MOLECULAR MECHANISMS OF RETINAL
SYNAPTOPHYSIN LOSS IN EXPERIMENTAL DIABETES

A Dissertation in
Neuroscience
by
Travis Savio D’Cruz

© 2013 Travis D’Cruz

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2013
The dissertation of Travis D'Cruz was reviewed and approved* by the following:

**Alistair J. Barber**  
Associate Professor of Ophthalmology and Cellular and Molecular Physiology  
Dissertation Advisor  
Chair of Committee

**Kirsteen N. Browning**  
Assistant Professor of Neural and Behavioral Sciences

**Scot R. Kimball**  
Professor of Cellular and Molecular Physiology

**Ralph L. Keil**  
Associate Professor of Biochemistry and Molecular Biology

**Patricia Grigson-Kennedy**  
Professor of Neural and Behavioral Sciences  
Director of Neuroscience Graduate Program

*Signatures are on file in the Graduate School
Diabetic retinopathy (DR) is a complication of diabetes that compromises the structure and function of the retina causing deficits in vision. It is the leading cause of new cases of blindness in working-age Americans and affects 4.2 million people with diabetes aged 40 years or older. Clinically, DR is diagnosed by identifying abnormalities in retinal vasculature. DR also has a neuronal component that can go undetected during a clinical examination due to the transparency of neural tissue in the retina.

The neurodegenerative process in DR includes structural and functional changes in retinal neurons that lead to symptoms such as delayed dark adaptation and deficits in visual acuity and contrast sensitivity. Changes in the composition of synapses can be important factors in compromised retinal function. Previous studies have shown that synaptophysin, a presynaptic protein, is reduced in retinas of experimentally diabetic rats. This protein is found exclusively in both plexiform layers and may be particularly important for retinal function given its role in synaptic vesicle fusion and recycling. The aim of this research was to test the hypothesis that diabetes alters specific mechanisms that regulate synaptophysin content in the rat retina.

The mRNA translation, N-glycosylation, and degradation of synaptophysin were identified as potential mechanisms. Male Sprague-Dawley rats, made diabetic by
a single intraperitoneal STZ injection, were used for this research. mRNA translation was measured using a $^{35}$S-methionine labeling assay followed by synaptophysin immunoprecipitation and autoradiography. A pulse-chase study was used to determine the depletion of newly-synthesized synaptophysin. Depletion of total synaptophysin was determined after treatment with cycloheximide. N-glycosylation was studied by quantifying mannose-rich (immature) synaptophysin and measuring the activity of $\alpha$-mannosidases responsible for de-mannosylating the protein.

The $^{35}$S-methionine labeling assay showed that mRNA translation of retinal synaptophysin was significantly increased by diabetes. In the pulse-chase study, diabetes significantly accelerated the degradation of newly-synthesized synaptophysin in the rat retina. Total synaptophysin degradation also sped up compared to control, but only after 2 months of STZ-diabetes. The results from the glycosylation studies indicated that newly glycosylated mannose-rich synaptophysin accumulates in retinas of diabetic rats due to reduced activity of $\alpha$-mannosidases responsible for de-mannosylation. Inhibition of $\alpha$-mannosidases in healthy retinas specifically accelerated depletion of mannose-rich synaptophysin, mirroring the effect of diabetes on the newly-synthesized protein. Collectively, these findings suggest that diabetes disrupts N-glycosylation of synaptophysin to trigger an early degradation of its immature form and cause a reduction in synaptophysin protein content.
This study specifically characterizes diabetes-induced irregularities in retinal synaptophysin metabolism, and further suggests an abnormality in post-translational glycosylation that could affect other N-glycosylated proteins. In doing so, this work provides new knowledge on the molecular deficits that may contribute to retinal dysfunction during diabetes. Thus, the findings reported here are important contributions to the larger goal of rescuing functional changes in the retina, and ultimately preserving vision during the early stages of DR.
Chapter 1 Literature Review

1.1. Overview of Diabetic Retinopathy ................................................................. 2
  1.1.1. Diabetes and Its Prevalence ..................................................................... 2
  1.1.2. Diabetes and the Retina .......................................................................... 3
    1.1.2.1. Occurrence of Diabetic Retinopathy ............................................. 3
    1.1.2.2. Detection of Diabetic Retinopathy .............................................. 4
  1.1.3. Treatment of Diabetic Retinopathy ........................................................ 5
    1.1.3.1. Current Treatments ....................................................................... 5
    1.1.3.2. New Therapies ............................................................................ 7

1.2. Cellular Changes in Diabetic Retinopathy ...................................................... 12
  1.2.1. Vascular Pathology ............................................................................... 12
  1.2.2. Neuronal Component .......................................................................... 13

1.3. Rodent Models of Diabetes ............................................................................. 14
  1.3.1. Streptozotocin Rat as a Model of Diabetes ........................................... 14
  1.3.2. Vision Impairment in Diabetic Rodents ............................................... 15

1.4. An Overview of Presynaptic Proteins in Diabetes ........................................... 16
  1.4.1. In the Pancreas ..................................................................................... 17
  1.4.2. In the Brain .......................................................................................... 20
  1.4.3. In the Retina ......................................................................................... 23

1.5. Synaptophysin ................................................................................................ 25
  1.5.1. Function and Interaction ...................................................................... 25
  1.5.2. The Metabolism of Synaptophysin ....................................................... 28
  1.5.3. Retinal Synaptophysin and Diabetes ................................................... 29

1.6. Rationale and Hypothesis ............................................................................. 32

Chapter 2 Materials and Methods

2.1. Animals ........................................................................................................ 37

2.2. Experimental Procedures ............................................................................. 38
  2.2.1. Western blot Analysis .......................................................................... 38
  2.2.2. Synaptosome Isolation ........................................................................ 39
  2.2.3. Autoradiography and Scintillation Counting ...................................... 41
  2.2.4. Cell Death ELISA ............................................................................... 42
  2.2.5. ATP assay ............................................................................................ 43
  2.2.6. Immunoprecipitation .......................................................................... 44
  2.2.7. Radioactive Pulse-Chase and CHX Chase ......................................... 44
  2.2.8. PNGase F and Endo H Treatment ...................................................... 45
  2.2.9. α-Mannosidase Activity Assay ............................................................ 45
  2.2.10. Swainsonine Treatment of Retinal Explants and Degradation Studies 46

Chapter 3 Diabetes Increases Retinal Synaptophysin mRNA Translation

Translation
Chapter 4 Diabetes Disrupts Post-Translational Processing and Accelerates Degradation of Retinal Synaptophysin 70

4.1. Introduction ................................................................................................................ 71
4.2. Degradation of Synaptophysin .................................................................................. 75
4.2.1. Depletion of \( {}^{35}\)S-Synaptophysin is Accelerated after 1 and 2 Months of STZ-Diabetes........................................................................................................... 75
4.2.2. Depletion of Total Synaptophysin is Accelerated after 2 Months, but not 1 Month, of STZ-Diabetes ............................................................................................................. 76
4.2.3. Proteasome Subunits are Detectable in Whole Retina and Isolated Synaptosomes ................................................................................................................................. 78
4.3. Synaptophysin is N-Glycosylated in Retinas of Control and STZ-Diabetic Rats ...... 79
4.4. Mannose Rich Retinal Synaptophysin is Increased after 1 Month, but Not 2 Months, of STZ-Diabetes ............................................................................................................... 81
4.5. Depletion of Mannose Rich Retinal Synaptophysin is Not Altered by STZ-Diabetes ................................................................................................................................. 82
4.6. Discussion ................................................................................................................. 85

Chapter 5 Reduced \( \alpha \)-Mannosidase Activity Alters Post-Translational Processing and Expression of Retinal Synaptophysin 94

5.1. Introduction ................................................................................................................ 95
5.2. Retinal Golgi \( \alpha \)-Mannosidase II Content is Unaltered in STZ-Rats ....................... 98
5.3. Retinal \( \alpha \)-Mannosidase Activity is reduced in STZ-Rats ....................................... 98
5.4. Inhibition of Retinal \( \alpha \)-Mannosidase ....................................................................... 99
5.4.1. Swainsonine Inhibits \( \alpha \)-Mannosidase Activity in Retinal Lysates ................. 99
5.4.2. \( \alpha \)-Mannosidase Inhibition Reduces Retinal Synaptophysin Content .......... 100
5.4.3. \( \alpha \)-Mannosidase Inhibition Accelerates Mannose-Rich Synaptophysin Degradation in the Rat Retina ................................................................. 102
5.5. Discussion ................................................................................................................. 104

Chapter 6 Closing Discussion 109

6.1. Diabetes Alters Mechanisms of Retinal Synaptophysin Metabolism ....................... 110
6.2. Implications for Vision Loss ..................................................................................... 116
List of Figures

Figure 1. Overview of synaptophysin’s role at the presynaptic terminal .................................. 27
Figure 2. Diabetes reduces the content of retina synaptophysin ........................................... 32
Figure 3. Gap in knowledge ................................................................................................. 35
Figure 4. Synaptosomes were isolation by differential centrifugation ............................... 40
Figure 5. Synaptic protein enrichment in the synaptosome (P2) fraction .......................... 41
Figure 6. Assay setup for measuring mRNA translation in retinal explants .................... 51
Figure 7. Validation of the radioactive amino acid incorporation assay .......................... 54
Figure 8. Diabetes transiently increases retinal mRNA translation .................................. 56
Figure 9. Diabetes does not alter eIF content in retinas and retinal synaptosomes .......... 58
Figure 10. Diabetes reduces 4E-BP1 hyperphosphorylation in rat retinas ..................... 59
Figure 11. Diabetes reduces retinal synaptophysin content ............................................. 61
Figure 12. Diabetes increases retinal synaptophysin mRNA translation ....................... 62
Figure 13. The role of 4E-BP1 hyperphosphorylation in mRNA translation .................... 69
Figure 14. Degradation Assay Set up .................................................................................. 74
Figure 15. Diabetes accelerates newly-synthesized retinal $^{35}$S-synaptophysin degradation .......................................................................................................................... 76
Figure 16. Degradation of total retinal synaptophysin is accelerated after 2 months of diabetes ...................................................................................................................... 77
Figure 17. Proteasome subunits are present in whole retina and synaptosomes ............. 79
Figure 18. All retinal synaptophysin is N-glycosylated in diabetic and control rats .......... 80
Figure 19. Diabetes induces mannose rich (endo H-sensitive) retinal synaptophysin accumulation ......................................................................................................................... 82
Figure 20. Diabetes does not alter the degradation of endo H-sensitive synaptophysin in the retina ......................................................................................................................... 84
Figure 21. Mannose-rich (immature) N-glycosylated proteins are sensitive to Endo H deglycosylation ..................................................................................................................... 90
Figure 22. Synaptophysin degradation was measured in control retinas during α-mannosidase inhibition.................................................................................................................. 97
Figure 23. Diabetes reduces α-mannosidase activity in the retina .................................... 99
Figure 24. Swainsonine inhibits α-mannosidase activity in retinal lysates ....................... 100
Figure 25. Inhibition of α-mannosidase activity reduces total synaptophysin and increases endo H-sensitive synaptophysin content in the rat retina ........................................ 101
Figure 26. Inhibition of α-mannosidase accelerates the degradation of endo H-sensitive synaptophysin .................................................................................................................. 103
Figure 27. Diabetes alters mechanisms of retinal synaptophysin metabolism ............... 115
Figure 28. ER stress markers in the retina from diabetic and control rats ....................... 126
Figure 29. An overview of the ER stress response pathways. .......................................................... 127
List of Tables

Table 1. Abbreviated version of the ETDRS scale used in the DCCT and UKPDS .............. 4
Table 2. Weight and blood glucose of rats at time of sacrifice ................................................ 38
Table 3. Antibodies ............................................................................................................... 39
Table 4. Experimental outcomes after 1 and 2 months of diabetes ................................. 116
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E-BP1</td>
<td>4E-binding protein 1</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End Products</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CNT</td>
<td>Control</td>
</tr>
<tr>
<td>CNX</td>
<td>Calnexin</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factors</td>
</tr>
<tr>
<td>Endo H</td>
<td>Endoglycosidase H</td>
</tr>
<tr>
<td>Endo H-Syn</td>
<td>Endo H-sensitive Synaptophysin</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER associated degradation</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment of Diabetic Retinopathy Scale</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>2 N-Acetylg glucosamine</td>
</tr>
<tr>
<td>GRP78</td>
<td>78 kDa Glucose-Regulated Protein</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin Growth Factor</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol-Requiring Enzyme 1</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-Term Potentiation</td>
</tr>
<tr>
<td>Met/Cys</td>
<td>Methionine/Cystine</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-Proliferative Diabetic Retinopathy</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric Acid Precipitation</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR Like ER Kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>Peptide-N-Glycosidase F</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosomal Associated Protein 25</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SV</td>
<td>Synaptic Vesicle</td>
</tr>
<tr>
<td>Sw</td>
<td>Swainsonine</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin Proteasome System</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>VAMP-2</td>
<td>Vesicle Associated Membrane Protein 2/Synaptobrevin II</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>XBP-1</td>
<td>X-Box Binding Protein 1</td>
</tr>
</tbody>
</table>
Acknowledgements

First and foremost, I would like to thank my family and friends for their continued moral support throughout my time as a graduate student pursuing a doctoral degree. Second, I would like to extend my most sincere gratitude to Dr. Alistair Barber along with my other committee members, Dr. Ralph Keil, Dr. Scot Kimball, and Dr. Kirsteen Browning for their patience, guidance, excellent mentorship, and vital contribution towards my development as a scientist. I would also like to thank the colleagues I have worked with through the years, particularly; Dr. William Robinson, Brittany Weibley, Wade Edris, and Dr. Paul Titchenell. Their several contributions have greatly benefited my experience in graduate school.

Lastly, I wish to thank Lydia Kutzler for her technical assistance with the development of the $^{35}$S-met/cys incorporation assay and eIF western blot analysis and Brittany Weibley for her assistance with the STZ injections and maintenance of rats used in this study. The projects in this dissertation were funded by grants from the Juvenile Diabetes Research Foundation, the American Diabetes Association, and the PA Lions Sight Conservation and Eye Research Foundation.
Chapter 1

Literature Review
1.1. Overview of Diabetic Retinopathy

1.1.1. Diabetes and Its Prevalence

Diabetes mellitus is a chronic metabolic disease in which a person has elevated blood glucose levels, either because the body does not produce enough insulin (Type 1 diabetes), or because cells do not respond to the insulin that is produced (Type 2 diabetes). Type 1 diabetes is induced by autoimmune destruction of insulin-producing pancreatic β-cells and accounts for approximately 10% of the estimated 346 million diabetes mellitus cases worldwide (WHO, 2011). The remaining 90% of cases are attributed to Type 2 diabetes. Genetics and environment play an important role in the development of the disease. The influence of genetics on the initiation of Type 1 diabetes has been extensively studied and as many as eighteen different chromosome regions showed some positive evidence of linkage to the disease (Davies et al., 1994). On the other hand, research has found that factors such as obesity have a strong contribution to the development of Type 2 diabetes (Leong and Wilding, 1999; Mokdad et al., 2003). While Type 1 diabetes results in the loss of insulin production, Type 2 diabetes results in hyperinsulinemia in the initial stages as the pancreas attempts to compensate for decreased insulin sensitivity. Increased insulin resistance can reduce insulin secretion, resulting in decreased insulin signaling and hyperglycemia (Stumvoll et al., 2005; Pietropaolo et al., 2007).
1.1.2. Diabetes and the Retina

1.1.2.1. Occurrence of Diabetic Retinopathy

The United Kingdom Prospective Diabetes Study (UKPDS) reported that 63% of patients do not have any retinopathy during the initial diagnosis of Type 2 diabetes. After 6 years of diabetes, however, 22% of these developed some form of retinopathy, while 29% of patients with retinopathy at diagnosis progressed by two or more steps on the Early Treatment of Diabetic Retinopathy Scale (ETDRS; Table 1) (Stratton et al., 2001). In Type 1 diabetes, some degree of DR is seen in approximately 95% of patients within 20 years of diagnosis, making DR the leading cause of new cases of blindness in working adults (Amos et al., 1997; Davis et al., 1998). More recent reports from The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) show that between 2005 and 2008, 4.2 million people with diabetes above the age of 40 had DR, and of these, 0.7 million (4.4% of patients with diabetes) had advanced DR that could lead to severe vision loss (NIDDK Fact Sheet, 2008). An epidemiological study in Type 1 diabetic patients, monitored over a period of 25 years showed that an increased risk of visual impairment is significantly associated with more severe retinopathy (Klein et al., 2010).
Table 1. Abbreviated version of the ETDRS scale used in the DCCT and UKPDS

<table>
<thead>
<tr>
<th>Level</th>
<th>Severity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>No retinopathy</td>
<td>Diabetic retinopathy absent</td>
</tr>
<tr>
<td>20</td>
<td>Very mild NPDR (Non-proliferative DR)</td>
<td>Micro-aneurysms only</td>
</tr>
<tr>
<td>35</td>
<td>Mild NPDR</td>
<td>Cotton wool spots, and/or mild retinal hemorrhages</td>
</tr>
<tr>
<td>43</td>
<td>Moderate NPDR</td>
<td>Moderate retinal hemorrhages</td>
</tr>
<tr>
<td>47</td>
<td>Moderate NPDR</td>
<td>Severe retinal hemorrhages, or venous beading in one quadrant</td>
</tr>
<tr>
<td>53</td>
<td>Severe NPDR</td>
<td>Severe retinal hemorrhages in four quadrants, or venous beading in at least two quadrants</td>
</tr>
</tbody>
</table>

1.1.2.2. Detection of Diabetic Retinopathy

The primary technique for diagnosing DR in the clinic is ophthalmoscopy (funduscopy), which detects vascular abnormalities including microaneurysms and hemorrhages (Klein et al., 1998). More recently, adaptive optics scanning laser ophthalmoscopy (AOSLO), a technique that simultaneously assesses several features of the retinal capillary network, has enabled the detection of disruptions in arteriovenous channels in the retina, even before the onset of obvious vascular deficits (Tam et al., 2011).
Even with the availability of these advanced techniques, any existing neuropathology proves difficult to observe due to the transparency of neural tissue in the retina. Moreover, clinical symptoms resulting from any neural deficit are not easily apparent to the patient at the earliest stages of the disease. Consequently, DR is diagnosed when vascular lesions are detectable during a fundus exam or by fluorescein isothiocyanate (FITC) angiography.

1.1.3. Treatment of Diabetic Retinopathy

1.1.3.1. Current Treatments

Prior to necessary clinical treatment, the best form of prevention is tight glycemic control and intensive insulin therapy (DCCT, 1993, 1995a, b, 1998). The primary and most efficient approach to inhibit the progression of DR that threatens vision is laser photocoagulation, a process during which laser burns are administered on specific locations (focal photocoagulation) or over a wide area (scatter/pan-retinal photocoagulation) of the retina to prevent further neovascularization. This procedure is performed on the peripheral regions of the retina in order to preserve the central retina, and hence central vision (Yoon et al., 1996). However, laser photocoagulation has significant disadvantages. First, the treatment can only be administered in advanced stages of DR. Second, damage from the laser burns results in inevitable loss of vision in the peripheral retina.
Electroretinogram (ERG) studies in treated patients showed increases in implicit time and decreases in amplitude, compared to pretreatment values (Greenstein et al., 2000). Laser energy is absorbed primarily by the pigment epithelium and the adjacent photoreceptor layer in the outer regions of the retina, suggesting functional changes in addition to physical damage caused by the treatment. Finally, photocoagulation is only a temporary solution for 50% of patients who receive it. In these patients, growth of new blood vessels into the retinal space will require more laser burns, and this repetitive treatment will continue to damage the retinal tissue. In light of this drawback, the development of additional, and more importantly, earlier therapy is necessary to prevent vision loss before an advanced stage of the disease is reached. The primary challenge, however, is identifying the changes during the early stages of DR as they often go unnoticed due to their subtlety, transparency of retinal tissue, and the patient's difficulty in describing the symptoms.

