Identification of the Effects of Diabetes Mellitus on the Brain

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IDENTIFICATION OF THE EFFECTS OF DIABETES MELLITUS 
ON THE BRAIN

by

TRYPHINA ADEL MIKHAIL

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, FL

Spring Term, 2016

Thesis Chair: Mohtashem Samsam, M.D., Ph.D.
ABSTRACT

As more studies accumulate on the impact of diabetes mellitus on the central nervous system, they resound with the same conclusion - diabetes has a detrimental effect on cognition regardless of the presence of comorbidities. Less consistent however, are the specific mental processes wherein these declines are noticeable, and the structural changes that accompany these reductions in mental capacity. From global atrophy to changes in the volume of gray and white matter, to conflicting results regarding the effects of hypo- and hyperglycemic states on the development of the hippocampus, the studies display a variety of results. The goal of this research is to link the structural and compositional changes occurring in the diabetic brain with the clinical and behavioral findings highlighted in the literature, as well as to explore the potential mechanisms behind the pathologic brain state of diabetic encephalopathy.

Using diabetic (OVE26) and non-diabetic wild type (FVB) mice as models, differences in the number of hippocampal neurons in the dentate gyrus, and cornu ammonis areas 1, 2, and 3 were investigated through Nissl staining. Neurodegeneration was confirmed in those cells determined to be hyperchromatic in the diabetic model through staining with Fluoro-Jade C. Finally, the presence of progenitor cells in the hippocampus was compared in the diabetic and non-diabetic models using Musashi-1 antibodies, to determine whether neurogenesis in these areas is affected by diabetes. These experiments were performed to better understand the effect of DM on learning and memory, and could potentially explain the linkage between diabetes mellitus and the increased prevalence of Alzheimer’s disease, vascular dementia, and depression in this subset of the population.
DEDICATION

To Baba, Mama, and Samuel, my pillars of strength and my role models,
To Gido Samuel and Gido Aziz, whose lives have inspired me,
To my friends who will always seem more like family,
And to Pupsy and Mish-Mish, who were always there to listen.

“Therefore humble yourselves under the mighty hand of God, that He may exalt you in due time, casting all your care upon Him, for He cares for you.” 1 Peter 5: 6-7
ACKNOWLEDGMENTS

I would like to express the utmost gratitude to those whose contributions made the completion of this thesis possible. My greatest thanks to Dr. Hatcher for teaching me how to perform Nissl staining and for help with the Fluoro-Jade visualization and the antibody protocol, for taking the time out of your busy workday to supervise my experiments in the lab, for giving me helpful feedback, and especially for teaching me to have patience with myself because mistakes are a part of learning. I would also like to thank Dr. Cheng for the use of his lab and for his donation of mouse brain tissue, and Dr. Hoffman-Ruddy for serving on my committee. Thank you to Dr. Chen for mentoring me in the art of sectioning and in the use of a cryostat, skills I never thought I would obtain as an undergraduate. To Dr. Samsam especially, for giving me guidance and direction, and for always finding a window whenever some unexpected obstacle lay in the path of obtaining our objectives. None of this could have been possible without your support.

I would also like to thank my friends for their incredible belief in me, for their faith when mine was running low, and for their encouragement, and to thank my family who acted as cheerleaders all along the way. Samuel you gave me the love only a little brother could give, Mom you were always there to redirect my focus to the next practical step, and Dad you reminded me that He who had started with me would finish the good work He had begun (Philip 1:6). You were lifelines I could not have done without during this process. Lastly, I would like to thank God for stretching me beyond my small limits, and teaching me to do my best and leave the rest up to Him.
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INTRODUCTION

