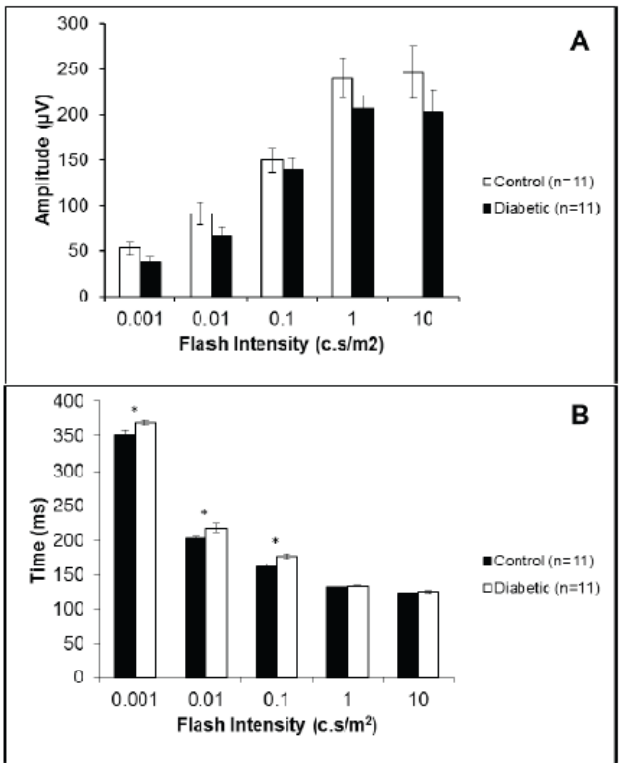


Figure 6 shows data for the amplitude of the scotopic threshold response, indicating a significant reduction in this response in the diabetic mice. There was no significant change in the implicit time of this response (data not shown).

While there are a small number of papers showing reductions in the scotopic threshold response in humans and rats with diabetes, this is the first time that a change in this response has been demonstrated in diabetic mice. The result suggests an early loss of inner retina function due to diabetes.

As predicted by results from STZ-diabetic rats and humans with diabetes, there were no significant deficits in the a-wave, which is derived from rod photoreceptors (data not shown). Also the amplitude and implicit time of the b-wave were not altered by diabetes (data not shown), suggesting no slowing of the glutamatergic neurotransmitter response in these mice. These parameters may be expected to change after longer durations of diabetes, based on results from STZ-diabetic rats and humans.

Finally, there was no significant reduction in the summated amplitude of the oscillatory potentials (Figure 7A), which are thought to be derived from amacrine cells and are a series of sine waves carried on the b-wave. The implicit time of the oscillatory potentials was significantly increased in Ins2^{Akita} diabetic mice compared to controls (Figure 7B). This is considered to be one of the most robust effects of diabetes on the ERG response, and these data conform to other recordings from humans, rats and mice with diabetes.



The data from Aim 2 show that some parameters of the ERG, specifically the implicit time of the oscillatory potentials and the amplitude of the scotopic threshold response, are altered within the first 2 months of diabetes in the Ins2^{Akita} diabetic mouse. These deficits indicate primarily inner retina dysfunction, affecting the amacrine and ganglion cell response. Electrophysiological measures of photoreceptor function were not altered, despite a reduced expression of rhodopsin, indicating that the loss of photopigment may have no detectable functional consequences.

Overall, the results of this project establish that the Ins2^{Akita} mouse can be used to model some of the early functional deficits in vision, associated with diabetes, including spatial frequency threshold (acuity), contrast sensitivity; and electrophysiological parameters such as the oscillatory potentials and scotopic threshold response. When possible, we performed correlational analyses between functional measures and blood glucose or cell death, but found that none of these parameters were significantly associated; implying that early loss of function may not be directly due to cell death or hyperglycemia. Insulin studies also suggest that functional deficits may be reversible to some extent, at least during the first 1-2 months of diabetes.