Because DR can progress to irreversible stages with relatively few symptoms, the most favorable time for treatment is before visual acuity is impaired. Studies have confirmed that screening patients as early as possible results in better treatment outcomes. The DCCT studies have further shown that prevention of complications due to early intensive diabetes control persist for years, even with subsequent hyperglycemia. Therefore, regular screening for DR can potentially delay visual deficits and also reduce the cost of treatment.
1.1.3.2. New Therapies

Recent studies have provided insight into potential therapeutic targets and many compounds have shown promise in the laboratory setting (Sima et al., 1992; Aiello et al., 2006; El-Remessy et al., 2006). Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that has been implicated in normal vascular development, ovulation, and tumor angiogenesis (Plate et al., 1992; Shweiki et al., 1995). Studies in the human eye have reported that elevated levels of vitreous and aqueous VEGF strongly correlate with retinal-ischemia-associated neovascularization observed in DR (Adamis et al., 1994). In recent years, anti-VEGF agents have emerged as new treatment approaches. Pegaptanib (Macugen), ranibizumab (Lucentis), and bevacizumab (Avastin) are currently available anti-VEGF agents. Ranibizumab and bevacizumab block all VEGF isoforms, while Pegaptanib only blocks the VEGF\textsuperscript{165} isoform (Yasukawa et al., 2004).

Several clinical studies have reported promising results with VEGF inhibitors for the treatment of DR. Studies using ranibizumab and bevacizumab have found that retinal neovascularization is rapidly halted, visual acuity is improved, and retinal thickness is decreased in DR patients, including those that are unresponsive to conventional therapies (Chun et al., 2006; Oshima et al., 2006; Diabetic Retinopathy Clinical Research et al., 2007; Arevalo et al., 2009). In a double-blind trial that included 172 patients with diabetes-induced macular
edema, pegaptanib treatment had better outcomes with regard to visual acuity and reduced retinal thickness. More importantly, these subjects were also less likely to need additional panretinal photocoagulation at follow-up (36 weeks post-treatment) (Cunningham et al., 2005). Intravitreal injection is an effective means of delivering anti-VEGF drugs to the retina. However, endophthalmitis, traumatic injury of the lens, and retinal detachment are the most common adverse effects of this technique (Rosenfeld et al., 2006). While the incidence of serious complications is low, patients who require multiple treatments over many years may be at significant risk for these adverse effects.

The role of protein kinase C (PKC) and PKC inhibitors in the development of vascular abnormalities during DR have also been the focus of recent research. Specifically, PKC-β has been identified as the predominant isoform activated in vascular tissue in response to hyperglycemia (Inoguchi et al., 1992; Koya and King, 1998). This kinase has an important role in regulating endothelial cell permeability and is an important signaling component for VEGF (Nagpala et al., 1996; Xia et al., 1996). Transgenic animals overexpressing PKC-β in vascular tissues develop retinal abnormalities similar to those seen in human patients with DR (Takahara et al. 1999). Inhibition of all PKC isoforms is likely to cause unacceptable levels of toxicity in humans. This was the case with PKC412, an inhibitor of the α, β, and γ isoforms of PKC (Seo et al., 1999; Fabbro et al., 2000). This compound showed promise in animal models of neovascularization by inhibiting ischemia-induced angiogenesis as well as retinal vessel formation.
during development (Seo et al., 1999). However, early pharmacodynamic studies resulted in some adverse outcomes, raising doubts as to whether this compound will be useful for treating human patients (Propper et al., 2001). Ruboxistaurin, a specific inhibitor of PKC-β, on the other hand, has been shown to prevent and reverse microvascular complications in animal models of diabetes (Ishii et al., 1996). Further, it blocks neovascularization associated with retinal ischemia, and inhibits VEGF, reducing retinal permeability and endothelial cell growth (Aiello et al., 1997; Danis et al., 1998). In patients with non-proliferative DR, ruboxistaurin reversed retinal blood flow abnormalities and was accompanied with minimal side effects (Aiello LP, 1999), thus becoming a very promising drug for the treatment of DR. Most recently, a combined analysis of two phase 3 trials determined that ruboxistaurin significantly reduced sustained moderate visual loss (defined as a 15-letter or more reduction from baseline visual acuity sustained for a patient’s last 6 months of study participation) by 50% compared to placebo (Sheetz et al., 2013).

In the presence of high glucose, carbohydrates interact with protein side chains in a nonenzymatic fashion to subsequently form an excessive amount of advanced glycation end products (AGEs) (Brownlee et al., 1984; Friedman, 1999). This event has been proposed as another biochemical link between diabetes and the development of microvascular complications. AGEs may affect such functions as enzyme activity, binding of regulatory molecules, and susceptibility of proteins to proteolysis (Brownlee et al., 1984). In vitro, the AGE
and AGE-specific receptor interaction has been associated with oxidative stress and the activation of nuclear factor-κB, leading to activation of several proinflammatory cytokines (Singh et al., 2001). This may result in damage to vascular endothelial cell structure. Investigations into preventive therapies for vascular complications caused by diabetes include efforts to reduce AGEs in the absence of maintaining normal blood glucose levels (Friedman, 1999). Compounds such as aminoguanidine, a diamine oxidase and nitric oxide synthase inhibitor that reduces levels of AGEs by interacting with 3-deoxyglucosone, have been successful at blocking AGE accumulation in the retinal capillaries of diabetic rats (Hammes et al., 1991). The reduction in AGE accumulation was also associated with a reduced number of acellular capillaries and pericyte loss (Hammes et al., 1991). In another rodent model of DR, aminoguanidine reduced retinal oxidative stress and PKC activity caused by diabetes (Kowluru et al., 2000). While recent work has suggested that aminoguanidine is unable to prevent endothelial and microcirculation abnormalities in primate models of Type 1 diabetes (Brooks et al., 2008), recent studies in STZ–rats suggest that the inhibitor effectively clears AGEs in the retina and restores hyperemia (flickering light-induced dilation of retinal arterioles and increased retinal blood flow) (Mishra and Newman, 2011; Luo et al., 2012) Hence, the use aminoguanidine for treatment of DR remains controversial.

Another promising area of new therapies against DR is oxidative stress and use of antioxidant compounds. Reactive oxygen species (ROS) have been implicated
in the development of diabetic complications (Giugliano et al., 1996). The suggested role of ROS in the biochemical processes such as PKC activation pathways (Nishikawa et al., 2000) leading to microvascular damage has driven research towards antioxidants as preventive therapy (Bursell et al., 1999). Tocopherol, a lipid-soluble antioxidant, inhibits hyperglycemia-induced PKC activation and prevents retinal abnormalities in diabetic rats (Kunisaki et al., 1995). Retinal blood flow was significantly increased with tocopherol treatment in patients with Type 1 diabetes with little or no retinopathy (Bursell and King, 1999). More recently, the anti-oxidative properties of hesperetin, a bioflavonoid, was tested in diabetic rat retinas. Hesperetin-treated diabetic rats showed significantly lower levels of retinal pro-inflammatory cytokines as compared to untreated diabetic rats (Kumar et al., 2013). Treatment also had an inhibitory effect on caspase-3 expression suggesting an effective therapy for the prevention of diabetes induced neurovascular complications such as DR (Kumar et al., 2013). These encouraging results warrant additional evaluation of such antioxidants for treatment and prevention of DR in humans.

Despite significant strides in research on alternative therapies, panretinal photocoagulation remains the gold standard of treatment for DR. Continued investigations on molecular pathways affected by the disease will eventually make way for more effective therapies.
1.2. Cellular Changes in Diabetic Retinopathy

1.2.1. Vascular Pathology

In order to understand the symptoms diagnosed in DR, the cellular mechanisms leading to these symptoms have been extensively researched. Fundus images show that the vasculature of patients with DR is significantly compromised. In the non-proliferative form of the disease, the appearance of aneurysms and fluid/protein deposits around the macular is observed. The resulting inflammation causes the most common form of non-proliferative DR, macular edema. Proliferative DR is characterized by angiogenesis of vasculature into the retinal space.

Hemorrhaging of the blood vessels also leads to disrupted vision and discomfort. At the cellular level, the presence of acellular capillaries (devitalized basement membrane remnants), loss of pericytes (mesenchymal-like smooth muscle cells associated with the walls of small blood vessels), leukostasis (abnormal aggregation of white blood cells at the vasculature) and tight junction breakdown are some of the elements responsible for the vascular pathology seen in the diabetic retina (Kern and Engerman, 1996; Arevalo, 2007).
1.2.2. Neuronal Component

Studies in rodent models suggest that diabetes causes neurodegeneration in the retina. In situ DNA terminal dUTP nick end labeling (TUNEL) identified a 10-fold increase in the frequency of apoptosis in whole-mounted rat retinas after 1, 3, 6, and 12 months of diabetes (Barber et al., 1998). In this study, most apoptotic cells were not associated with blood vessels suggesting that retinal neural cell death is increased in diabetes. After 7.5 months of hyperglycemia, the thickness of the inner plexiform layer was reduced by 22% of control and the inner nuclear layer was reduced by 14% of controls (Barber et al., 1998). A significant decrease in the thickness of the whole retina and the inner and outer nuclear layers was also seen in 10-week diabetic mice (Martin et al., 2004). In retinas from rats after 2, 8, and 16 weeks of experimental diabetes, 2-6% of caspase-3 immunoreactive cells were double-labeled for tyrosine hydroxylase immunoreactivity, indicating that amacrine cells were apoptotic. The same study also reported a 20% decrease in the number of cholinergic and a 16% decrease in dopaminergic amacrine cells in retinas from Ins2^-Akita^-diabetic mice, compared with the non-diabetic controls (Gastinger et al., 2006).

Apart from cellular changes, diabetes is known to induce functional changes in retinal neurons that lead to disrupted vision (Ng et al., 2004). Several studies suggest that the neurological function of the retina is compromised in rodents with experimental diabetes, because their ERG and scotopic threshold response
are diminished (Hancock and Kraft, 2004; Kohzaki et al., 2008). Furthermore, behavioral optokinetic studies suggest that diabetic animals have visual deficits (Kirwin et al., 2011; Akimov and Renteria, 2012) (Barber AJ et al, IOVS 2010; 51: ARVO E-abstract 109). In these rodent models, visual function deficits precede vascular lesions in the retina (Bai et al., 2006), suggesting that compromised vision may go undetected in human clinical exams during the early stages of the disease. The molecular disparities that contribute to these early functional abnormalities in the retina are not clearly understood. Further research on diabetes-induced alterations in biochemical pathways that likely maintain retinal function will shed more light on how hyperglycemia may compromise vision during the early stages of the disease.

1.3. Rodent Models of Diabetes

1.3.1. Streptozotocin Rat as a Model of Diabetes

Streptozotocin (STZ) is a naturally occurring toxic compound. It is approved by the U.S. Food and Drug Administration (FDA) for treating metastatic cancer of the pancreatic islet cells. Since it carries a substantial risk of toxicity and rarely cures the cancer, it is only used to reduce tumor size when surgery is not an option (Brentjens and Saltz, 2001). STZ is used as a diabetogenic agent that is selectively taken up into pancreatic β-cells through the high-affinity GLUT 2 glucose transporter, causing cell stress and subsequent β-cell apoptosis (Junod
et al., 1967). The STZ-diabetic rat is commonly used as a rodent model of diabetes. STZ-rats have been used to study several complications of diabetes and have provided us with a better understanding of the cellular and molecular processes that contribute to the development and progression of DR in humans. The basic cellular composition and organization of the mammalian retina is conserved. This allows the comparison of functional changes in humans with diabetes with those observed in STZ-rats. Further, the model has contributed to a variety of findings with regard to vascular, glial and neuronal components of DR (Lieth et al., 1998; Antonetti et al., 2006; Gastinger et al., 2008). The Sprague Dawley (SD) rat was used as the STZ rodent model in this project. Through the use of this model, a greater understanding of diabetes-induced molecular alterations in the neural retina may lead to new therapeutic targets to prevent and/or reverse neuronal dysfunction in an effort to diminish the progress of retinopathy in diabetic patients.

1.3.2. Vision Impairment in Diabetic Rodents

Disrupted retinal function within the first weeks of experimental diabetes in rodents is detectable with ERG studies. The a-wave, the leading edge of which is produced by photoreceptor activity, has a reduced amplitude in 1-month diabetic rodents, indicating retinal neural dysfunction in the early stages of the disease (Aizu et al., 2002; Phipps et al., 2004). In both rats and mice, oscillatory potential amplitudes are consistently depressed and delayed, beginning as early as two
days after the induction of diabetes (Yonemura et al., 1962). The amplitude of the b-wave also decreases after two to four weeks of diabetes in STZ-rats (Aizu et al., 2002). Further, optokinetic tracking studies suggest that tracking thresholds are significantly reduced in 1-month STZ-rats (Kirwin et al., 2011). While there is a distinct variation in ERG changes between strains of diabetic rodents, the broader conclusion to be drawn from these studies is that diabetes compromises the functional integrity of the neural retina, potentially causing significant visual deficits. Taken together, the ERG and behavioral data suggest a broad dysfunction of retinal neurons as well, involving various cell types of the retina. ERG abnormalities in rodents and humans develop in the early stages of diabetes even in the absence of clinically detectable retinopathy (Aizu et al., 2002; Klemp et al., 2005). Hence, rodent models of diabetes are suitable and clinically relevant models for investigating potential mechanisms that contribute to vision loss in human diabetes.

1.4. An Overview of Presynaptic Proteins in Diabetes

Neurotransmitter exocytosis occurs due to fusion of neurotransmitter-containing synaptic vesicles (SV) at the presynaptic nerve terminal. Previously thought to be involved exclusively in neurotransmitter release, presynaptic proteins are known to be essential components of small vesicle transport in several types of cells. In neurons, the synaptic proteins synaptophysin, synaptosomal-associated protein 25 (SNAP-25), and vesicle associated membrane protein 2 (VAMP-2) are known
to interact with each other at the presynaptic terminal membrane to facilitate SV fusion to the plasma membrane (Sollner et al., 1993; Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). Synaptophysin is also present in pancreas from 9 mammalian species- human, dog, mink, cow, rabbit, guinea pig, rat, mouse, and gerbil (Redecker et al., 1991). Co-immunoprecipitation and confocal microscopy studies indicated that VAMP-2, SNAP-25, syntaxin-4 and synaptotagmins are responsible for insulin granule secretion from β cells of the pancreas (Daniel et al., 1999; Spurlin and Thurmond, 2006). Given their presence in different tissue types, several aspects of presynaptic proteins and other molecules associated with their regulation have been studied in diabetes.

1.4.1. In the Pancreas

The release of granules containing insulin in response to glucose stimulation is of particular interest because Type 1 diabetes involves defects in insulin secretion and β cell death. The earliest studies in perfused rat pancreas determined that glucose stimulation results in a characteristic biphasic pattern of insulin secretion (Curry et al., 1968). Mathematical analysis of diabetic humans revealed that the biphasic secretory process consists of a rapid first phase followed by a longer second phase (Cerasi et al., 1974). The first phase results from the rapid fusion of granules that are pre-docked at the plasma membrane. Replenishment after the first phase, with the mobilization, docking and priming of previously non-
releasable granules occur in the second-phase of insulin secretion, at a rate of 5-40 granules per cell per minute (Barg et al., 2002).

Co-immunoprecipitation studies in mouse pancreatic βHC-9 cells and perfusion analyses in islets from syntaxin-4 heterozygous (-/+), knockout mice showed that VAMP-2, SNAP-25, synaptotagmin, and syntaxin-4 are responsible for the first phase of glucose-stimulated insulin secretion from islet β cells (Daniel et al., 1999; Spurlin and Thurmond, 2006). Treatment of pancreatic β cell line (HIT) and primary rat islet cells with botulinum neurotoxins causes SNAP-25 cleavage and subsequently Ca²⁺-dependent insulin granule release is inhibited (Sadoul et al., 1995). VAMP-2 is also reportedly cleaved by pretreatment of insulin-secreting HIT-T15 cells resulting in abolished Ca²⁺-induced insulin release (Regazzi et al., 1995). These data suggest that presynaptic proteins play an important role in the secretion of insulin granules and their function could be compromised during diabetes.

In the diabetic Goto-Kakizaki (GK) rat, a model which develops Type 2 diabetes early in life due to a neonatal deficit in β-cell mass, islet western blot analysis indicates reduced SNAP-25 and VAMP-2 expression (Gaisano et al., 2002). The authors of this study speculated that the reduction in SNARE proteins may have a direct hand in reduced insulin secretion since β cells containing the SNARE complex make up more than 65% of islet endocrine cells. In another study, ³H-proinsulin pulse-chase experiments showed that increased degradation of insulin
in the islets isolated from GK diabetic rats coincides with a decrease in SNAP-25 and syntaxin-1A by approximately 60% compared to controls (Nagamatsu et al., 1999). The insulin deficit is recovered by the over expression of these proteins in the isolated islet cells using recombinant adenovirus gene transduction. Partial reversal of SNARE protein depletion is also seen with phlorizin treatment of the hyperglycemic GK rat (Gaisano et al., 2002). Phlorizin, an inhibitor of renal glucose reabsorption, is used to study normoglycemic control of insulin sensitivity and islet β cell function. It is known to reverse insulin resistance by improving glucose transport in skeletal muscle (Krook et al., 1997; Ling et al., 2001). The depletion of the presynaptic proteins SNAP-25, VAMP-2 and syntaxin-1A caused by hyperglycemia is thought to be partly responsible for reduced insulin granule release from these secretory cells. This is because the partial normalization of the insulin secretory response in islets from phlorizin-treated diabetic rats is accompanied by increased expression of these presynaptic proteins.

Collectively, these data highlight the important role of presynaptic proteins in the pancreatic islet cells. Further, reduced expression of presynaptic proteins in this tissue leads to reduced insulin secretion. While the immediate physiological consequence of such changes is not clearly defined, the underlying message to be drawn from the literature is that deficits in presynaptic protein function, no matter how subtle, could exacerbate the diabetic condition and its complications.
1.4.2. In the Brain

The hippocampus plays a key role in cognition and memory. In a study on Type 1 diabetic children, lower scores on standardized tests for basic skills occurred when blood glucose was poorly controlled (HbA1c >10) suggesting subtle cognitive changes correlate with hyperglycemia (McCarthy et al., 2002). In an effort to understand underlying molecular mechanisms involved in hyperglycemia-induced learning deficits, previous studies have investigated neuronal damage in hippocampi from diabetic rodents and conducted learning tests in the same animals. Indicators of apoptosis such as DNA laddering patterns, elevated Bax/Bcl-x ratio, and increased caspase 3 activity are not present in 2-month diabetic rats but become evident after 8 months (Li et al., 2002). These findings correlate with increased latencies in the Morris water maze test in 8-month diabetic rats, indicative of impaired spatial learning and memory. Observed molecular and behavioral deficits are preceded by significant decreases in hippocampal expression of insulin growth factors (IGF-I, IGF-II) (Li et al., 2002). The reduction in anti-apoptotic action of IGFs and eventual increase in pro-apoptotic factors indicates that hippocampal neurons are vulnerable to damage for an extended period of time that may allow cell death and impair learning and memory.