Diabetes mellitus is a disease wherein glucose uptake from the bloodstream into cells is inhibited, leading to retinopathy, nephropathy, vasculopathy, and disturbances of the autonomic nervous system.\(^1\) Moreover, diabetes has been shown to have significant effects on cognition through neural structural changes; therefore the focus of our study was to determine the effects of diabetes mellitus on the brain. Evidence suggests that both type I and type II diabetics have an increased risk of acquiring vascular dementia and Alzheimer’s disease, and a greater chance of suffering cognitive impairments.\(^2\) Cognitive decline is also more likely to occur by 60-100% if an individual with diabetes has chronic hyperglycemia.\(^3\) The decline is characterized by “neural slowing, attention deficit, and [difficulties in] executive functioning,” and type I diabetics were shown to have lower verbal and overall IQ scores in bouts of acute hyperglycemia in which blood glucose concentrations were above 15 mM.\(^3\) This may be due to a regulatory mechanism found in type I diabetic patients, but not in their type II counterparts, in which cerebral blood flow increased during periods of hypoglycemia and decreased during periods of hyperglycemia.\(^2\) Possibly akin to the way in which cerebral blood flow is increased in cases of chronic hypoxia, or hypercapnia, by affecting capillary density and vasodilating blood vessels, the opposite effect may be taking place in the brain when blood glucose concentration is too high.\(^4\) Moreover, decreased cerebral blood flow has been linked to total brain volume atrophy in Voxel-based morphometry studies, and arterial spin labeling MRI studies confirm this by showing evidence of cortical and subcortical atrophy.\(^3\),\(^5\)
In type II diabetics, on the other hand, hippocampal atrophy associated with memory impairment is more prominent, which may be due to the fact that the onset of type II diabetes often occurs in older patients. Since type II diabetes is caused by insulin insensitivity, hippocampal atrophy may be explained by the fact that insulin prevents the binding of pathogenic Aβ oligomers to “sites localized at hippocampal nerve cell synapses,” and if proper insulin binding cannot occur, this CNS protection is no longer present. Extended episodes of severe hypoglycemia are also known to cause the direct necrosis of these neurons. Of the areas most severely affected, the limbic unci and cerebellar posterior lobe, structures associated with language, affect, and executive function, are linked to the greatest loss of gray matter density. Voxel-based morphometry also revealed some of the more subtle structural changes that occur at the earlier stages of type II diabetes, which included “global brain volume atrophy, hippocampal and amygdala atrophy, and gray and white matter atrophy in the right temporal lobe.” The subsequent cognitive dysfunction and dementia that was observed in tandem was accounted to cerebral hypoperfusion, since perfusion is one of the main indicators utilized in measuring tissue function. While this decrease in perfusion was observed in type II patients, in rodent studies involving Streptozotocin-injected animals, which simulate the type I diabetic model via toxic destruction of pancreatic β-cells, the number of BBB tight junction proteins decreased, thereby increasing the permeability and perfusion of the blood brain barrier. On the whole, the structural and neurophysiological modifications that have been found in the brains of type I and type II diabetic patients are significant enough to be considered a diabetic encephalopathy because of the cognitive deficits incurred as a result. The overall progression of the disease has
been succinctly summarized as “accelerated brain aging,” and both hypoglycemic and hyperglycemic episodes have been the suspected causes in varying studies.5, 8

The purpose of this research was to determine whether signs of neurodegeneration, as well as disturbed neurogenesis were present in diabetic versus wild type mice, as a model of potential findings for this disease state in humans. According to one study wherein hippocampal neurogenesis was impaired via irradiation, the mice later showed deficits in retention of spatial memory, and overall reduced cognition.9 Even age-related reductions in the ability to generate new neurons in the hippocampus have been implicated as the source of impaired learning.10 Therefore, we carried out a series of experiments using frozen leftover brain tissues from OVE26 (diabetic) and FVB (wild type) mice from Dr. Cheng’s prior experiments, to see whether these patterns of increased neurodegeneration and decreased neurogenesis were present.

The OVE26 mice that were used in these experiments are transgenic variants of the FVB mice, and portray an early type I diabetic model.11 The insulin calmodulin gene in these mice is overexpressed, resulting in β cell toxicity that is sufficient enough to induce type I diabetes, but allows a small proportion of cells to survive so that the mouse can live for at least a year without insulin treatment.11 In addition to type I diabetes, these mice typically display characteristics such as decreased glomerular filtration rate, hypoalbuminemia, and high blood pressure as complications of their underlying condition.11
MATERIALS AND METHODS

The specific aims of this research were as follows: 1) to determine changes in neurodegeneration between diabetic and nondiabetic mice in the dentate gyrus, CA1, CA2, and CA3 regions of the hippocampus by means of histological analysis, and 2) to determine whether there were marked differences in the level of neurogenesis occurring in the tissues of diabetic and normal mice. The tissues obtained for staining and experimentation were donated by Dr. Cheng’s lab as leftover frozen specimens from previous experiments. They used the procedure below for perfusing the mice, removing the brain, and preserving the tissues, prior to storage in a -80°C freezer for two to three years (IACUC 15-19).