Diabetic mice and STZ-diabetic rats have reduced long-term potentiation (LTP) in the perforant path of the hippocampus. This was evidenced by longer escape
latencies and failure to establish a direct route to the hidden platform in the water maze test (Stranahan et al., 2008). Since increased neurotransmitter release is one of the key mechanisms of LTP in the hippocampus (Dolphin et al., 1982; Sola et al., 2004), the reduced occurrence of LTP could be explained by a dysfunction in synaptic mechanisms that control neurotransmitter release in the perforant path of diabetic rodents. Further, lowering the levels of adrenal steroid corticosterone in the same insulin-deficient rats and insulin-resistant mice restores LTP at the perforant path and prevents the diabetes-induced impairment of learning and memory (Stranahan et al., 2008). While this demonstrates a pivotal role for corticosterone in correcting impaired synaptic plasticity, these findings also suggest that specific components of synapses may be compromised during hyperglycemia to cause hippocampal dysfunction.

Diabetes also alters the structure and the composition of presynaptic terminals in the hippocampus. Within the first week of STZ-diabetes in Sprague Dawley rats, the total area occupied by the presynaptic mossy fiber terminals (MFT) that form excitatory synaptic contacts with the proximal CA3 apical dendrites is increased. Moreover, SVs in the MFTs of diabetic rats are significantly depleted (Magarinos and McEwen, 2000). Synaptophysin expression is increased in the nerve terminals of the hippocampus of rats in as early as two weeks of STZ-diabetes (Gaspar et al., 2010). A second study suggests that one week of STZ-diabetes in Sprague Dawley rats increases synaptophysin expression specifically in the CA3 region of the hippocampus, and this effect is accompanied by increases in
radioimmunolabeling of MAP-2, a marker for hippocampal dendrites, and PSD-95 expression in the same region (Grillo et al., 2005). Increased synaptophysin could be a result of increased branching of the presynaptic MFT. It can be further speculated that increased MAP-2 and PSD-95 expression is indicative of an early phase of dendritic reorganization. Taken together, these studies suggest that increases in synaptophysin protein content coincide with reorganization of synaptic connections in the CA3 region of the hippocampus in experimental diabetes.

Syntaxin, a presynaptic protein found at the SNARE complex, is depleted in hippocampal homogenates from 1-month diabetic Wistar rats. The Y-maze task, an indicator of spontaneous alternation behavior, yielded a lower score from the same diabetic rats compared to controls (Nitta et al., 2002). Since syntaxin interacts with Ca\(^{2+}\) binding proteins, abnormal calcium influx in the hippocampal neurons of diabetic rats could explain the reduced expression of the protein (Rizo and Sudhof, 1998). There is no direct evidence linking syntaxin depletion to reduced memory. However, a low score in the Y-maze task indicates that the diabetes-induced loss of syntaxin is accompanied by altered function in the hippocampus.

Presynaptic protein transport in the hippocampus may also be disrupted by diabetes. In hippocampal cultures, elevated glucose increases the content of SNAP-25, synaptotagmin-1 and VGluT-1 (Gaspar et al., 2010). Specifically,
these proteins accumulate in hippocampal cell bodies after exposure to elevated glucose (Gaspar et al., 2010). Therefore, diabetes may disrupt the trafficking of these proteins in hippocampal neurons, possibly resulting in their absence at the presynaptic terminal.

1.4.3. In the Retina

Presynaptic proteins are abundant in the neurons of the retina but have a varied distribution. In the rat retina, isoforms of synapsin are found only within the inner plexiform layer but marked differences of synapsin I and synapsin II expression are observed in single terminals (Mandell et al., 1992). Immunocytochemistry with conventional light microscopy and confocal laser scanning microscopy in rabbit and rat retina demonstrated synaptophysin expression in the inner plexiform layer and photoreceptor terminals of the outer plexiform layer (Brandstatter et al., 1996). In situ hybridization in adult rat retinas suggests that SNAP-25 mRNA is mainly expressed by ganglion, amacrine, and horizontal cells, but not by photoreceptors and bipolar cells. SNAP-25 is most abundant in the inner part of the inner and outer plexiform layers and is also found in the ganglion cell axons (Catsicas et al., 1992). VAMP-2 immunoreactivity is apparent in the inner and outer plexiform layer in Sprague Dawley rat retina (VanGuilder et al., 2008). The differential distribution of these proteins among retinal neurons implies a complex network of presynaptic mechanisms.
It is speculated that altered presynaptic composition could explain visual deficits seen early in diabetes. DR leads to a loss in retinal function and consequently impaired vision. Significant contrast sensitivity losses are observed in Type 2 diabetic patients with no retinopathy (Dosso et al., 1996). Another study in humans showed that patients with non-insulin dependent diabetes and no retinopathy had abnormal contrast sensitivity at only one spatial frequency while patients with non-insulin dependent diabetes and background retinopathy had abnormal contrast sensitivity at all six spatial frequencies tested (Sokol et al., 1985). These data suggest that retinal dysfunction can be present before the onset of clinically detectable retinopathy, but the molecular discrepancies behind these changes are unclear.

STZ-diabetes in Sprague Dawley rats causes the reduction of retinal presynaptic proteins synaptophysin, synapsin I, VAMP-2, SNAP-25 and PSD-95 (VanGuilder et al., 2008). Reduced synaptophysin content in particular, is a recurrent finding in several studies on diabetic rats (Kurihara et al., 2008; Duarte et al., 2009; Gaspar et al., 2010; Jiang et al., 2010; Sasaki et al., 2010; D'Cruz et al., 2012). While disrupted presynaptic protein content may play a role in compromised function of retinal neurons, the mechanisms involving their diabetes-induced depletion in the rat retina are not known.
1.5. Synaptophysin

1.5.1. Function and Interaction

Synaptophysin is a transmembrane protein in presynaptic SVs of neurons (Jahn et al., 1985; Wiedenmann and Franke, 1985) (Figure 1). It is a glycoprotein with four transmembrane domains and both amino and carboxyl termini facing the cytoplasm (Leube et al., 1987; Sudhof et al., 1987; Johnston et al., 1989). Although the function of synaptophysin is not fully understood, studies have revealed several properties of this protein and its interactions with other proteins. Previous work has indicated that it is integral to the regulation of synaptic function. Synaptophysin forms highly stable hexamers throughout the SV plasma membrane, making it an important element in the stability of the vesicle structure (Rehm et al., 1986). The interaction of synaptophysin with VAMP-2 appears to be important for SV docking at the presynaptic terminal. VAMP-2 was demonstrated to interact directly with synaptophysin in SV fractions from the brain (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995), suggesting a role in vesicle docking and neurotransmitter release. The synaptophysin-VAMP-2 interaction may also provide a vesicular reserve of VAMP-2 that could be made available for SNARE docking during periods of increased synaptic activity (Becher et al., 1999).

Neuronal stimulation, even when insufficient to cause significant exocytosis, releases VAMP-2 and permits its interaction with t-SNARES at the terminal (Daly
and Ziff, 2002). However, synaptophysin forms a complex with VAMP-2 prior to VAMP-2’s entry into the SNARE complex, suggesting that the synaptophysin/VAMP-2 complex has, to some extent, control over SV exocytosis (Edelmann et al., 1995) and modulates SV fusion and recycling rates (Bacci et al., 2001; Yelamanchili et al., 2005). Other than its association with VAMP-2, synaptophysin has also been implicated in the recycling of SV by associating with dynamin I, a GTPase required for endocytosis (Daly and Ziff, 2002).
Figure 1. Overview of synaptophysin’s role at the presynaptic terminal.

Synaptophysin is essential for vesicle docking, fusion and recycling, thus ensuring proper neurotransmitter release at the synapses.

Although synaptophysin has been implicated in the maintenance of the SV pool, synaptophysin-knockout mice exhibit normal synaptic transmission in the brain (McMahon et al., 1996). However, other studies have highlighted the importance of synaptophysin in processes that require and include neurotransmission, further complicating the understanding of its role in neuron function. For instance, synaptophysin overexpression in *Xenopus* enhances neurotransmitter release (Alder et al., 1995), and electrophysiological recordings from the
hippocampal CA1 region show reduced long-term potentiation in synaptophysin knockout mice (Janz et al., 1999). Taken together, these data suggest that a reduction in synaptophysin may contribute to deficits in neuronal function by disrupting normal presynaptic events. However, its exact contribution to efficient neurotransmission is still unclear.

1.5.2. The Metabolism of Synaptophysin

Synaptophysin mRNA is expressed early in development (Bergmann et al., 1991; Mahata et al., 1993; Marazzi and Buckley, 1993), and synaptophysin protein levels increase significantly during periods of synapse formation (Leclerc et al., 1989). Since it is an N-glycosylated protein (Leube et al., 1987), its mRNA translation likely occurs in the rough endoplasmic reticulum (ER). It is speculated that the rate of translation initiation or the rate of peptide chain elongation is the mechanism that regulates synaptophysin synthesis (Daly and Ziff, 1997). Studies in the entorhinal cortex of aged and young rats suggest that this event is also regulated by changes in LTP (Mullany and Lynch, 1997).

During its post-translational processing, synaptophysin is N-glycosylated in the lumen of the ER (Fykse et al., 1993). The major biosynthetic steps for N-glycosylation of in vertebrates have been established (Kornfeld and Kornfeld, 1985; Schachter, 1991). The essential step of converting mannose-rich peptides to complex N-glycans occurs in the Golgi, where N-acetylglucosamine (GlcNAc)-
transferase I adds a GlcNAc residue to form a hybrid-type N-glycan, GlcNAc1Man5GlcNAc2 (Schachter et al., 1983). Golgi α-mannosidase II then removes two mannose residues to form GlcNAc1Man3GlcNAc2 (Tulsiani et al., 1982b; Moremen et al., 1994). The removal of these two mannose residues is also the final step in the transformation of synaptophysin from a mannose-rich transient state into a complex N-glycan (Cutler and Cramer, 1990; Akama et al., 2006). De-mannosylation of mannose-rich synaptophysin, like other N-glycans, is an important step in its post-translational processing.

Previous studies using yeast two-hybrid screening in rat brain suggest Siah proteins ubiquinate synaptophysin and target it for degradation by the proteasome (Wheeler et al., 2002). Synaptophysin degradation through the proteasome pathway has been suggested in cell culture as well (Kurihara et al., 2008). Further, the literature indicates that degradation of functional and mature synaptophysin may be preceded by another catabolic event. Protein misfolding or disrupted N-glycosylation could initiate quality-control-response mechanisms, including, but not limited to, rapid protein degradation (Elbein et al., 1983; Nakatsukasa et al., 2008).

1.5.3. Retinal Synaptophysin and Diabetes

Synaptophysin likely plays an important role in SV regulation, and may be particularly relevant to the function of the retina, where it is abundant throughout
the synapse-rich inner and outer plexiform layers in humans and rodents (Kivela et al., 1989; Nag and Wadhwa, 2001; VanGuilder et al., 2008). Several lines of evidence suggest that synaptophysin is important for photoreceptor function in the retina. While synaptophysin-knockout mice do not appear to have obvious behavioral deficits, the SVs of rod photoreceptors are reduced in number and have increased diameters (Spiwoks-Becker et al., 2001). The reduction in SV population is further heightened in knockout mice during the dark period. Retinal synaptic activity is increased in the dark, indicating that synaptophysin may play an important role in recycling vesicles after exocytosis in the rodent retina. An increase in clathrin-coated pits occurs during the decrease in SVs, and is further amplified after dark adaptation. This may be due to the absence of compensatory mechanisms such as synaptoporin’s action, and clathrin-mediated endocytosis in the mouse photoreceptors (Brand and Castle, 1993; Stenius et al., 1995; Singleton et al., 1997; Spiwoks-Becker et al., 2001). Together, these studies indicate that synaptophysin performs several important roles in the presynaptic terminals of retinal neurons. A loss of synaptophysin, in the absence of its compensators in the rodent retina, may disrupt photoreceptor synapses and neurotransmitter release, significantly impacting retinal function.

Reduction of synaptophysin at presynaptic terminals is observed in various neurodegenerative diseases. For example, synaptophysin immunoreactivity is also reduced in the hippocampus of brains from Alzheimer’s and Parkinson’s disease patients (Zhan et al., 1993; Heinonen et al., 1995). Similarly,
synaptophysin content is significantly reduced in the retina after one and three months of STZ-diabetes. During the same periods of diabetes, the immunoreactivity of inner and outer plexiform layer synaptophysin is also significantly reduced compared to age-matched controls (VanGuider et al., 2008) (Figure 2).

The functional significance of synaptophysin reduction in the retina is not yet established. It is clear that the neurological function of the retina is compromised in rodents with experimental diabetes, because their ERG and scotopic threshold response are diminished (Hancock and Kraft, 2004; Kohzaki et al., 2008). Furthermore, behavioral optokinetic studies suggest that diabetic animals have visual deficits (Kirwin et al., 2011; Akimov and Renteria, 2012). In synaptophysin-knockout mice, there are no detectible compensatory transcriptional changes in the retina (Bai et al., 2006). However, they do have an impaired optomotor response and a significant spatial learning deficit (Schmitt et al., 2009). This could indicate that reduced synaptophysin in the hippocampus influences synaptic function, and does not exclude the possibility that synaptophysin is needed for aspects of visual function that are not easily detected by the super-physiological ERG. Further studies are required to conclusively determine the significance of synaptophysin in retinal function. Rescue of retinal synaptophysin expression in diabetic mice treated with lutein, however, was accompanied by improved ERG measurements (Sasaki et al., 2010).
Figure 2. Diabetes reduces the content of retina synaptophysin.

A) In control tissue, punctate synaptophysin immunoreactivity was located in the inner plexiform layer (IPL) and outer plexiform layer (OPL). B) In 1-month STZ-diabetic rats, there was a reduction of synaptophysin immunoreactivity as compared to control. (Original figure from Van Guilder et al., 2008; copyright license for reuse acquired from John Wiley & Sons Ltd.)

1.6. Rationale and Hypothesis

There is an abundance of evidence suggesting that diabetes results in neurodegeneration in the retina (Barber et al., 1998; Asnaghi et al., 2003; Gastinger et al., 2006). ERG measurements in rats during the early stages of STZ-diabetes show marked changes in retinal ganglion cell and photoreceptor activity (Aizu et al., 2002; Kohzaki et al., 2008). Other studies have found a significant reduction in overall retinal thickness of the rat retina within 10 weeks of STZ-diabetes. Dendrite remodeling and a decrease in the number of retinal amacrine cells have been reported in Ins2<sup>Akita</sup>-diabetic mice compared to control (Gastinger et al., 2006; Gastinger et al., 2008). These diabetes-induced alterations in structure and function of the retina are likely to jeopardize
communication between retinal neurons, which results in problematic vision. While the evidence for disrupted retinal function during the early stages of diabetes is strong, there is little data on the molecular events that bring about these functional changes.

Between 2005 and 2008, the estimated prevalence of DR and vision-threatening DR was 28.5% and 4.4% among US adults with diabetes, respectively (Zhang et al., 2010). Between 40 and 45% of Americans diagnosed with diabetes have some stage of DR. After 20 years of diabetes, nearly all patients with Type 1 and more than 60% of patients with Type 2 diabetes have some degree of retinopathy and diabetes-related blindness costs the nation about $500 million annually (CDC National DR Factsheet, 2008). These statistics suggest that current therapeutic strategies are still unable to prevent visual impairment caused by diabetes. Further investigations into the molecular mechanisms that define the early stages of the DR will help identify targets for better therapies in the future.

While the consequence of presynaptic protein loss in the retina during diabetes is not well understood, the loss of synaptophysin is clearly part of the neurodegenerative process. To cause a reduction in retinal synaptophysin, diabetes may target molecular mechanisms responsible for synaptophysin regulation. Since the relative mRNA content of synaptophysin was unchanged after 1 month of diabetes (VanGuilder et al., 2008), the mRNA translation, protein
glycosylation and degradation in the retina were identified as potential mechanisms.

Examining these events in vivo poses a substantial difficulty at the technical level and introduces several uncontrolled variables in the experiments. Therefore, a retinal explant approach was used for some studies in this dissertation. The aim of this work was to determine the effect of diabetes on mRNA translation and post-translational processing of synaptophysin in the rat retina and to investigate the role of these mechanisms in the reduction of the presynaptic protein (Figure 3). Using the STZ-diabetic rat as a model, the research presented in this dissertation tested the hypothesis that a short period of diabetes alters mechanisms that regulate synaptophysin expression in the retina. Specifically, it was hypothesized that diabetes reduced the mRNA translation and accelerated the degradation of synaptophysin in the rat retina. It was also postulated that diabetes disrupts the post-mRNA translational N-glycosylation of synaptophysin in the rat retina. Insight into these pathways will provide a greater understanding of underlying molecular irregularities responsible for deficits in retinal presynaptic terminal composition, encouraging further research into their role in vision loss.
Figure 3. Gap in knowledge.

Previous work suggests that diabetes causes a reduction in synaptophysin content in the rat retina. However, the mechanisms responsible for this loss are not known. This dissertation examines multiple mechanisms that may be responsible for the expression of retinal synaptophysin and determines their role in this protein’s reduced content under diabetic conditions. mRNA translation, N-glycosylation, and degradation during and after N-glycosylation were determined to be potential mechanisms.
Chapter 2

Materials and Methods
2.1. Animals

All animal experiments were approved by the Penn State College of Medicine Institutional Animal Care and Use Committee and performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats, 150-175 g (Charles River Laboratories, Wilmington, MA, USA), were housed in the Penn State Hershey College of Medicine Animal Facility. At a weight of 270-300 g, diabetes was induced by single intraperitoneal STZ injection (65 mg/kg body weight in 10 mM sodium citrate, pH 4.5). Control rats received an equal volume of citrate buffer. Diabetes was confirmed 6 days later and before sacrifice by a blood glucose level > 250 mg/dL (Alphatrak, Abott Laboratories, Illinois, USA) in a drop of blood from the tail (Table 2). Rats were housed on a 12 hr light/dark schedule with ad libitum food and water. Immediately prior to sacrifice rats were weighed and their blood glucose levels were recorded. Animals were anesthetized (ketamine 66.7 mg/kg /xylazine 6.7 mg/kg, intramuscular) and killed by decapitation.
Table 2. Weight and blood glucose of rats at time of sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>1 month</th>
<th>2 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight (g)</td>
<td>Weight Gain (%)</td>
</tr>
<tr>
<td>CNT</td>
<td>523.7±10</td>
<td>79</td>
</tr>
<tr>
<td>STZ</td>
<td>326.5±8***</td>
<td>15</td>
</tr>
</tbody>
</table>

2.2. Experimental Procedures

2.2.1. Western blot Analysis

Retinas were sonicated in lysis buffer (100 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.2% sodium dodecylsulfate, 2 mM EDTA, 10 mM HEPES, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM benzamidine, 10 µM microcystin, and one Complete™ protease inhibitor tablet/10ml of buffer, pH 7.3) and then rocked for 15 min at 4°C. Samples were centrifuged (12 min at 12,500g, 4 °C) and supernatant protein concentrations were determined using the BCA protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were adjusted to equal volumes diluted in sample buffer (NuPAGE® LDS, Invitrogen, Carlsbad, CA, USA) and resolved on 4–12% Bis-Tris gels (NuPAGE® Novex). Protein bands were transferred to nitrocellulose membranes (Thermo Fisher, Waltham, MA, USA), blocked with 5% milk in Tris-buffered saline with 0.05% Tween-20, and incubated with primary antibody overnight at 4 °C. Blots were incubated with appropriate secondary antibodies for 1 hr at room temperature. Blots were
imaged with a Typhoon 8600 Variable Mode Imager (GE Healthcare®, Wisconsin, USA), and quantified using Image Quant software (Molecular Dynamics, California, USA), standardized to β-actin, and expressed as percentage of control ± SEM.

Table 3. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host Species</th>
<th>Source</th>
<th>Product #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophyisin</td>
<td>Rabbit</td>
<td>Novus Biologicals</td>
<td>NB100-2323</td>
</tr>
<tr>
<td>Synapsin I</td>
<td>Mouse</td>
<td>Synaptic Systems</td>
<td>106021</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Rabbit</td>
<td>Covance</td>
<td>SMI-81R</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>2498</td>
</tr>
<tr>
<td>eIF3A</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>2538S</td>
</tr>
<tr>
<td>eIF2Bε</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>3595</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>9452S</td>
</tr>
<tr>
<td>Golgi α-Mann II</td>
<td>Rabbit</td>
<td>Abgent</td>
<td>AP-12501C</td>
</tr>
<tr>
<td>Histone H3</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>9715</td>
</tr>
<tr>
<td>Proteasome α-sub</td>
<td>Mouse</td>
<td>Biomol</td>
<td>PW8195</td>
</tr>
<tr>
<td>B-Actin</td>
<td>Rabbit</td>
<td>Millipore</td>
<td>EP1123Y</td>
</tr>
</tbody>
</table>

2.2.2. Synaptosome Isolation

Synaptosomes were isolated from retinas of 1-month diabetic and age-matched control rats by a procedure adapted from previously published methods (Redburn and Thomas, 1979; Lombardini, 1993). Immediately after each animal was killed, both retinas were removed and combined in 10 ml of ice-cold sucrose buffer (320 mM sucrose, 4 mM Hepes, 1 mM Na₃VO₄, pH 7.4). Samples were washed three times with gentle vortexing and buffer replacement to remove photoreceptor outer segments, and then homogenized in 5 ml of fresh sucrose buffer.
Homogenates were centrifuged at 200g for 10 min at 4 °C to pellet nuclear material (pP1) (Figure 4). The resulting supernatant was centrifuged at 800g for 12 min at 4°C to isolate cytoskeletal elements (P1) (Figure 4). The synaptosome fraction (P2) was obtained by centrifuging the resulting supernatant at 25,000g for 14 min at 4 °C (Figure 4). Synaptosome pellets were rinsed twice in 2 ml of ice cold sucrose buffer to remove contaminating material, and recollected by centrifugation (12 min at 25,000g, 4 °C). Enrichment of synaptosomes in the P2 fraction was confirmed using synaptic markers synapsin I, synaptophysin, and SNAP-25. In order to verify minimal contamination from nuclei, all synaptosome fractions and whole retina lysates (R) were also probed for histone H3 (Figure 5).

![Figure 4. Synaptosomes were isolation by differential centrifugation.](image)

The homogenized retinas were centrifuged at 200g, 800g, and 25,000g to obtain a final P2 pellet containing synaptosomes.
Figure 5. Synaptic protein enrichment in the synaptosome (P2) fraction.

The whole retina (R), nuclear (pP1), cytoskeletal (P1), and synaptosome (P2) fractions each were immunoblotted for synaptic proteins synaptophysin, SNAP-25, and histone H3. The P2 fraction was an increased expression of synaptic proteins suggesting an enrichment of synaptosomes. Histone H3 detection was diminished in the P2 fraction suggesting that nuclear contamination was minimal.

2.2.3. Autoradiography and Scintillation Counting

Retinas were rapidly extracted and incubated in 1ml of methionine-free DMEM (Sigma Aldrich) in a 12-well plate for 20 min in a cell culture incubator (37°C). After the pre-incubation period, 100 µCi of 35S-methionine/cystine protein labeling mix (EasyTag™ EXPRESS, Perkin Elmer, Waltham, Massachusetts, USA) was added to the media and incubated for 30 min. For autoradiography, samples were sonicated and the supernatants were run on 10% Bis-Tris gels (NuPAGE® Novex). The gel was fixed (30% methanol, 10% glycerol) for 1 hr and dried overnight at 55 °C. Dried gels were placed in phospho-screens for 72 hours and quantified. For measurement of total mRNA translation, whole retina lysates were run on the gel followed by autoradiography.
For scintillation counting, 250 µl of retinal lysate was added to 1.25 ml of 1 N perchloric acid and the sample was boiled for 15 min. The resulting precipitate was washed twice by centrifugation (10 min at 3,200g, 4 °C) followed by a wash with 1.5ml of 1:2:1 chloroform: ethanol: ether and lastly with 1.5ml of ether only. Samples were allowed to dry overnight before being resuspended in 1.5 ml of 0.1 N NaOH and boiled for 10 min. 500 µl of sample and 5ml of scintillation fluid (Formula 989, Perkin Elmer, Waltham, Massachusetts, USA) were mixed in scintillation vials and counted (LS 6500 Beckman Coulter, California, USA). Data were expressed as CPM (counts per min)/µg protein/ hr.

2.2.4. Cell Death ELISA

Following incubation times of 0, 30, 60, 90, 120 or 240 min, retinas were homogenized using a Pellet Pestrle Motor® for 10 short pulses and then put on ice. Samples were vortexed for 5 sec and incubated on a rocker at room temperature for 30 min. Samples were then centrifuged (12 min at 12,500g, 4 °C) and 100 µl of the supernatant were transferred to a new tube. A Cell Death Detection ELISA kit (Roche Diagnostics, Basel, Switzerland) was used to quantify nucleosomes (histone-bound DNA fragments) in the samples. Briefly, anti-histone antibody is fixed on the wall of the microplate provided in the kit. 20 µl of sample is added to each well followed by 80 µl of immunoreagent. During a 2 hr incubation, the nucleosomes contained in the sample bind via their histone
component to the immobilized anti-histone antibody. Anti-DNA-peroxidase is used to create a reaction with the DNA part of the nucleosome. The wells are rinsed three times with 200 µl incubation buffer to remove unbound peroxidase conjugate. Finally, the amount of peroxidase retained in the immunocomplex is photometrically determined following incubation with 100 µl of substrate solution (prepared as per kit instructions) for 15 min. The raw O.D. values were then obtained using a plate reader and the data was expressed as O.D./mg of wet tissue weight.

2.2.5. ATP assay

A luciferase based luminescent assay (Promega, Fitchburg, WI, USA) was used to measure ATP levels in explant retinas following incubation in DMEM. After 0, 30, 60, 90, or 120 min, retinas were sonicated, rocked for 15 min, and centrifuged (12 min at 12,500g, 4 °C). 20 µl of each lysate (in triplicates) was added to the wells of a 96-well plate. In separate wells, 20 µl of each standard provided with the kit (ranging from 0.001 to 1 µM) was added in triplicates. 20 µl of reaction buffer was pipetted into all the wells. After a 30 min reaction time, the luminescence was measured on a plate reader. Using the equation derived from the standard values, the data were expressed as nM/µg protein.
2.2.6. Immunoprecipitation

The retinas were homogenized in IP buffer (50 mM HEPES, 137.5 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Na₂H₂P₂O₇, 10 mM EDTA, 10 mM NaF, 2 mM PMSF, 0.16% benzamidine, 10% glycerol, 1% NP-40, and one Complete™ protease inhibitor tablet for every 10ml of buffer) and centrifuged at 12,500g at 4 °C for 10 min. The protein concentration was determined and synaptophysin was immunoprecipitated using 450 µg of lysate and 20 µg of rabbit polyclonal anti-synaptophysin (Novus Biologicals, Littleton, Colorado, USA), rotated overnight at 4 °C. The following day, 150 µl of protein A sepharose beads (Sigma Aldrich) were added to the lysate/ antibody mix and rotated for 3 hours at room temperature. The beads were washed three times with IP buffer, resuspended in 40 µl of 1X NuPAGE® LDS sample buffer (Invitrogen®, California, USA), and boiled for 5 min. Samples were centrifuged and run on 10% Bis-Tris gels (NuPAGE® Novex). The gel was fixed (30% methanol, 10% glycerol) for 1 hr and dried overnight at 55°C. Dried gels were placed in phospho-screens for 72 hours and quantified.

2.2.7. Radioactive Pulse-Chase and CHX Chase

Retinas were rapidly extracted and incubated in 1ml of methionine-free DMEM (Sigma Aldrich) in a 12-well plate for 20 min in a cell culture incubator (37 °C). After the pre-incubation period, 100 µCi of ³⁵S-methionine/cystine protein labeling mix (EasyTag™ EXPRESS, Perkin Elmer, Waltham, Massachusetts, USA) was
added to the media and incubated for 30 min. The retinas were then washed and incubated in DMEM containing 1 mM methionine (Sigma Aldrich) for 0, 30, 60, 90, or 120 min. This was followed by immunoprecipitation of synaptophysin and autoradiography as described above. To measure the loss of total synaptophysin in the retina, explants were incubated in DMEM containing methionine along with 30 mM cycloheximide (CHX) (Sigma Aldrich) for 30, 60, or 120 min. The retinas were then sonicated and a western blot analysis was performed.

### 2.2.8. PNGase F and Endo H Treatment

6 µl of 1X denaturing buffer (New England Biolabs, Ipswich, MA, USA) was added to 30 µg of retinal lysate and boiled for 7 min. 15 µg was taken from each sample and incubated with endoglycosidase H (endo H) or PNGase F and 1X ‘G5’ reaction buffer (New England Biolabs, Ipswich, MA, USA) for 1 hr at 37 °C. The reaction was stopped by the addition of sample buffer (1X NuPAGE® LDS, Invitrogen®, California, USA) and heated at 70 °C for 15 min. The samples were probed for synaptophysin through western blot analysis.

### 2.2.9. α-Mannosidase Activity Assay

The activity of α-mannosidases was measured using a procedure adapted from previously published methods (Rabouille et al., 1999). The retinas were homogenized in IP buffer (50 mM HEPES, 137.5 mM NaCl, 1 mM MgCl₂, 1 mM
CaCl₂, 10 mM Na₂H₂P₂O₇, 10 mM EDTA, 10 mM NaF, 2 mM PMSF, 0.16% benzamidine, 10% glycerol, 1% NP-40, and one Complete™ protease inhibitor tablet for every 10 ml of buffer) and centrifuged at 12,500g at 4 °C for 10 min. The protein concentration was determined using the BCA protein assay (Bio-Rad Laboratories, Hercules, CA, USA). α-mannosidase activity measurements were carried out in the presence of 40 mM MES buffer, pH 5.75, and 5 mM p-nitrophenyl α-D-mannopyranoside (PNP-Man, Sigma Aldrich, St Louis, MO, USA) using a reaction volume of 50 µl in 96-well clear bottom plates. Varying concentrations of the α-mannosidase small molecule inhibitor, swainsonine, were added to the appropriate wells prior to addition of protein lysate (80 µg). The plates were sealed with Parafilm to prevent evaporation and incubated in 37 °C for 1 hr. The reaction was stopped by the addition of 50 µl of 500 mM sodium carbonate and the absorbance measured at 405 nm using a plate reader. Blanks contained only MES buffer and lysate without the substrate PNP-Man. All reactions and controls were carried out in triplicate and the results averaged. The data was expressed as a % of the samples not treated with swainsonine.

2.2.10. Swainsonine Treatment of Retinal Explants and Degradation Studies

Retinas were extracted from healthy rats and incubated in DMEM with 50 µM of swainsonine (Sigma Aldrich) for at least 30 min. Synaptophysin and mannose rich N-glycosylated synaptophysin were quantified by western blot analysis. To
measure the degradation of total synaptophysin and endo H sensitive synaptophysin during the inhibition of Golgi α-mannosidase in the retina, explants were incubated in DMEM containing 50 μM swainsonine along with 30 mM CHX (Sigma Aldrich) for 30, 60, or 120 min.

2.2.11. Data Analysis

Data are expressed as mean ± SEM, as a percent of control average. Statistical comparisons between control and diabetic groups were made by Student’s two-tailed homoscedastic t-test (Microsoft Excel; Microsoft, Redmond, WA, USA). Two-way ANOVA followed by Bonferroni multiple comparisons test was used for the newly-synthesized and total synaptophysin depletion assays (Prism; Graphpad, La Jolla, CA, USA). A p-value < 0.05 was interpreted as a significant difference.
Chapter 3

Diabetes Increases Retinal Synaptophysin mRNA Translation
3.1. Introduction

Recent investigations have revealed an important role for mRNA translation as an independent mechanism of regulation for protein expression, particularly in diabetes (Kahvejian et al., 2005; Schrufer et al., 2010). In the present study, the total retinal mRNA translation (all proteins detectable within a 30 min incubation time) was measured in STZ-diabetic and control rats. While mRNA translation is mediated by ribosomes, which act as RNA-based enzymes to facilitate peptide bond formation, the process also requires several other additional proteins, which are known as initiation factors. Changes in content of factors that regulate all stages of the process - initiation, elongation, and termination, will significantly impact overall mRNA translation. Eukaryotic initiation factors or eIFs were measured in whole retinas as well as isolated synaptosomes from diabetic and control animals in this study.

The regulation of mRNA translation due to phosphorylation of eIFs has been closely studied in another complication of diabetes, diabetic nephropathy. High glucose induced hypertrophy of glomerular epithelial cells in vitro (Lee et al., 2007). This was associated with an increase in phosphorylation of 4E-BP1 (4E-binding protein 1) and a decrease in Thr56 phosphorylation of elongation factor eEF2 indicating both the initiation phase and elongation phases of translation were stimulated. Increased 4E-BP1 hyperphosphorylation is also seen in mouse
models of DR (Schrufer et al., 2010). In this chapter, the hyperphosphorylation of 4E-BP1 is examined in retinas from STZ-diabetic rats and compared to controls.

While previous works suggest abnormal regulation of translation factors alters mRNA translation in diabetic rodent retinas, the metabolic process has not been directly examined. By developing a radioactive explant assay, a more definitive measurement of total and synaptophysin mRNA translation can be made. The aim of the research presented in this chapter was to test the hypothesis that diabetes reduces synaptophysin mRNA translation in the rat retina. mRNA translation was measured using a $^{35}$S-methionine labeling assay followed by synaptophysin immunoprecipitation and autoradiography (Figure 6). Total mRNA translation was measured using autoradiography as well as scintillation counting. Contrary to the expected outcome, the data indicate that diabetes increases mRNA translation of synaptophysin in the retina. The reason for this increase is not clear and diabetes-induced retinal synaptophysin loss is likely a result of a different metabolic abnormality. The total mRNA translation was also increased, but only at the 1-month duration. Western blot analysis in this group indicated that eIF content was unchanged in the rat retina and likely not responsible for the observed increase in total mRNA translation.
Figure 6. Assay setup for measuring mRNA translation in retinal explants.

Rat retinas were extracted and placed in DMEM with a radioactive Met/Cys labeling mix for 30 min at 37 °C. Retinas were homogenized and the lysates or immunoprecipitated synaptophysin were run on a gel followed by autoradiography.
3.2. Assay Validation

3.2.1. CHX Treatment

An explant radioactive metabolic labeling assay was developed to measure mRNA translation in the rat retina. CHX inhibits mRNA translation by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome) thus blocking translational elongation (Siegel and Sisler, 1963). Explant retinas were incubated in DMEM with different concentrations of CHX for 30 min. Amino acid incorporation was completely inhibited by CHX when retinas were incubated with 50, 100, and 200 µCi $^{35}$S-Met/Cys confirming that the radioactive incorporation was due to mRNA translation (Figure 7A).

3.2.2. DNA Fragmentation

Among other events, apoptosis is characterized by the activation of endogenous Ca$^{2+}$- and Mg$^{2+}$-dependent endonucleases that cleave double-stranded DNA at the most accessible internucleosomal linker region, generating nucleosomes-DNA tightly complexed with the core histones H2A, H2B, H3, and H4 (Wyllie et al., 1980). These are discrete multiples of a 180 bp subunit, detected as a "DNA ladder" on agarose gels after extraction. The lysates from retinal explants incubated in DMEM were tested for nucleosomes in order to determine cell death levels at multiple incubation times. Nucleosome levels were not different between
explants at 0, 30, 60, 90, and 120 min of incubation (Figure 7B). However, levels significantly increased at 240 min of incubation compared to the other time points ($p<0.05$; Figure 7B). Therefore, all future studies did not exceed 120 min of incubation in DMEM.

3.2.3. ATP Levels

Depletion of energy resources during explant incubation may be a serious limitation of the explant assay and could affect observed outcomes. Therefore, retinal explant viability in cell culture medium was also evaluated for ATP levels at multiple incubation time points using a luciferase based luminescent assay. There was no significant difference in ATP concentration (nmoles/μg protein) between explants over 120 min of incubation in DMEM (Figure 7C).
Figure 7. Validation of the radioactive amino acid incorporation assay.

The assay was validated using CHX treatment. Viability of the retina was determined by a cell death ELISA and an ATP assay. A) Explants were incubated in 50, 100, and 200 µCi of $^{35}$S-met/cys. Addition of 30 mM CHX to the medium abolished the mRNA translation of total protein. B) There was a significant increase in the nucleosome content at the 240 min time point compared to all the other time points (n=4; *p< 0.05). C) There was no change in ATP levels between the 0, 30, 60, 90, and 120 min time points (n=4).
3.3. \(^{35}\text{S}\)-Met/Cys Incorporation into Protein is Transiently Increased by STZ-Diabetes

3.3.1. Incorporation is Increased after 1 Month of STZ-Diabetes

mRNA translation was measured in retinas from 1-month STZ-diabetic rats and age-matched controls followed by autoradiography of lysates (Figure 8A). \(^{35}\text{S}\)-met/cys incorporation was significantly elevated in retinas from 1-month STZ-diabetic rats compared to controls (2.31 fold; \(p<0.001\); Figure 8B). Incorporation was also measured by PCA precipitation and the average CPM in samples from 1-month STZ-diabetic rats was significantly more than age-matched controls (2,255 CPM/µg protein/hr vs. 1,531 CPM/µg protein/hr) (\(p<0.05\); Figure 8C).

3.3.2. Incorporation is Unchanged after 2 Months of STZ-Diabetes

\(^{35}\text{S}\)-met/cys incorporation was measured in retinas from 2-month STZ-diabetic and age-matched control rats (Figure 8D). Incorporation was not significantly different in retinas from 2-month STZ-rats compared to controls (Figure 8E). The average CPM was also not significantly different (1,530 CPM/µg protein/hr in 2-month STZ-diabetic rats compared to 1,827 CPM/µg protein/hr in controls) (Figure 8F).
Figure 8. Diabetes transiently increases retinal mRNA translation.

Explant retinas were incubated with $^{35}$S-met/cys for 30 min to label newly translated proteins. Autoradiography and PCA precipitation were performed on explants from 1- and 2-month diabetic and control rats following $^{35}$S-met/cys incubation. A) $^{35}$S-met/cys incorporated into many proteins in 1-month diabetic and age-matched control explants. B) $^{35}$S-met/cys incorporation was increased in retinal explants from 1-month STZ-diabetic rats (n=15) compared to age-matched controls (n=13) (**p<0.001). C) The average CPM in explants from 1-month STZ-diabetic rats was significantly increased compared to age-matched controls (n=9; *p<0.05). D) $^{35}$S-met/cys incorporation in explants from 2-month diabetic and age-matched control rats. E) $^{35}$S-met/cys incorporation was not significantly different in retinal explants from 2-month STZ-diabetic rats compared to age-matched controls (n=9). F) The average CPM was not significantly different in explants from 2-month STZ-diabetic rats compared to age-matched controls (n=9).
3.4. eIF Content is Unaltered in Retinas 1 Month of STZ-Diabetes

In order to determine if initiation factor content was responsible for the observed increase in retinal mRNA translation during STZ-diabetes, eIF2α, eIF2Bε, eIF3A, and eIF4G proteins were quantified in retinal lysates from 1-month STZ-diabetic and age-matched control rats (Figure 9). These initiation factors are essential for ribosome complex assembly, and any changes in their content could significantly impact mRNA translation. eIF2α resolved as a single 38 kDa band, eIF2Bε as a single 75 kDa band, eIF3A as a single 166 kDa band, and eIF4G as a single 220 kDa band (Figure 9A). In all cases, the content of these proteins was not significantly different in retinas from 1-month STZ-diabetic rats compared to controls (Figure 9B).