Method for Perfusion, Removal, and Preservation of Mouse Tissues

1. Three-month old FVB (wild type normal) and OVE26 (diabetic) mice were injected with a sub-lethal dose of sodium pentobarbital (i.p., 100ug/g) sufficient to induce deep anesthesia. Suitable depth of anesthesia was determined by the absence of the hind-paw pinch withdrawal reflex.

2. An incision was made bilaterally from the tip of the sternum to the axillary region of the mouse, and the flap of skin was gently removed upwards to expose the ribcage.

3. The tip of the sternum was lifted using a small pair of forceps, and an incision was made near the base of the sternum and continued along the base of the ribcage toward the spine on both sides, stopping approximately 2 centimeters short of the spine.

4. The tip of the sternum was clamped with a small pair of hemostats, and the rib cage was cut bilaterally approximately 1.5 cm from the midline towards the clavicle. The central
part of the ribcage was then lifted and held out of the way by weight of the hemostat to expose the heart and lungs.

5. 0.1 mL heparin solution was injected into the left ventricle, and the animal was left alone for 1 minute to allow heparin to circulate. The thoracic cavity was kept moist with warm PBS during this procedure.

6. While the heart was still beating, a 22-gauge needle was inserted into the left ventricle and stabilized in position using a hemostat. The inferior vena cava was then located and cut, opening the circulatory system for drainage. Warm PBS was then introduced into the circulation through the left ventricle using a peristaltic pump at a low flow rate in order to flush blood from the circulatory system. Blood and PBS coursed through the circulatory system, and exited through the cut inferior vena cava on the way back to the heart, draining into a collecting basin beneath the mouse.

7. When fluid draining from the IVC turned clear, a switch was made to cold Zamboni’s fixative (2% paraformaldehyde, 15% picric acid in PBS, pH 7.4). Perfusion with Zamboni’s fixative continued until the feet, tail, and tissues around the nose and mouth picked up a yellow tint from the Zamboni’s fixative, and the limbs/tail stiffened. Perfusion at this point was discontinued.

8. Using a small pair of scissors, an incision was made at the bridge of the nose between the eyes of the mouse.

9. Using a small pair of forceps, the front part of the skull was alternately peeled away or broken starting at the incision between the orbits. The bottom prong of the forceps was
gently worked into the space between the brain and skull as the skull was carefully removed to expose the brain. Care was taken not to damage the brain tissue.

10. The occipital lobe was removed last, with great caution taken to avoid damaging the cerebellum or brainstem.

11. The head of the mouse was flexed forward to allow insertion of the bottom prong of the forceps into the spinal canal starting with the first cervical vertebra. The forceps were used to expose the brainstem and spinal cord, vertebra by vertebra, down to the level of the 2\textsuperscript{nd} to 3\textsuperscript{rd} thoracic vertebra.

12. The meninges covering the brain and spinal cord were identified, then gently peeled away.

13. Using the forceps, remaining bits of skull and vertebrae were removed from around the brain and spinal column. The forceps were used to feel for sharp edges of bone prior to removal of the brain.

14. Using a dull scalpel, the brain was gently separated from the base of the skull by inserting the scalpel underneath the brain, gently severing cranial nerves and vessels, slowly lifting the brain, working from the olfactory bulbs back to the brain stem.

15. Once the brain and spinal cord were separated to approximately the 2\textsuperscript{nd} or 3\textsuperscript{rd} thoracic vertebrae, the spinal cord was cut as low as possible and the brain removed.

16. The freshly excised brain was placed into cold Zamboni’s fixative and allowed to postfix overnight.

17. After postfixing, the brain was transferred into a 30% sucrose solution for cryoprotection. Initially, the brain would float near the surface of the sucrose solution.
18. After the brain had sunk to near the bottom of the sucrose solution, it was removed and a razor was used to cut away the cerebellum and brainstem. The cerebellum was then separated from the brainstem and discarded.

19. The brainstem and cortical/midbrain tissues were then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, USA) and frozen at -80°C.

**Nissl Staining**

_Trial 1_

Three month-old FVB and OVE26 mice brains were used in this study, and the tissues were transferred to a cryostat (HM 505N) and mounted on a plate using freezing medium at a setting of -25°C. 12 μm coronal sections of the hippocampus and olfactory bulb were cut. Mice classified with the letter “L” were normal, while mice classified with the letter “D” were diabetic.