3.5. eIF Content is Unaltered in Retinal Synaptosomes after 1 Month of STZ-Diabetes

eIF2α, eIF2Bε, eIF3A, and eIF4G proteins were further quantified in retinal synaptosome lysates from 1-month STZ-diabetic and age-matched control rats. eIF2α resolved as a single 38 kDa band, eIF2Bε as a single 75 kDa band, eIF3A as a single 166 kDa band, and eIF4G as a single 220 kDa band (Figure 9C). In all cases, the content of these proteins was not significantly different in retinas from 1-month STZ-diabetic rats compared to controls (Figure 9D).
Figure 9. Diabetes does not alter eIF content in retinas and retinal synaptosomes.

eIF2α, eIF2Bε, eIF3A, and eIF4G were quantified in whole retina lysates from 1-month STZ-diabetic and age-matched control rats. A) eIF2α resolved as a single 38 kDa band, eIF2Bε as a single 75 kDa band, eIF3A as a single 166 kDa band, and eIF4G as a single 220 kDa band. B) The content of each of the eIFs was not significantly different in the whole retina from 1-month STZ-diabetic rats compared to controls ($n=10$). C) In retinal synaptosomes, eIF2α resolved as a single 38 kDa band, eIF2Bε as a single 75 kDa band, eIF3A as a single 166 kDa band, and eIF4G as a single 220 kDa band. D) The content of each of the eIFs was not significantly different in the retinal synaptosomes from 1-month of STZ-diabetic rats compared to controls ($n=10$).
3.6. Retinal 4E-BP1 Hyperphosphorylation is Reduced after 1 Month of STZ-Diabetes

Like eIF content, phosphorylation changes in these proteins are important events in translation regulation. To determine if 4E-BP1 hyperphosphorylation was altered by diabetes, 4E-BP1 phosphorylation was measured in retinas from 1- and 2-month STZ-diabetic and age-matched control rats. 4E-BP1 resolved as 3 bands (α, β, and γ) between 15 and 20 kDa (Figure 10A). Hyperphosphorylation was calculated by inputting α, β, and γ band intensity values in the formula \[\frac{\gamma}{(\alpha+\beta+\gamma)}\]. The data was then expressed as % CNT average. After 1 and 2 months of STZ diabetes the amount of phosphorylated 4E-BP1 in the rat retina was significantly less than controls \((p < 0.001; \text{Figure 10B})\).

![4E-BP1 bands](image)

Figure 10. Diabetes reduces 4E-BP1 hyperphosphorylation in rat retinas.

Hyperphosphorylated 4E-BP1 content was also measured in 1- and 2-month STZ-diabetic and age-matched control rat retinas. Hyperphosphorylation was calculated by inputting α, β, and γ band intensity values in the formula \[\frac{\gamma}{(\alpha+\beta+\gamma)}\]. The data was then expressed as % CNT average. A) 4E-BP1 resolved as 3 bands...
between 15 and 20 kDa. B) Phosphorylated 4E-BP1 was significantly reduced in the rat retina after 1 month and 2 months of STZ-diabetes compared to controls (n=10; ***p < 0.001)

3.7. Retinal Synaptophysin Content is Reduced by STZ-Diabetes

Synaptophysin protein content was measured by western blot analysis in retinal lysates from 1- and 2-month STZ-diabetic and age-matched control rats (Figure 11A, B). There was significantly less synaptophysin in the 1- and 2-month STZ-diabetic rat retinas compared to controls (p<0.05; Figure 11C, D), confirming previously published results (VanGuilder et al., 2008).

3.8. \textsuperscript{35}S-Met/Cys Incorporation into Retinal Synaptophysin is Increased by STZ-Diabetes

Synaptophysin mRNA translation in the retinal explants was determined by 100 µCi \textsuperscript{35}S-Met/Cys incorporation for 30 min, followed by synaptophysin immunoprecipitation and autoradiography (Figure 12A, B). \textsuperscript{35}S-met/cys synaptophysin in 1-month STZ-diabetic rat retinas was significantly greater than controls (2.3 fold; p<0.01; Figure 12C). \textsuperscript{35}S-met/cys incorporation into synaptophysin in 2-month STZ-diabetic rat retinas was also significantly greater than controls (2.3 fold; p<0.05; Figure 12D).
Figure 11. Diabetes reduces retinal synaptophysin content.

A) Retinal synaptophysin resolved as a single 38 kDa band in lysates from 1-month STZ-diabetic rats and age-matched controls. B) Retinal synaptophysin also resolved as a single 38 kDa band in lysates from 2-month STZ-diabetic rats and age-matched controls. C) Retinal synaptophysin was significantly less in 1-month STZ-diabetic rats \( (n=15) \) compared to age-matched controls \( (n=13) \) \( (*p<0.05) \). D) Retinal synaptophysin content was also significantly less in 2-month STZ-diabetic rats compared to age-matched controls \( (n=9; *p<0.05) \).
Figure 12. Diabetes increases retinal synaptophysin mRNA translation.

35S-synaptophysin content was measured in retina lysates by autoradiography after a 30 min incubation followed by immunoprecipitation. Whole lysates (interpreted as total mRNA translation and quantified in Figure 8) corresponding to each immunoprecipitated synaptophysin sample were run on the same gel for autoradiography. A) 35S-met/cys synaptophysin bands were detected at 38 kDa in retinas from 1-month STZ and control rats. B) 35S-met/cys synaptophysin bands were also detected at 38 kDa in retinas from 2-month STZ and control rats. C) 35S-met/cys synaptophysin significantly increased in retinas from 1-month STZ-diabetic rats (n=15) compared to age-matched controls (n=13) (**p<0.01). D) 35S-met/cys synaptophysin was significantly increased in retinas from 2-month STZ-diabetic rats compared to age-matched controls (n=9; *p<0.05).
3.9. Discussion

This study examined the mRNA translation of synaptophysin in the rat retina during experimental diabetes. An explant radiolabeling assay was developed to test mRNA translation in the retina. The mRNA translation of synaptophysin was increased after 1 and 2 months of STZ-diabetes while mRNA translation of all proteins synthesized within 30 min was transiently increased only at the 1-month duration.

Retinal explant viability in the radiolabeling assay developed for this study was examined by measuring cell death and ATP content. The data from the cell death study determined that histone-bound fragments of DNA (nucleosomes) were significantly increased after 120 min of incubation, while the ATP levels were unchanged for up to 240 min of incubation time. These findings imply that a consistent amount of ATP was available for mRNA translation for 240 min in an ex vivo environment. However, the significant increase of cell death at 240 min compared to all other time points limited the assay to a maximum duration of 120 min. CHX treatment was used to confirm that the assay measured mRNA translation. The inhibition of radioactive amino acid incorporation by CHX indicated that the assay correctly measured mRNA translation in the retinal explants. It must be noted that treating retinas in an explant system has other technical obstacles. Most importantly, retinal function in response to light stimulation cannot be controlled while performing this assay. The explants are
exposed to light during dissection, and darkness when being incubated. While this issue can be resolved by conducting all aspects of the experiment the absence of light, the handling of radioisotopes in a dim lit room is not advisable.

The total mRNA translation was significantly increased in retinal explants after 1 month of diabetes. After 2 months of diabetes, however, the total mRNA translation was not different from controls. In addition to autoradiography, these results were replicated using PCA precipitation. In order to confirm that total mRNA translation was not increased due to changes in eIF expression, eIFs particularly important in the assembly of the initiation complex prior to the beginning of mRNA translation were quantified in retinas from 1-month STZ-rats and controls. The primary role of eIF2 in translation initiation is to transfer Methionyl-tRNA to the 40S ribosomal subunit and its α-subunit plays an important role in the regulation of mRNA translation through modulation of the interaction between eIF2 and eIF2B (Pain, 1996; Kimball, 1999). eIF2B, a guanine nucleotide exchange factor, is composed of 5 subunits, the largest of which is eIF2Bε. Multiple phosphorylation sites on this subunit have significant implications on initiation (Wang et al., 2001). eIF3 is a large translation initiation complex with 10 to 13 different subunits. It interacts with eIF4G, which may be responsible for the association of the 40S ribosomal subunit with mRNA (Masutani et al., 2007). The content of eIF2α, eIF2Bε, eIF3A, and eIF4G was not different between retinas from STZ-diabetic rats and age-matched controls.
confirming that the expression of these eIFs was unchanged and likely not a factor influencing the observed increase in mRNA translation.

Previous studies in Ins2\(^{Akita}\)-mice and STZ-rats showed a significant reduction in 4E-BP1 hyperphosphorylation, implying that diabetes reduces cap-dependent mRNA translation in the retina (Schrufer et al., 2010). While the results presented here are contrary to the expected outcome according to published literature, it is important to note that no direct measurement of mRNA translation in the retina of STZ-rats had been made prior to this study. Also, the mRNA translation detected using the explant assay includes cap-dependent and cap-independent translation. Therefore, it is possible that 4EBP-1 does not regulate the translation of a subset of proteins detected in this assay. This could explain the discrepancy between conclusions drawn from previous work and the increased mRNA translation measured in this study. Furthermore, the amount of methionine present in retinas from diabetic and control rats prior to incubation in methionine-free media may vary, and hence, influence the outcome of the assay. The quantity of methionine in the explants used in this study was not measured, and addressing this issue could provide a better explanation of the observed results.

While the evidence for protein synthesis at the presynaptic terminal is not abundant, a proteomic investigation of metabolically labeled presynaptic synaptosomes from the squid optic lobe confirmed the \textit{de novo} synthesis of approximately 80 protein species, including nuclear-encoded mitochondrial
proteins, cytoskeletal proteins, and various enzymes (Jimenez et al., 2002). Also, pure preparations of regenerating axons separated from the dorsal root ganglion cell bodies can actively synthesize proteins in vitro and contain ribosome-bound β-actin and neurofilament mRNAs (Zheng et al., 2001). These examples indicate that spatial separation of mRNA translation in neurons could allow location-specific regulation. More recently, presynaptic protein mRNA has been detected in isolated retinal synaptosomes from rats, and the synaptic proteins synapsin I, synaptophysin, VAMP-2, SNAP-25, and PSD-95 are significantly reduced in synaptosomes from STZ-rats (VanGuilder et al., 2008). This suggests that translation mechanisms may be present at the presynaptic terminals of retinal neurons. In order to determine if initiation factors are detectable and altered in the retinal presynaptic terminals during diabetes, eIFs were also quantified in isolated retinal synaptosomes from diabetic and control rats. The content of eIF2α, eIF2Bε, eIF3A, and eIF4G was not different between isolated synaptosomes from STZ-diabetic rats and age-matched controls. These data indicate that factors responsible for mRNA translation are present at the presynaptic terminals of retinal neurons. While this finding strengthens the possibility that presynaptic mRNA translation does occur in the retina, the content of these proteins was not altered by diabetes and does not explain altered mRNA translation.

Several past studies have found increased mRNA translation of select proteins in response to elevated glucose. In pre-diabetic BB rat (Biobreeding rat that
spontaneously develops Type 1 diabetes) pancreas, nitric oxide synthase mRNA translation is increased in areas of inflammation (Kleemann et al., 1993). Cell culture studies on renal epithelial cells indicate that synthesis of laminin-β1, a glycoprotein that contributes to renal extracellular matrix expansion in diabetes, is increased within minutes of exposure to high glucose (Mariappan et al., 2007). In the present study on rat retinas, STZ-diabetes significantly increased synaptophysin mRNA translation compared to age-matched controls. Evidently, while the increase in total mRNA translation in retinas from 1-month STZ-rats appears to equal controls after 2 months, synaptophysin mRNA translation continues at a higher rate. The reason for increased synaptophysin mRNA translation is not clear. Early studies in cultured embryonic hippocampal neurons suggest that an increase in the rate of incorporation of $^{35}$S-met/cys into synaptophysin protein, without a corresponding increase in the level of synaptophysin mRNA occurs by an increase in the rate of translation initiation, demonstrated by its mRNA being associated with a greater number of ribosomes (Hershey, 1991; Daly and Ziff, 1997). However, discrepancy between the 4E-BP1 study and the radioactive incorporation assay in this dissertation together with other literature (discussed below) suggest that synaptophysin mRNA translation may not be regulated by initiation.

There was a significant decrease in the amount of phosphorylated 4E-BP1 in the rat retina after 1 and 2 months of diabetes compared to controls. In the hypophosphorylated state, 4E-BPs compete with eIF4G for binding to eIF4E
(Figure 13) and prevent initiation complex assembly leading to a decrease in cap-dependent mRNA translation (Haghighat et al., 1995; Mader et al., 1995). In the current study, however, mRNA translation of synaptophysin was increased by diabetes, suggesting that the retinal mRNA translation detected in this assay is not completely subjected to regulation by 4E-BP1. mRNAs whose translation is regulated by their 5' untranslated region (5' UTR) and initiation factors often contain a considerably longer than average 5' UTR (Kozak, 1987). These mRNAs with long and structured UTRs are translated more slowly and are very inefficient in assembling the initiation complex (Kozak, 1989; Van der Velden and Thomas, 1999; Ringner and Krogh, 2005). Conversely, 5' UTRs predicted to be shorter lead to higher translation rates. Synaptophysin is reported to have a relatively short 5' UTR (Leube et al., 1987), and radioactive amino acid incorporation into the protein is detectable as early as 30 min along with the other rapidly synthesized proteins this study. Therefore, the reduced hyperphosphorylation of 4E-BP1 reported here is less likely to have a major regulatory effect on the translation of retinal synaptophysin and the other rapidly synthesized proteins during diabetes.
Figure 13. The role of 4E-BP1 hyperphosphorylation in mRNA translation.

4E-BP1 binds to eIF4E and inhibits its interaction with the ribosomal complex resulting in the inhibition of cap-dependent mRNA translation. Hyper-phosphorylation of 4E-BP1 results in the release of eIF4E, consequently allowing translation to proceed.

The total amount of synaptophysin was reduced by diabetes, as shown previously (VanGuider et al., 2008), while the mRNA translation of newly-synthesized $^{35}$S-synaptophysin was elevated (Figure 12). These data introduce a dilemma that can be explained by the synaptophysin degradation studies described in detail in Chapter 4. Diabetes significantly depleted the newly-synthesized $^{35}$S-synaptophysin compared to controls, offering a potential explanation.
Chapter 4

Diabetes Disrupts Post-Translational Processing and Accelerates Degradation of Retinal Synaptophysin
4.1. Introduction

Protein catabolism is a fundamental cellular process that occurs on a regular basis in every cell type. Small alterations in the regulation of this catabolic process, can contribute to severe physiological damage (Hu et al., 2007). The results from the Chapter 3 suggest that loss of synaptophysin in the retinas of STZ-diabetic rats does not occur via reduced mRNA translation. On the contrary, translation of synaptophysin mRNA is increased in the retina of diabetic rats. Therefore, the research presented in this chapter explores the degradation rate of retinal synaptophysin as a potential mechanism of protein loss during diabetes.

Degradation mechanisms such as the ubiquitin-proteasome system (UPS) and lysosomal degradation have become associated with several disease states, including diabetes. Increased proteasomal degradation of muscle proteins is seen in acutely diabetic mice (Hu et al., 2007). In retinal endothelial cells exposed to high glucose, the downregulation of interleukin-1 type I receptor is attributed to increased lysosomal degradation (Aveleira et al., 2010). ER associated degradation (ERAD) is yet another degradation pathway that has gained attention in the context of diabetes (Li et al., 2009). To ensure proper processing of proteins within the ER, eukaryotic cells employ a quality control mechanism to recognize and degrade aberrantly folded proteins, and prevent the aggregation and/or delivery of potentially dysfunctional or cytotoxic proteins.
(Plemper and Wolf, 1999). Diabetes-induced changes in any or all of these mechanisms may have significant implications for retinal synaptophysin metabolism.

The studies presented in this chapter test the hypothesis that diabetes accelerates the degradation of synaptophysin in the rat retina. A pulse-chase study was used to determine the depletion of $^{35}$S-met/cys synaptophysin (Figure 14A). However, this approach only measures the degradation of the newly-synthesized population of synaptophysin. Hence, depletion of total synaptophysin was determined by using a CHX incubation assay (Figure 14B). The results showed that diabetes accelerated depletion of the newly-synthesized protein in the retina after 1 and 2 months of STZ-diabetes. However, the degradation of total synaptophysin was accelerated only at the latter time point. These data suggest that two separate degradation mechanisms may regulate synaptophysin in the retina and they may also be affected differently by diabetes. Since newly-synthesized synaptophysin is subject to N-glycosylation in the ER, this post-translational modification was also examined in retinas from diabetic rats.

Previous studies have suggested that polypeptide structure influences how rapidly proteins are broken down within cells (Goldberg and Dice, 1974). In healthy mouse livers, proteins of larger molecular weight tend to degrade more rapidly than smaller ones and non-glycosylated proteins undergo slower degradation than glycosylated peptides (Gurd and Evans, 1973). Newly-
synthesized synaptophysin is N-glycosylated in the lumen of the ER (Rehm et al., 1986). This mannose-rich form of the protein is then exported and processed through several compartments of the Golgi apparatus. In the trans Golgi, the removal of 2 mannose residues by Golgi α-mannosidase II is the final step in the transformation of synaptophysin to its mature form before being released from the Golgi (Cutler and Cramer, 1990). In this study, PNGase F treatment was used to quantify the total amount of N-glycosylated synaptophysin the rat retina during diabetes. Similarly, Endo H treatment was used to measure the content of mannose-rich retinal synaptophysin, the transient immature form of the protein, in diabetic and control rats. It was postulated that diabetes alters the post-translational N-glycosylation of synaptophysin in the rat retina. The PNGase study determined that under diabetic conditions, all retinal synaptophysin was N-glycosylated. However, Endo H treatment showed that 1 month, but not 2 months, of STZ-diabetes caused an accumulation of the transient mannose-rich form of the presynaptic protein in the rat retina. Together, the data in this chapter imply that the diabetes-induced increase in retinal mannose-rich synaptophysin content is an indication of irregular post-translational N-glycosylation of synaptophysin. Importantly, it can only be speculated that the newly-synthesized synaptophysin detected in the pulse-chase study is mannose-rich. However, the accumulation of mannose-rich synaptophysin and rapid loss of the newly-synthesized protein after 1 month of STZ-diabetes suggests that these two events may explain the previously observed deficit in synaptophysin content.
Figure 14. Degradation Assay Set up.

A) Degradation of newly-synthesized synaptophysin was measured using a pulse-chase approach. Retinas were incubated with 35S-met/cys for 30 min, followed by 1 mM cold methionine for 30, 60, 90, or 120 min followed by autoradiography. B) Degradation of total synaptophysin was measured in the presence of CHX. Synaptophysin content was measured by western blot analysis on retinas incubated with CHX for 30, 60, or 120 min.
4.2. Degradation of Synaptophysin

4.2.1. Depletion of $^{35}$S-Synaptophysin is Accelerated after 1 and 2 Months of STZ-Diabetes

The loss of newly-synthesized synaptophysin in whole explant retinas from 1-month STZ-diabetic and age-matched control rats was measured using a pulse-chase approach. After the incubation of retinal explants in $^{35}$S-met/cys for 30 min, which was then replaced by non-radioactive methionine for 0, 30, 60, 90, or 120 min, $^{35}$S-met/cys-synaptophysin was quantified by immunoprecipitation and autoradiography. There was significantly less $^{35}$S-met/cys synaptophysin in retinas from 1-month of STZ-diabetic rats after 120 min compared to age-matched controls (CNT (120 min): 67.3 ± 6.01%, STZ (120 min): 21.1 ± 3.23%) (p<0.05; Figure 15A, B). A similar result was also found in the 2-month STZ-diabetic rats compared to age-matched controls (CNT (120 min): 90.7 ± 13.91%, STZ (120 min): 31.4 ± 8.28%) (p<0.05; Figure 15C, D). The depletion of newly-synthesized synaptophysin between 1- and 2-month age-matched controls was also examined. After 120 min of chase time, there was no difference in $^{35}$S-met/cys synaptophysin between the two control groups. Lastly, when the data from the 30 min time point was expressed as a % control, there was a significant increase in $^{35}$S-met/cys-synaptophysin in the 1-month STZ group compared to control (CNT (30 min): 100.0 ± 11.23%, STZ (30 min): 221.5 ± 15.31%). This was also true in the 2-month study (CNT (30 min): 100.0 ± 8.21%, STZ (30 min): 215.7 ± 14.94%). These data, as expected, confirmed the results from Figure 12.
Figure 15. Diabetes accelerates newly-synthesized retinal $^{35}$S-synaptophysin degradation.

Retinas were incubated with $^{35}$S-met/cys for 30 min, followed by 1 mM cold methionine for 30, 60, 90, or 120 min followed by autoradiography. A) $^{35}$S-met/cys synaptophysin resolved as a single 38 kDa band in retinas from 1-month STZ-diabetic and age-matched control rats. B) $^{35}$S-met/cys synaptophysin was significantly less in retinas from 1-month STZ-diabetic rats compared to controls after 120 min (n=4; **p<0.05). C) $^{35}$S-met/cys synaptophysin resolved as a single 38 kDa band in retinas from 2-month STZ-diabetic and age-matched control rats. D) $^{35}$S-met/cys synaptophysin was also significantly less in retinas from 2-month STZ-diabetic rats compared to controls after 120 min (n=4; **p<0.05). A comparison between the 1- and 2-month control groups revealed no difference in $^{35}$S-met/cys synaptophysin content after 120 min.

4.2.2. Depletion of Total Synaptophysin is Accelerated after 2 Months, but not 1 Month, of STZ-Diabetes

Retinas from 1- and 2-month STZ-diabetic and age-matched control rats were treated with CHX for 30, 60, or 120 min, and synaptophysin was quantified by western blot analysis. The amount of synaptophysin in retinas from 1-month STZ-diabetic rats was not significantly different compared to age-matched controls after 120 min (Figure 16A, B). The amount of synaptophysin was
significantly less in retinas from 2-month STZ-diabetic rats compared to controls after 120 min of protein synthesis inhibition (CNT (120 min): 84.6 ± 8.42%, STZ (120 min): 69.7 ± 2.71%) (p<0.05; Figure 16C, D). The depletion of total synaptophysin between 1- and 2-month age-matched controls was also examined. After 120 min of CHX incubation, there was no difference in total synaptophysin content between the two control groups.

Figure 16. Degradation of total retinal synaptophysin is accelerated after 2 months of diabetes.

Synaptophysin content was measured by western blot analysis on retinas incubated with CHX for 30, 60, or 120 min. A) Synaptophysin resolved as a 38 kDa band in retinas from 1-month STZ-diabetic rats and age-matched controls after 30, 60, and 120 min. B) There was no significant difference in synaptophysin content between retinas from 1-month STZ-diabetic rats and controls after 120 min (n=6). C) Synaptophysin resolved as a 38 kDa band in retinas from 2-month STZ-diabetic rats and age-matched controls after 30, 60, and 120 min. D) There was significantly less synaptophysin in retinas from 2-month STZ-diabetic rats compared to controls after 120 min (n=6;*p<0.05). A comparison between the 1- and 2-month control groups revealed no difference in synaptophysin content after 120 min of CHX incubation.
4.2.3. Proteasome Subunits are Detectable in Whole Retina and Isolated Synaptosomes

In order to confirm the presence of degradation mechanisms in the retina and retinal synaptic terminals, a western blot analysis specific for α subunits of the proteasome was performed in whole retinal lysates and isolated synaptosomes. The 20S core of a proteasome is made up of four rings. Each of the inner two rings is formed by seven β subunits while each outer ring has seven α subunits. These proteasome α subunits were detectable in whole retina and synaptosomes (Figure 17). The western blot analysis revealed 2 bands (28 kDa and 17 kDa). For each sample, the two bands were summed and the data was expressed as % control. However, subunit presence in whole retinal tissue or synaptosomes was not changed after 1-month of STZ-diabetes compared to age matched controls (Figure 17).
Figure 17. Proteasome subunits are present in whole retina and synaptosomes.

A) The western blot analysis yielded two bands between 28 and 17 kDa. B) α subunits were detectable in whole retina and synaptosomes. Subunit content in whole retinal tissue or synaptosomes were not different between 1-month STZ-diabetic rats and age-matched controls (n=4). For each sample, the two bands were summed and the data was expressed as % control.

4.3. Synaptophysin is N-Glycosylated in Retinas of Control and STZ-Diabetic Rats

The N-Glycosylation of synaptophysin in the rat retina was measured by treating retinal lysates with PNGase F, an enzyme that is able to deglycosylate all N-glycosylated proteins. After PNGase F treatment, retinal lysates from 1- and 2-month STZ-diabetic and age-matched control rats were western blotted for
synaptophysin. There was a significant decrease in the amount of PNGase F-sensitive synaptophysin in the retinas from 1-month STZ-diabetic rats compared to age-matched controls \( (p<0.05; \text{Figure 18A, B}) \). This was also true in 2-month STZ diabetic rats compared to controls \( (p<0.05; \text{Figure 18C, D}) \). However, there was no detectable amount of PNGase-insensitive synaptophysin in after 1 and 2 months of STZ-diabetes or age-matched control samples (Figure 18A, C).

Figure 18. All retinal synaptophysin is N-glycosylated in diabetic and control rats.

Retinal lysates were treated with PNGase F to quantify all N-glycosylated synaptophysin. A) PNGase F treated samples from 1-month STZ-diabetic rats and controls revealed a band at 35 kDa. Untreated samples revealed a band at 38 kDa. There was no detectable level of PNGase-insensitive synaptophysin in either diabetic or control samples. B) There was significantly less PNGase F-sensitive synaptophysin in the retinal lysates from 1-month STZ-diabetic rats compared to age-matched controls \( (n=8; \ast p<0.05) \). There was significantly less synaptophysin in untreated lysates from 1-month diabetic rats compared to control \( (n=8; \ast p<0.05) \). C) PNGase F treated samples from 2-month STZ-diabetic rats and controls revealed a band at 35 kDa. Untreated samples revealed a band at 38 kDa. There was no detectable level of PNGase-insensitive synaptophysin in either diabetic or control samples. D) There was significantly less PNGase F-sensitive synaptophysin in the retinal lysates from 2-month STZ-diabetic rats compared to age-matched controls \( (n=8; \ast p<0.05) \).
4.4. Mannose Rich Retinal Synaptophysin is Increased after 1 Month, but Not 2 Months, of STZ-Diabetes

Endo H was used to cleave mannose rich oligosaccharides from synaptophysin. Retinal lysates from 1- and 2-month STZ-diabetic and age-matched control rats were treated with endo H and western blotted for synaptophysin. There was significantly more endo H-sensitive synaptophysin (lower band) in the retinas from 1-month STZ-diabetic rats compared to age-matched controls ($p<0.05$; Figure 19A, B). There was no significant difference in the amount of endo H-sensitive synaptophysin in the retinas from 2-month STZ-diabetic rats compared to age-matched controls (Figure 19C, D). As observed in Chapter 4 and previous literature, there was significantly less synaptophysin in untreated lysates from 1- and 2-month diabetic rats compared to control ($p<0.05$; Figure 19).
Figure 19. Diabetes induces mannose rich (endo H-sensitive) retinal synaptophysin accumulation.

Retinal lysates were treated with endo H to quantify mannose rich glycosylated synaptophysin. A) Endo H treated samples from 1-month STZ-diabetic rats and controls had an additional band at 35 kDa below the 38 kDa synaptophysin band. Untreated samples revealed a band at 38 kDa. B) There was significantly more endo H-sensitive synaptophysin in the retinal lysates from 1-month STZ-diabetic rats compared to age-matched controls (n=8; *p<0.05). There was significantly less synaptophysin in untreated lysates from 1-month diabetic rats compared to control (n=8; *p<0.05). C) Endo H treated samples from 2-month STZ-diabetic rats and controls also had an additional band at 35 kDa below the 38 kDa synaptophysin band. D) There was no significant difference in endo H-sensitive synaptophysin between retinal lysates from 2-month STZ-diabetic rats and age-matched controls (n=8). There was significantly less synaptophysin in untreated lysates from 2-month diabetic rats compared to control (n=8; *p<0.05).

4.5. Depletion of Mannose Rich Retinal Synaptophysin is Not Altered by STZ-Diabetes

Retinas from 1- and 2-month STZ-diabetic and age-matched control rats were treated with CHX for 30, 60, or 120 min. Lysates were then treated with endo H and synaptophysin was quantified by western blot analysis. The amount of endo
H-sensitive synaptophysin in retinas from 1-month STZ-diabetic rats was not significantly different compared to age-matched controls after 120 min (Figure 20A, B). The amount of endo H-sensitive synaptophysin was not significantly different in retinas from 2-month STZ-diabetic rats compared to controls after 120 min of protein synthesis inhibition (p<0.05; Figure 20C, D). The depletion of endo H-sensitive synaptophysin between 1- and 2-month age-matched controls was also compared. After 120 min of CHX incubation, there was no difference in endo H-sensitive synaptophysin content between the two control groups. It must be noted that when the data from the 30 min time point was expressed as % control, there was a significant increase in endo H-sensitive synaptophysin in the 1-month STZ group compared to control (CNT (30 min): 100.0 ± 5.67%, STZ (30 min): 143.6 ± 5.46%). This replicated the previously observed increase in mannose-rich synaptophysin after 1-month of STZ-diabetes (Figure 19A). However, there was no change in endo H-sensitive synaptophysin at the 30 min time point between the 2-month STZ group and controls.
Figure 20. Diabetes does not alter the degradation of endo H-sensitive synaptophysin in the retina.

Endo H-sensitive synaptophysin content was measured by western blot analysis on retinas incubated with CHX for 30, 60, or 120 min. A) Endo H-sensitive synaptophysin resolved as a 35 kDa band below the usual 38 kDa band in retinas from 1-month STZ-diabetic rats and age-matched controls after 30, 60, and 120 min. B) There was no significant difference in endo H-sensitive synaptophysin content between retinas from 1-month STZ-diabetic rats and controls after 120 min (n=6). C) Endo H-sensitive synaptophysin resolved as a 35 kDa band below the usual 38 kDa band in retinas from 2-month STZ-diabetic rats and age-matched controls after 30, 60, and 120 min. D) There was no significant difference in endo H-sensitive synaptophysin in retinas from 2-month STZ-diabetic rats compared to controls after 120 min (n=6).
4.6. Discussion

This study examined the post-translational mechanisms of synaptophysin processing in the rat retina during experimental diabetes. A pulse-chase approach in retinal explants was implemented in order to measure the degradation of newly-synthesized synaptophysin. Total synaptophysin degradation was measured using CHX treatment in retinal explants. The degradation of newly-synthesized synaptophysin was accelerated after 1 and 2 months of STZ-diabetes while total synaptophysin depletion was accelerated only after 2 months.

Previous studies provide indirect evidence that accelerated degradation is responsible for retinal synaptophysin loss during diabetes. Angiotensin II, the final product of the renin angiotensin system (RAS), increases in human surgical samples from eyes with DR (Funatsu et al., 2002). The RAS has already been implicated in diabetes induced retinal vascular complications such as leukocyte adhesion in diabetic C57BL/6 mice using concanavalin A lectin perfusion labeling, and impaired blood flow in STZ-diabetic rats using video fluorescein angiography (Horio et al., 2004; Nagai et al., 2007). It is also involved in the loss of synaptophysin in retinal neurons from diabetic mice. Blocking its receptor with inhibitors valsartan and telmisartan in 6-week diabetic C57BL/6 mouse retina leads to reversal of oscillatory potential changes measured by ERG and reduction of synaptophysin content (Kurihara et al., 2008). Treating neuronal
PC12D cells with angiotensin II results in enhanced poly-ubiquination of synaptophysin and this effect is attenuated by inhibition of the receptor (Kurihara et al., 2008). Apart from the role of angiotensin II, these findings also implicate degradation as a mechanism of synaptophysin loss in the retina during diabetes. In this study, a more direct measurement of this pathway was made using a retinal explant system.

One and two months of diabetes significantly depleted the newly-synthesized $^{35}$S-synaptophysin compared to controls. Degradation of total synaptophysin in 2-month diabetic rats was also accelerated, but did not become significant in retinas from 1-month diabetic rats, at least during the short 2 hr time course used for this study, suggesting that the rate of degradation of total synaptophysin increases with extended duration of diabetes. Together with the findings from Chapter 3, the data suggest that the mRNA translation for synaptophysin is elevated within the first month of diabetes, but that there is also an increase in its degradation during an early stage of protein maturation, possibly during its post-translational processing. The poly-ubiquination of synaptophysin in previous cell culture studies (Kurihara et al., 2008) suggests that the ubiquitin proteasome system (UPS) may be the mode of this increased degradation. While the proteasome is able to degrade transmembrane proteins if they are extracted from the membrane into the cytosol, lysosomal degradation is also likely for most membrane proteins (Luzio et al., 2007; Nakatsukasa et al., 2008; Tai and
Schuman, 2008). Hence, the mode of retinal synaptophysin degradation reported here is not yet known.

The content of proteasomal subunits was measured in retinas and isolated synaptosomes from 1-month diabetic and control rats. Since synaptophysin degradation is accelerated by diabetes, and previous work implies that the protein is degraded via the proteasome, it is important that STZ-diabetes does not alter the content of proteasomal α subunits; which serve as docking domains for the regulatory particles and form a gate that blocks unregulated access of substrates to the interior cavity (Smith et al., 2007). The retinal proteasome α subunits were detectable but, as expected, not different between diabetic and control rats, indicating that diabetes did not alter proteasome content in the retina. α subunits were also detected in isolated retinal synaptosome. This finding suggests that the proteasome is present at the presynaptic terminal in the retina of diabetic and control rats. This is particularly relevant for synaptophysin as it susceptible to degradation soon after mRNA translation and upon reaching full functionality at the presynaptic terminal.

The increased degradation of newly-synthesized $^{35}$S-synaptophysin after 1 month of diabetes could be a result of irregular processing of the glycoprotein in the ER and/or Golgi apparatus, because failure of glycoproteins to fold correctly during post-translational processing can trigger early degradation (Molinari et al., 2003). Folding and proper processing of a glycoprotein is dependent on its
glycosylation soon after mRNA translation. Increases in synaptophysin mRNA translation in the retina after diabetes prompted an investigation into synaptophysin glycosylation. Soon after mRNA translation, synaptophysin is N-glycosylated in the lumen of the ER with a 14 sugar molecule consisting of 2 N-acetylglucosamine (GlcNAc), 9 mannose, and 3 glucose residues (Rehm et al., 1986). The mannose rich synaptophysin is then exported and processed through several compartments of the Golgi apparatus. In the trans Golgi, the removal of 2 mannose residues by Golgi α-mannosidase II is the final step in the transformation of synaptophysin from its ‘immature” form into a highly complex N-glycan (Cutler and Cramer, 1990). The “mature” synaptophysin is then released from the trans Golgi. A disruption in this process could trigger an early degradation mechanism such as ER-associated degradation (ERAD) (Nakatsukasa et al., 2008).

While endo H reduces the molecular weight of the protein by specifically cleaving asparagine-linked mannose-rich oligosaccharides from N-glycosylated proteins, peptide-N-glycosidase F (PNGase F) is an amidase that cleaves oligosachrides of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Maley et al., 1989). The reasons for the specificity of these enzymes towards different N-glycosylated proteins are not abundantly clear. However, a very early biochemistry study suggests that a single structural addition to the oligosaccharide results in the insensitivity of a glycosylated protein to Endo H. The addition of an (α1→6)-linked fucose (a hexose deoxy sugar) to
the proximal core GlcNAc completely blocks the action of Endo H on complex N-glycans (Tarentino and Maley, 1975) (Figure 21A). The fucosyltransferase which adds fucose to growing oligosaccharide chains is present in the Golgi apparatus, and the addition of fucose is part of the terminal processing event during the transformation of a N-glycosylated protein from its “immature” form into a highly complex N-glycan (Kornfeld and Kornfeld, 1985). PNGase F, however, cleaves between the innermost GlcNAc and asparagine residues, enabling it to cleave all types of N-glycosylated proteins (Maley et al., 1989) (Figure 21B). Therefore, mannose-rich and mature synaptophysin are both sensitive to deglycosylation by PNGaseF. This enzyme was used in this study to measure changes in overall N-glycosylation of synaptophysin.

Since synaptophysin is significantly reduced in retinas from STZ-rats compared to controls, PNGase F-sensitive synaptophysin was also significantly reduced by diabetes. The more important interpretation here is that all retinal synaptophysin in diabetic rats and age-matched controls is N-glycosylated (Figure 18). Non-N-glycosylated synaptophysin would not be sensitive to PNGase F. Hence, hyperglycemia does not cause retinal synaptophysin to be glycosylated through a different mechanism nor does it cause the protein to skip the glycosylation process all together. “Immature” glycosylated synaptophysin was significantly increased in rat retinas after 1 month of STZ-diabetes compared to age-matched controls (Figure 19). The accumulation of mannose-rich synaptophysin could be
due to improper post-translational processing of the protein. It is speculated that this irregularity increases the activity of synaptophysin degradation mechanisms.

Figure 21. Mannose-rich (immature) N-glycosylated proteins are sensitive to Endo H deglycosylation.

A) Endo H cleaves between the two GlcNAc residues of the oligosaccharide, generating a truncated sugar molecule with one GlcNAc residue remaining on the protein. B) PNGase F, however, cleaves between the first GlcNAc residue and the protein. The addition of a fucose to the first GlcNAc residue (found primarily on complex N-glycosylated proteins) blocks Endo H action on the N-glycosylated protein.
ER stress is a possible explanation for the observed results in this study. Once proteins have entered the ER, protein folding followed by glycosylation and disulfide bond formation commences. Proteins unable to fold correctly cause ER stress and activate the unfolded protein response (UPR). Activation of the UPR transcriptionally upregulates an array of genes required for protein folding, ER expansion, ER-Golgi trafficking, and ERAD, which all act collectively to relieve stress within the ER (Travers et al., 2000). ERAD is a three-step process that involves 1) identification of misfolded/misprocessed proteins in the ER, 2) translocation of these proteins to the cytoplasm, and 3) protein degradation via the ubiquitin-proteasome system (UPS) (Schroder and Kaufman, 2005). Studies have provided ample evidence that ER stress causes retinal vascular and neuronal cell death in DR (Shimazawa et al., 2007; Li et al., 2009). Multiple ER stress markers, including 78 kDa glucose-regulated protein (GRP78), and phospho-eIF2α are significantly upregulated in the retinas of animal models of type 1 diabetes and oxygen-induced retinopathy (Li et al., 2009; Yan et al., 2012). Diabetes increases the activity of inositol-requiring enzyme 1α (IRE1α) and PKR like ER kinase (PERK), two major transmembrane transducers sensing ER stress, in retinas of Ins2\(^{Akita}\)-mice compared to wild-type litter mates (Li et al., 2009). The results presented in the appendix of this dissertation do not demonstrate the presence of ER stress in the retinas from 1-month STZ-diabetic rats (Figure 28). Defective ERAD, however, is a stronger possibility. This phenomenon has been reported in pancreatic tissue from humans with type 2
diabetes, further complicating the already compromised β-cell function (Laybutt et al., 2007; Costes et al., 2011). In the retina, it is clear that immature synaptophysin is vulnerable to accelerated degradation mechanisms during glycosylation in the ER after 1 month of STZ diabetes. Therefore, the corrective ability of retinal ERAD may be altered during diabetes, causing this rapid degradation to occur.

The depletion of mannose-rich synaptophysin in retinas from diabetic rats over the 2 hr time course was not altered compared to controls in the 1- and 2-month study. The time constraint of 120 min (due to the increase in the cell death after 240 min of incubation reported in Figure 7) is the most probable cause for the lack of a significant difference between the two groups. Further, subtle changes in degradation may not be detectable by the assay. It was speculated that inducing a greater accumulation of mannose-rich synaptophysin, ex vivo, would make the assay more sensitive to changes in degradation rates within the restricted time course. By demonstrating this concept, a more definitive argument for the involvement of disrupted N-glycosylation in the eventual loss of synaptophysin during diabetes can be made (Chapter 5).

Incubation with the enzyme endo H, identified the transient mannose rich form of synaptophysin. While the increase in endo H sensitive synaptophysin after 1-month of diabetes could explain the accelerated degradation of $^{35}$S-synaptophysin at this time point, the lack of change in endo H-sensitive
synaptophysin in the retinas of 2-month diabetic rats does not. These data suggests that early post-translational degradation occurs in 1-month animals as a result of excessive mannosylation, while the reduced content of synaptophysin after 2-months may be triggered by a different mechanism. One possible explanation is the mRNA transcript levels of synaptophysin, previously shown to be reduced after 3 months of STZ-diabetes compared to age-matched controls (VanGuilder et al., 2008).

In conclusion, the data presented in this chapter suggest that mechanisms of synaptophysin catabolism and its post-translational processing machinery in the rat retina are compromised by experimental diabetes. The reduction of synaptophysin is likely due to overcompensation by its degradation mechanism in response to the accumulation of “immature” glycosylated synaptophysin content. It is possible that diabetes significantly affects factors responsible for the de-mannosylation of mannose-rich retinal synaptophysin. This concept is explored in Chapter 5, by further investigating synaptophysin availability and degradation due to disrupted post-translational glycosylation in the rat retina.
Chapter 5

Reduced α-Mannosidase Activity Alters Post-Translational Processing and Expression of Retinal Synaptophysin
5.1. Introduction

N-linked glycosylation is initiated by the transfer of a mannose-rich oligosaccharide from a membrane-bound dolichol phosphate anchor to a asparagine-X-Serine/Threonine residue in the newly-synthesized polypeptide chain (Kornfeld and Kornfeld, 1985). The protein shuttles through several cycles of de- and reglucosylation by α-glucosidase II and uridine diphosphate (UDP)-glucose:glycoprotein glucosyl transferase (UGGT) activities (Ellgaard et al., 1999). UGGT preferentially recognizes the unfolded conformation. The protein then undergoes de-mannosylation by α-mannosidase I (Ellgaard et al., 1999). If folded correctly, the mannose-trimmed protein is exported to the Golgi complex for further de-mannosylation. An important step in the de-mannosylation process occurs when Golgi α-mannosidase II removes two mannose residues from GlcNAc1Man5GlcNAc2 to form GlcNAc1Man3GlcNAc2 (Tulsiani et al., 1982b; Moremen et al., 1994). The removal of these two mannose residues is also the final step in the transformation of synaptophysin from its mannose-rich transient state into a complex N-glycan (Cutler and Cramer, 1990; Akama et al., 2006).

Interruptions, abnormal folding, or reduced activity of enzymes responsible for de-mannosylation could jeopardize the expression of mature and functional synaptophysin. Based on the results from Chapter 4, it was hypothesized that diabetes induced accumulation of mannose-rich synaptophysin in the retina is caused by disrupted post-translational N-glycosylation. To test for this irregularity
α-mannosidase content and activity was measured in the retina. Golgi α-mannosidase II content was quantified in lysates from STZ-diabetic rats compared to control rats using western blot analysis. A colorimetric assay was used to measure α-mannosidase activity. There was no change in Golgi α-mannosidase II expression in the rat retina. However, the data showed that diabetes reduced retinal α-mannosidase activity. Further, total synaptophysin and mannose-rich synaptophysin were quantified in whole retinas explanted from healthy rats treated with an α-mannosidase inhibitor, swainsonine (Sw). The results suggested that inhibiting α-mannosidase had a similar effect on the content of synaptophysin and its mannose-rich form as diabetes.

In order to determine if the reduced α-mannosidase activity altered synaptophysin depletion mechanisms, the degradation of synaptophysin and its mannose-rich form were measured in the presence of Sw (Figure 22). The results showed that inhibition of α-mannosidase accelerates the degradation of mannose-rich synaptophysin. Together, the findings in this chapter suggest that rapid degradation of mannose-rich synaptophysin is due to disrupted N-glycosylation. This dysfunction is likely responsible for the previously observed synaptophysin reduction in the retina during diabetes.
Figure 22. Synaptophysin degradation was measured in control retinas during α-mannosidase inhibition.

Retinal explants were incubated in CHX and Sw for 30, 60, and 120 min. Lysates were then treated with or without Endo H and western blot analyses were performed for synaptophysin.
5.2. Retinal Golgi α-Mannosidase II Content is Unaltered in STZ-Rats

Golgi α-mannosidase II content was measured in retinal lysates from 1- and 2-month STZ-diabetic rats and age-matched controls. The western blot analysis revealed an expected single 91 kDa band (Figure 23A). The content of retinal golgi α-mannosidase II was not different between lysates from 1-month diabetic rats compared to controls (Figure 23B).

5.3. Retinal α-Mannosidase Activity is reduced in STZ-Rats

Retinal α-mannosidases, a family of mannosidases that includes Golgi α-mannosidase II, are important for proper N-glycosylated synaptophysin processing. α-mannosidase activity was measured in retinal lysates from 1-month STZ-diabetic and age-matched control rats using a colorimetric activity assay with (p-Nitrophenyl α-D-mannopyranoside) PNP-man as a substrate. α-mannosidase activity was significantly reduced in retinal lysates from 1-month STZ-diabetic rats compared to age-matched controls (3.37 pmol/min/μg vs. 4.18 pmol/min/μg) (p<0.05;Figure 23C).
Figure 23. Diabetes reduces α-mannosidase activity in the retina.

A) Western blot analysis revealed a single 91 kDa band. (B) Retinal Golgi α-mannosidase II content was not significantly different in 1 and 2-month STZ-diabetic rats compared to respective controls (n=8). C) α-mannosidase activity was significantly decreased in retinal lysates from 1-month STZ diabetic rats compared to controls (n=8). *p<0.05

5.4. Inhibition of Retinal α-Mannosidase

5.4.1. Swainsonine Inhibits α-Mannosidase Activity in Retinal Lysates

α-Mannosidase activity was inhibited with the reversible competitive drug, swainsonine (Sw). The basal activity of α-mannosidase in untreated lysates was 4.52 pmol/min/µg. Activity was reduced to 2.69 pmol/min/µg in lysates treated with 50 µM of Sw, and 1.83 pmol/min/µg in lysates treated with 400 µM of Sw (Figure 24).
Figure 24. Swainsonine inhibits α-mannosidase activity in retinal lysates.

α-Mannosidase activity was reduced to 2.69 pmol/min/μg by 50 μM of Sw and further reduced to 1.83 pmol/min/μg by 400 μM of Sw. α-Mannosidase activity in untreated retina lysates was 4.52 pmol/min/μg (n=3).

5.4.2. α-Mannosidase Inhibition Reduces Retinal Synaptophysin Content

Retina explants were treated with Sw (50 μM) for 30 min. Lysates from the explants were treated with and without endo H and western blotted for synaptophysin. Retinas treated with Sw had significantly less synaptophysin compared to controls (p<0.001; Figure 25A, B). In contrast, Sw-treated retinas had significantly more endo H-sensitive synaptophysin compared to controls (p<0.05; Figure 25A, B). Sw treatment did not significantly change synapsin I or β-actin content (Figure 25A, B).
Figure 25. Inhibition of α-mannosidase activity reduces total synaptophysin and increases endo H-sensitive synaptophysin content in the rat retina.

A) Retina explants incubated in Sw were treated with or without endo H. Syn resolved as a single 38 kDa band, endo H treated samples had an additional band at 35 kDa, Synapsin I resolved as a 80kDa double band. B) There was significantly less Syn in Sw-treated (Sw(+)) samples compared to controls (Sw(-)) (n=4; *p<0.001), while there was significantly more endo H-Syn compared to Sw(-) (n=4; *p<0.05). There was no significant difference in Synapsin I and β-actin content in Sw(+) retinas, compared to Sw(-).
5.4.3. \( \alpha \)-Mannosidase Inhibition Accelerates Mannose-Rich Synaptophysin Degradation in the Rat Retina

Rat retinas were treated with or without Sw for 30, 60, or 120 min, after CHX was used to block \textit{de novo} protein synthesis. Endo H-sensitive synaptophysin and synaptophysin were quantified by western blot analysis. There was no significant reduction of total synaptophysin content in Sw-treated retinas after 120 min of CHX inhibition (Figure 26A, B). It should be noted that when the data from the 30 min time point were expressed as \% Sw (-), there was significantly less synaptophysin in the Sw (+) group compared to the Sw (-) group (Sw (-): 100.0 ± 2.66\%, Sw(+): 72.5 ± 0.42\%, \( p<0.001 \)), as in the previous experiment. There was significantly less endo H-sensitive synaptophysin in Sw-treated retinas compared to controls during the same time period (\( p<0.05 \); Figure 26C, D). Note also that when the 30 min time point data were expressed as \% Sw (-), there was significantly more Endo H-Syn in the Sw (+) group compared to the Sw (-) group (Sw (-): 100.0 ± 6.79\%, Sw (+): 139.8 ± 1.41\%, \( p<0.001 \)), further confirming results from Figure 19.
Figure 26. Inhibition of α-mannosidase accelerates the degradation of endo H-sensitive synaptophysin.

Syn and endo H-Syn content was measured by immunoblot analysis of retinas incubated in CHX with or without the addition of Sw for 30, 60, or 120 min. A) Syn resolved as a 38 kDa band and β-actin resolved at 47 kDa. B) There was no significant difference in Syn content due to Sw after 120 mins of CHX (n=4). C) Endo H-Syn resolved as an additional 35 kDa band below the 38 kDa Syn band. β-actin resolved at 47 kDa and its content was not significantly altered by Sw treatment. D) There was significantly less endo H-Syn in Sw(+) retinas compared to control (Sw(-)) after 120 min of CHX (n=4; *p<0.05).
5.5. Discussion

Diabetes causes the accumulation of immature synaptophysin in the rat retina. The colorimetric assay used in this study revealed that the activity of α-mannosidases responsible for de-mannosylating the immature form of the protein is also reduced. In order to demonstrate that these two events cause the eventual loss of mature synaptophysin, healthy retinal explants were treated with an α-mannosidase inhibitor and the effects on synaptophysin content and degradation were measured. The results suggest that the inhibition of α-mannosidase has a similar affect on retinal synaptophysin as diabetes.

N-glycosylation in the ER and Golgi apparatus is an important step in the production of the functional form of an N-glycosylated protein. This post-translational modification serves several purposes in the protein folding process. First, due to the hydrophilic nature of carbohydrates, glycosylation increases the solubility of glycoproteins and defines the attachment area for the surface of the protein. Second, due to their large hydrated volume oligosaccharides shield the attachment area from surrounding proteins. Third, oligosaccharides interact with the peptide backbone and stabilize its conformation (Wormald and Dwek, 1999). While there is no substantial evidence of any N-glycosylation mechanism being disrupted in the retina of diabetic rodents, recent investigations indicate increases in the activity of major ER stress sensing proteins in retinas of diabetic mice (Li et al., 2009). This suggests that diabetes may induce subtle irregularities
in the machinery responsible for N-glycosylation, making the mechanism an important one to investigate in the context of retinal synaptophysin loss during diabetes.

Synaptophysin is N-glycosylated in the lumen of the rough endoplasmic reticulum (ER) soon after its mRNA is translated (Cutler and Cramer, 1990; Atkins et al., 1999). Its transient mannose-rich form undergoes several enzymatic steps in the ER and Golgi apparatus, including de-mannosylation. The final complex N-glycan form of synaptophysin is released from the Golgi, embedded in SV membranes (Griffiths et al., 1999). Western blot and immunohistochemistry studies indicate that experimental diabetes reduces synaptophysin content in retinas of Sprague-Dawley rats (VanGuilder et al., 2008). In Chapter 4, the relative portion of mannose-rich synaptophysin was estimated using endo H, which reduces the molecular weight of the protein by specifically cleaving asparagine-linked mannose-rich moieties (Fujita et al., 2000). The results suggest that the content of mannose-rich synaptophysin was significantly elevated in retinas from 1-month diabetic rats compared to age-matched controls (Figure 19).

Since, α-mannosidase activity was found to be reduced by diabetes, it was hypothesized that α-mannosidase inhibition increases the content of mannose-rich synaptophysin in the rat retina. Inhibition of this enzyme using a specific small molecule inhibitor, swainsonine (Sw) (Tulsiani et al., 1982a; Bucan et al., 2009), reduced the expression of mature synaptophysin, while increasing the
content mannose-rich synaptophysin. Also, the inhibition did not alter non-glycosylated synapsin I and actin expression. These observations suggest that the diabetes-induced accumulation of mannose-rich synaptophysin in the retina is likely caused by suppressed α-mannosidase activity.

The rapid removal of mannose-rich synaptophysin would be necessary in order to prevent further accumulation due to α-mannosidase inhibition. It was, therefore, hypothesized that α-mannosidase inhibition accelerates the degradation of mannose-rich synaptophysin in the rat retina. Degradation was measured in the presence of CHX to stop de novo protein synthesis. The results show that mannose-rich synaptophysin was depleted significantly faster over 120 min compared to controls. These data imply that reduced α-mannosidase activity accelerates an early degradation mechanism for the mannose-rich synaptophysin. The depletion of synaptophysin over 120 min, however, was not altered by inhibition of α-mannosidase with Sw. Although there was no significant change in the degradation rate of mature synaptophysin, it may be that over a period of time longer than 120 min the trend towards reduced synaptophysin content would also become significant, due the reduced expression of the mature form of the protein that is released from the Golgi.

Sw is a specific inhibitor of α-mannosidase and does not inhibit other acid glycosidases including β-mannosidase, a glycosidase commonly localized in organelles such as lysosomes (LaBadie and Aronson, 1973; Kang and Elbein,
More specifically, Sw inhibits α-mannosidase II, which removes the α-(1→3) and α-(1→6) mannose residues from the GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub> protein, but Sw has no effect on the mannosidases that remove the first four α-(1→2) linked mannose units (Tulsiani et al., 1982a). Therefore, the results of this study are most likely due to inhibition of Golgi α-mannosidase II activity.

Irregular post-translational processing of synaptophysin could be an indication of ER stress. There is published evidence for ER stress in the retinas of other diabetic models. The activity of inositol-requiring enzyme 1α (IRE1α) and PKR like ER kinase (PERK), two major transmembrane transducers sensing ER stress, in retinas of diabetic Ins2<sup>Akita</sup>-mice compared to wild-type litter mates (Li et al., 2009). Regardless of the ER stress level, disrupted N-glycosylation protein processing could initiate quality control response mechanisms, including but not limited to rapid protein degradation (Wang and Hebert, 2003; Nakatsukasa et al., 2008). It is unclear if ER stress is responsible for the altered post-translational processing of synaptophysin reported here, but it is one potential explanation. Furthermore, the observed reduction in retinal α-mannosidase activity during the first month of STZ-diabetes could mark the beginnings of broader ER dysfunction as the disease progresses.

Several previous studies have reported diminished expression of retinal synaptophysin in diabetes (Kurihara et al., 2008; VanGuilder et al., 2008; Gaspar et al., 2010; Jiang et al., 2010; Sasaki et al., 2010). The results presented here
suggest that expression of the protein in the retina is determined by the activity of α-mannosidase during the late stages of post-translational processing. Inhibition of α-mannosidase in retinas from healthy rats had a similar effect on the glycosylation and expression of synaptophysin as the induction of STZ-diabetes. Therefore, reduced activity of α-mannosidase and the consequent acceleration of mannose-rich synaptophysin degradation is a mechanism for the reduction of this presynaptic protein in the retina during diabetes.
Chapter 6

Closing Discussion
6.1. Diabetes Alters Mechanisms of Retinal Synaptophysin Metabolism

The research presented in this dissertation details potential mechanisms responsible for the loss of the presynaptic protein, synaptophysin, in the retina of experimentally diabetic rats. mRNA translation, N-glycosylation, and protein degradation were identified as potential pathways affected by diabetes. Post-transcriptional mechanisms were specifically investigated because previous work suggested that during the first month of STZ-diabetes the relative mRNA levels of synaptophysin were unaltered compared to controls (VanGuilder et al., 2008). In order to determine if the normal function of these molecular pathways was affected by diabetes, an ex vivo approach was used. Retina samples were obtained from STZ-diabetic and age-matched control Sprague Dawley rats. As opposed to an in vivo model, the use of explanted retinas to measure mRNA translation and degradation was advantageous in that the samples were exposed to fewer uncontrolled variables.

The aim of this dissertation was to identify post-transcriptional mechanisms of retinal synaptophysin loss during diabetes and then determine if these mechanisms coordinate in any way to bring about this reduction. The results suggest that mRNA translation, N-glycosylation, and degradation of retinal synaptophysin were all altered by STZ-diabetes. However, the effect of diabetes on these mechanisms was not uniform. The turnover of synaptophysin was significantly different in retinas from diabetic animals compared to controls. It was
hypothesized that STZ-diabetes reduces the mRNA translation and accelerates the degradation of retinal synaptophysin. While the data indicated that degradation was faster due to diabetes, synaptophysin mRNA translation also increased in the rat retina (Figure 27A). Further, the mRNA translation of all rapidly synthesized proteins was increased in retinas from diabetic rats compared to controls. Increased mRNA translation was unexpected and the true reason for this change is unknown. One possible explanation is that diabetes may induce a shift in the steady state of rapidly synthesized proteins, including synaptophysin, however there is no robust evidence for this in the literature. The only other recent study to report an increase in mRNA translation of a selective protein due to acute hyperglycemia was done in kidneys from STZ-mice. In that study, VEGF protein but not mRNA was increased in renal cortex of hyperglycemic mice, suggesting that regulation of VEGF synthesis occurs at the level of mRNA translation, rather than gene transcription. A polysome assay confirmed that VEGF mRNA translation initiation is increased by hyperglycemia (Day et al., 2010). However, these measurements were made within the first week of STZ-diabetes in the mice. Using a luciferase-based assay, another recent study in livers from STZ-mice suggests that diabetes causes a downregulation of cap-dependent mRNA translation and simultaneous increase in cap-independent mRNA translation (Dennis et al., 2013). Therefore, it can also be speculated that the mRNA translation detected in the explant assay is largely cap-independent. Lastly, the elevated synaptophysin mRNA translation in 2-
month STZ-rats suggests that this mechanism is likely not responsible for reduced retinal synaptophysin expression in diabetes.

In the degradation studies, the data showed that newly-synthesized synaptophysin is degraded rapidly in the retina during diabetes (Figure 27C). Given that synaptophysin is an N-glycosylated protein that undergoes several quality control checkpoints in the ER and Golgi, it was important to determine if this accelerated degradation was exclusive to the newly-synthesized polypeptide. Interestingly, the non-radioactive explant assay (using CHX) suggested that total synaptophysin depletion is not affected by diabetes at the 1-month time point, but it is accelerated at 2 months compared to control. This finding implied that the degradation mechanisms of total and newly-synthesized synaptophysin are regulated differently. In order to determine if faulty glycosylation was responsible for the accelerated degradation of newly-synthesized synaptophysin, the content of the mannose-rich (immature) form of the protein was quantified in diabetic and control animals. Furthermore, the enzymatic activity of \(\alpha\)-mannosidases responsible for converting this immature form to mature protein was measured. The data indicated that immature synaptophysin accumulated in the rat retina after 1 month of diabetes (Figure 27B). However, this was not the case at the two month time point. As expected, 1 month of diabetes also reduced the \(\alpha\)-mannosidase activity in the retina. Firstly, these results imply a subtle but significant dysfunction in the overall N-glycosylation process in the retina. Disrupted protein processing through the ER and Golgi as a result of reduced \(\alpha\)-
mannosidase activity could significantly impact a broad range of N-glycosylated proteins. Secondly, immature synaptophysin accumulation due to reduced α-mannosidase activity could explain the accelerated depletion of the newly-synthesized protein in the retina. Therefore, it was postulated that inhibited α-mannosidase activity increases immature synaptophysin content in the rat retina and accelerates its degradation.

In order to test this hypothesis, whole retinas explanted from healthy rats, treated with or without an α-mannosidase inhibitor, were probed for total and immature synaptophysin. Synaptophysin content was reduced due to α-mannosidase inhibition while the immature form of the protein accumulated in the retina, replicating the effects of diabetes. Also, the disruption of N-glycosylation in the retina sped up immature synaptophysin degradation. These data imply that the diabetes-induced increase in immature synaptophysin and reduced α-mannosidase activity cause the rapid depletion of synaptophysin before the protein reaches its functional stage.

Taken together, the work detailed in this dissertation conclusively demonstrates that diabetes inhibits α-mannosidase activity in the retina, causing a disruption in overall post-translational N-glycosylation. This irregularity results in an accumulation of mannose rich (immature) glycosylated synaptophysin, most likely in the rough ER and/or Golgi apparatus. Another possibility is that immature synaptophysin is accumulated due to increased synaptophysin mRNA translation.
during the same time point of diabetes. The accelerated degradation of immature synaptophysin is an attempt by a quality control mechanism to dispose of excess immature synaptophysin that could potentially harm or stress the ER. These events are collectively responsible for the previously observed net reduction in retinal synaptophysin during diabetes. It is also important to note that synaptophysin degradation resulting from disrupted N-glycosylation may only occur during the first month of STZ-diabetes, since the data show that immature synaptophysin is not accumulated in the rat retina at the 2-month time point. Other mechanisms that were not investigated in this work may have a role in synaptophysin reduction as well, particularly in later stages of the disease. The different outcomes for experiments performed in 1- and 2-month groups (Table 4) further suggest that pathways that regulate synaptophysin in the retina are affected by diabetes in a time dependent manner.
Figure 27. Diabetes alters mechanisms of retinal synaptophysin metabolism.

A) Synaptophysin is rapidly translated by the ribosome at the Rough ER. Diabetes increases retinal synaptophysin mRNA translation. B) Newly-synthesized synaptophysin undergoes N-Glycosylation in the ER and is de-mannosylated by α-mannosidases in the ER and Golgi. Golgi α-mannosidase II converts mannose-rich (immature) synaptophysin to its complex N-glycan (mature) form. Diabetes causes immature synaptophysin to accumulate in the retina. Golgi α-mannosidase II activity is also reduced. C) Irregularities in the N-glycosylation process triggers a rapid degradation of newly-synthesized synaptophysin, possibly via the proteasome.
Table 4. Experimental outcomes after 1 and 2 months of diabetes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1 month</th>
<th>2 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synaptophysin Content</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>mRNA Translation</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>Synaptophysin mRNA Translation</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Newly-synthesized Synaptophysin Degradation</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Total Synaptophysin Degradation</td>
<td>–</td>
<td>↑</td>
</tr>
<tr>
<td>Mannose-Rich Synaptophysin Content</td>
<td>↑</td>
<td>–</td>
</tr>
<tr>
<td>α-Mannosidase Activity</td>
<td>↓</td>
<td>N/A</td>
</tr>
</tbody>
</table>

(↑: increase, ↓: decrease, -: no change, N/A: did not test).

6.2. Implications for Vision Loss

These studies have identified mechanisms of diabetes-induced synaptophysin loss in the retina. Previous work has indicated that the retina may be particularly sensitive to this deficit. Transcriptional analysis of retinas from synaptophysin-null animals suggests the absence of any compensatory gene expression due to synaptophysin loss (Bai et al., 2006). Thus, disrupted synaptophysin expression with diabetes could potentially have a significant effect on photoreceptor function in the retina and lead to compromised vision. The results in this dissertation advance knowledge of the metabolism of synaptophysin in retinas from diabetic rats. With this new information, several avenues of retinopathy research can be pursued, with the eventual goal of identifying molecular targets to rescue synaptophysin loss. However, the functional importance of synaptophysin, and several other presynaptic proteins reduced during diabetes, is yet to be clearly
defined. With that in mind, future approaches must be designed to address this issue in addition to investigating molecular pathways that regulate presynaptic proteins in the retina.

6.3. Potential Future Directions

6.3.1. Impact of Retinal Synaptophysin Reduction

The functional consequence of a reduction in synaptophysin expression in the retina is not yet established. However, there is abundant evidence that the retinal neurological function and visual ability of rodents with experimental diabetes is compromised, as demonstrated by ERG and behavioral optokinetic studies, respectively. Measureable changes in presynaptic components resulting from synaptophysin loss will greatly increase the clinical significance of the findings reported in this dissertation. Moreover, future studies examining the functional relevance of synaptophysin knockdown in the retina will specifically determine if the protein plays an important role in preserving healthy vision.

6.3.1.1. Neurotransmitter Vesicle Morphology and Quantity

Synaptophysin is suggested to regulate endocytosis to ensure vesicle availability during and after sustained neuronal activity (Kwon and Chapman, 2011). Primary cortical cultures derived from synaptophysin knock-out mice exhibited a more diffuse distribution of VAMP-2, suggesting that the synaptophysin is required for
normal SV targeting at the synaptic terminal (Gordon et al., 2011). In the retina, alterations in SV number and morphology occur in rod photoreceptors of synaptophysin-deficient mice (Spiwoks-Becker et al., 2001). Synaptophysin loss in the retina during diabetes may be particularly important for the maintenance of proper neurotransmitter transport at the presynaptic terminal. Rod photoreceptors in rodents are known to lack synaptoporin, a protein closely related to synaptophysin and overlapping its function (Spiwoks-Becker et al., 2001). Given the reduction of retinal synaptophysin in diabetic rats, it can be hypothesized that diabetes alters SV size and quantity in the rat retina. To measure such changes, electron microscopy techniques can be employed on retinas from diabetic and control rats. This approach, however, will not specifically test synaptophysin involvement in diabetes-induces changes in SVs.

6.3.1.2. Visual Function

Although previous studies have shown that rescued synaptophysin expression is accompanied by improved ERG (Fujita et al., 1999; Kurihara et al., 2008), the significance of synaptophysin in retinal function is not yet understood. Mouse hippocampal function has been measured in the absence of synaptophysin and its compensatory family members. The synaptogyrin/synaptophysin double knockout mouse displays major changes in the regulation of neurotransmitter release and impaired LTP (Janz et al., 1999). Currently, it is clear that the neurological function of the retina is compromised in rodents with experimental
diabetes, because their ERG and scotopic threshold response are diminished (Hancock and Kraft, 2004; Kohzaki et al., 2008). In contrast to expected outcomes, ERG studies in synaptophysin knockout mice have shown no change in scotopic single-flash ERG amplitudes (representing rod function only at low stimulus intensities). Furthermore, the b-wave recovery after light adaptation was not different between knockout and wild-type mice, suggesting that the absence of synaptophysin does not induce any changes in photoreceptor function during dark adaptation (Spiwoks-Becker et al., 2001). The literature goes on to explain that the lack of detectable change in these studies emphasizes the limitation of the ERG method in measuring specific photoreceptor function. While several additional ERG measurements can be made to address this issue, synaptophysin’s role in the maintenance of vision will still be unclear. To test the hypothesis that synaptophysin loss impairs vision, alterations in visual acuity and contrast sensitivity thresholds can be measured. The visual acuity threshold is known to be significantly reduced 1 month after the induction of diabetes in Long Evans rats, suggesting that diabetes impairs vision (Johnsson, 1959). Changes in optokenetic responses in knockout mice can be interpreted as impaired vision ability due to synaptophysin absence. This study will likely strengthen the clinical relevance of the previously observed synaptophysin reduction during diabetes and highlight the importance of rescuing the protein to preserve vision.
6.3.2. ER Associated Degradation in Diabetic Rats

6.3.2.1. EDEM and Calnexin

The ER possesses a quality-control mechanism that correctly identifies folded proteins from misfolded proteins (Fewell et al., 2001). The misfolded proteins are diverted to the cytosol and degraded by the UPS in a process known as ER-associated degradation (ERAD) (Kopito and Sitia, 2000). The studies in this dissertation suggest that retinal synaptophysin is accumulated in the ER during diabetes. Consequently, the newly-synthesized protein undergoes an early and rapid degradation. While it is unknown if this is due to misfolding, it is likely that ERAD mechanisms are involved in this process. It has been established that ER degradation-enhancing α-mannosidase-like protein (EDEM) is an ER stress inducible membrane protein with homology to α-mannosidase but lacking mannosidase activity (Oda et al., 2003). Specifically, in vitro studies have shown that EDEM forms a complex with calnexin (CNX), another ER protein. During the interaction, a mannose rich protein may be transferred from CNX to EDEM, followed by translocation to the cytosol for degradation by the proteasome (Molinari et al., 2003). Overexpression of EDEM greatly accelerates this process (Hosokawa et al., 2001).

During diabetes, the specifics of ERAD function are not clearly understood. Using the new knowledge from this dissertation, a further investigation into EDEM interaction and consequent proteasomal degradation in experimental diabetes
can be carried out to test the hypothesis that diabetes increases EDEM expression and complex formation with CNX in the retina. Co-immunoprecipitation studies can be employed to investigate the formation of the EDEM/CNX complex in retinas from STZ-diabetic rats. Since synaptophysin appears to be a strong candidate for ERAD, the protein’s interaction with EDEM can also be examined in this model. Further, investigating the role of CNX in mannose-rich synaptophysin degradation will provide additional insight into the mechanism of retinal synaptophysin reduction during diabetes. Synaptophysin likely interacts with CNX during its post-translational N-glycosylation. The use of alkaloids such as castanospermine has proven to be very effective in disassociating misfolded proteins from CNX (Molinari et al., 2003). An ex vivo study on the degradation of mannose rich synaptophysin using explants from diabetic rats treated with and without castanospermine may confirm CNX’s role in synaptophysin loss. Inhibiting CNX binding ability should slow the degradation of mannose-rich synaptophysin in the retina during diabetes.

6.3.2.2 The Ubiquitin Proteasome System

Studies on ERAD function in the retina may renew or strengthen interest in the role of the UPS in retinal synaptophysin loss. However, attempts to co-immunoprecipitate synaptophysin with ubiquitin to demonstrate that the UPS is indeed the mode of synaptophysin degradation in the rat retina have been technically challenging and unsuccessful thus far. The development of plate
based assays may provide a more suitable alternative in tackling this issue. Angiotensin II enhanced poly-ubiquinated synaptophysin in PC12D neuronal cells. Further, the angiotensin II-induced depletion of synaptophysin is rescued by MG132 (proteasome inhibitor) treatment (Kurihara et al., 2008). This study and others have implicated the UPS in synaptophysin degradation (Wheeler et al., 2002; Bingol and Schuman, 2005). The present research suggests that mannose-rich retinal synaptophysin undergoes rapid degradation during diabetes. Since degradation during this phase is likely proteasomal, it can be postulated that diabetes increases the UPS mediated degradation of mannose-rich synaptophysin in the retina. To test this hypothesis, co-immunoprecipitation or plate based immunoassays may be used to determine the ubiquination of mannose-rich synaptophysin in the rat retina. MG132 may also be used to inhibit the proteasome in retinas from STZ-rats, either by intravitreal injection or by explant incubation, followed by a western blot analysis of mannose-rich synaptophysin. The predicted outcome of these experiments is that poly-ubiquination of retinal immature synaptophysin is increased during diabetes. If so, proteasomal inhibition should result in further accumulation of mannose-rich synaptophysin in the rat retina.
Appendix

Additional Research
ER Stress Markers Are Not Upregulated in Retinas from Diabetic Rats

A1.1. Introduction

The activation of ER stress mechanism is evidenced in previous work on the retinas of diabetic rodent models. ER stress markers, 78 kDa glucose-regulated protein (GRP78), phospho-eIF2α, phospho-IRE1, and ATF-4 are upregulated in Ins2Akita-diabetic mouse retinas, compared with the non-diabetic controls (Li et al., 2009). During ER stress, GRP78 is released from its complex with IRE1 to support protein folding (Kaufman, 1999). IRE1 oligomerises and activates its ribonuclease domain through auto-phosphorylation and catalyzes the excision of a 26 nucleotide intron from the mRNA for X-box binding protein 1 (XBP-1), a transcription factor. Splicing of this intron causes a frame shift in the XBP-1 coding sequence resulting in the translation of a 376 amino acid, 54 kDa, spliced XBP-1 (XBP-1s) isoform rather than the 261 amino acid, 33 kDa, XBP-1 isoform (Yoshida et al., 2003).

The data presented in this dissertation prompted a pilot investigation on ER stress markers in STZ-diabetic rats. Since synaptophysin, an N-glycosylated protein, is accumulated in the retina of diabetic rats, it was postulated that ER stress mechanisms are active. Therefore, it was expected that GRP78 content would increase and XBP-1s would be detected in retinas from STZ-diabetic rats. However, the result suggested that diabetes does not change the expression level of GRP78 and does not cause XBP-1 splicing in the rat retina.
A1.2. Results

A1.2.1. GRP78 Content is Not Altered in Retinas from STZ-Diabetic Rats

In order to detect the presence of ER stress in the rat retina, GRP78 was quantified through western blot analysis on retinal lysates. GRP78 resolved as a single 80 kDa band (Figure 28A). The content of GRP78, however, was unaltered in retinas from 1- and 2-month STZ-diabetic rats compared to age-matched controls (Figure 28B).

A1.2.2. XBP-1 mRNA was Unspliced in Retinas from STZ-Diabetic Rats

XBP-1 splicing is an important event during ER stress since it induces the activation of resident ER chaperone proteins and has the ability to upregulate ERAD (Yoshida et al., 2003). Using reverse transcriptase-polymerase chain reaction (RT-PCR), XBP-1 splicing was measured in retinal lysates from STZ-diabetic rats and age-matched controls. Rat liver injected with tunicamycin was used as a positive control. The XBP-1s was detectable in the tunicamycin treated liver samples but was not observed in the retinas from STZ-diabetic rats (Figure 28C).
A) Western blot analysis for GRP78 content revealed a band above the 75 kDa marker. B) Retinal GRP78 content was not significantly different in 1 and 2-month STZ-diabetic rats compared to respective controls. (C) Spliced XBP-1 mRNA (XBP-1s) was not detected in retinas from 1-month diabetic and age-matched control rats. XBP-1s was detected in liver from tunicamycin injected rats.

**A1.3. Discussion**

GRP78 is a prominent ER-resident chaperon, which binds to the three ER stress sensors- IRE1, ATF6, and PERK (Figure 29) (Ron and Walter, 2007). The disassociation of GRP78 from its complex is a key feature of the ER stress response. Thus, upregulation of GRP78 is one of the most commonly used markers of ER stress. The splicing of transcription factor XBP-1 is a downstream effect of GRP78 disassociation (Figure 29) (Yoshida et al., 2003). Immature
synaptophysin accumulation during its post-translational N-glycosylation could signify ER stress in the retina. Therefore, a brief study on GRP78 content and XBP-1 splicing was conducted in retinas from STZ-diabetic rats and age-matched controls. However, the results showed that diabetes caused no detectable change in either of these markers in the rat retina.

Figure 29. An overview of the ER stress response pathways.

In light of the present data, it can be speculated that the accumulation of immature synaptophysin in the absence of any detectable upregulation of GRP78 is an event that contributes to eventual ER stress reported in previous studies (Li
et al., 2009). Since ER stress has several time-dependent stages (Schroder and
Kaufman, 2005), it is also likely that the markers are regulated in a time-sensitive
manner. Therefore, a robust and detectable change in the GRP78 protein and/or
XBP-1 mRNA may occur only at a later time point of diabetes. In order to
address this issue and thoroughly explore ER stress in the STZ-rat retina, all
three pathways must be investigated after different durations of experimental
diabetes.
References


Akama TO, Nakagawa H, Wong NK, Sutton-Smith M, Dell A, Morris HR, Nakayama J, Nishimura S, Pai A, Moremen KW, Marth JD, Fukuda MN


Brand SH, Castle JD (1993) SCAMP 37, a new marker within the general cell surface recycling system. EMBO J 12:3753-3761.


patients--association with polymorphisms of vitamin D-receptor, TNF, Neuro-D and IL-1 receptor 1 genes. Coll Antropol 33 Suppl 2:99-105.


system in type 2 diabetes mediated by islet amyloid polypeptide-induced UCH-L1 deficiency. Diabetes 60:227-238.


DCCT (1995a) Effect of intensive therapy on the development and progression of diabetic nephropathy in the Diabetes Control and Complications Trial. The


Gaspar JM, Castilho A, Baptista FI, Liberal J, Ambrosio AF (2010) Long-term exposure to high glucose induces changes in the content and distribution


flow and acetylcholine-induced vasodilatation in normotensive diabetic rats. Diabetologia 47:113-123.


Johnsson S (1959) [The importance of a strict diabetes therapy for the prevention of nephropathy & retinopathy; comparative follow-up of 2 groups of patients treated according to different principles]. Nord Med 62:1005-1009.


Tulsiani DR, Hubbard SC, Robbins PW, Touster O (1982b) alpha-D-Mannosidases of rat liver Golgi membranes. Mannosidase II is the GlcNAcMAN5-cleaving enzyme in glycoprotein biosynthesis and mannosidases Ia and IB are the enzymes converting Man9 precursors to Man5 intermediates. J Biol Chem 257:3660-3668.


VITA
Travis D'Cruz

Education

Penn State Hershey Medical Center
Doctor of Philosophy in Neuroscience
Hershey, PA
2013

Drexel University
Bachelor of Science in Biological Sciences
Philadelphia, PA
2007

Honors and Awards

AFER/Retina Research Foundation/Joseph M. and Eula C. Lawrence Travel Grant
2012
Penn State Hershey Eye & Vision Research Day Presentation Award
2012
Placement on the Drexel University Dean's List
2003-2007
Three nominations for Global Young Leaders Conference (GYLC)
2002

Publications


T. D'Cruz, A.J. Barber. Disruption of the Post-Translational N-Glycosylation Process Accelerates Mannose-Rich Synaptophysin Degradation in the Rat Retina. (Submitted)

Select Presentations