**Table 1: Sections from Normal and Diabetic Mice**

<table>
<thead>
<tr>
<th>Mouse Identification</th>
<th>Hippocampus Slices</th>
<th>Olfactory Slices</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>L18</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>L121</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>D101</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>D107</td>
<td>Yes</td>
<td>No</td>
<td>Olfactory bulb was very dry and kept crumbling; unsalvageable</td>
</tr>
<tr>
<td>D110</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

Nissl staining using cresyl violet was used to visualize the morphology of the neurons and perform cell counts on sections capturing the hippocampus and olfactory bulb. The staining was performed as follows. Slides containing the sections were permitted to air dry for 15 - 30 minutes prior to immersion into a series of twelve solutions for staining: 95% ETOH for 15
minutes, 70% ETOH for 1 minute, 50% ETOH for 1 minute, DH$_2$O for 2 minutes, DH$_2$O for 1 minute, Cresyl Violet stain for 15 minutes, DH$_2$O for 1 minute, 50% ETOH for 1 minute, 50% ETOH for 1 minute, 70% acid ETOH for 2 minutes, 95% ETOH for 2 minutes, 95% ETOH for a few dips, and finally 100% ETOH for 1 minute.\textsuperscript{12}

\textit{Trial 2}

The protocol in trial 1 presented many challenges to data collection, in that the brain tissues had inherent damage, and the procedure for dehydrating the tissues proved too extreme and contributed to small perforations seen under the microscope, especially since the sections were only 12 μm thick. Therefore the protocol was modified and optimized, and applied to newly cut 30 μm slices. Instead of dipping the slides into a series of solutions, a pipette was used to deliver the solutions to each slide gently, so that minimal disruption would occur to the tissues. Each slide was treated with cresyl violet for eight minutes, and then rinsed with 1x PBS buffer two to three times. Then each slide underwent three rounds of treatment with Histoclear for three minutes each, rinsing with 50% EtOH twice in between. Finally the slide was rinsed in 1x PBS buffer and allowed to air dry prior to being fitted with a coverslip using mounting media. The slides were viewed at 200X magnification, and individual photos of each field of view were taken and compiled into mosaics via the software ImageJ.\textsuperscript{13} The DG, CA1, CA2, and CA3 regions of the hippocampus were identified, and the neurons in five different 200 μm$^2$ areas were counted using Inkscape and averaged.
Fluoro-Jade C

Fluoro-Jade C is a form of fluorescent histological staining used to highlight neurons in the process of degeneration.\textsuperscript{14} Slides containing 12 µm thick hippocampal slices of OVE26 and FVB mice were utilized. 1% NaOH in 80% ethanol was pipetted onto each slide for five minutes, followed by 2 minutes of 70% alcohol, and then 2 minutes of diH\textsubscript{2}O. Then 0.06% potassium permanganate was placed on the slides for 10 minutes, before the slides were rinsed with distilled water for 2 minutes. 0.0001% Fluoro-Jade C in 0.1% acetic acid was then added to the slide for 10 minutes, and the slide was incubated in the dark during this interval. Six consecutive
1 minute washes in distilled water followed, and then the slides were allowed to air dry for 5 minutes. Lastly, the slides were cleaned with Histoclear for 5 minutes, allowed to dry, and then cover-slipped for visualization at 488 nm on the confocal microscope.

**Immunohistochemistry: Musashi-1**

Musashi-1 is a mouse RNA-binding protein that is highly expressed in the stem cells of the central nervous system. Because it is expressed highly in neuronal and glial precursor cells, and then its presence virtually disappears once the cell has fully differentiated, it is a prime marker for detecting neurogenesis in tissue samples. For the preparation of each solution, refer to Appendix B.

First, sections of the hippocampus were selected and hydrated by pipetting 50% EtOH onto the slides for 30 seconds. Then the tissues underwent six 1.5-minute washes in PBS buffer while being agitated on a rotating shaker. The excess moisture was removed from the slides, and then a Super Pap Pen was used to draw circles around each individual section so that when the antibodies were added they would concentrate on the sections themselves, and not migrate to unoccupied areas on the slide. 15 mL of blocking solution containing BSA and normal donkey sera was added to the slides, and they were incubated on a rotating shaker at room temperature for two hours. Then 1.5 mL of primary antibody (1 μg/mL) were incubated overnight for approximately 16-18 hours at 4°C in a humidified chamber. This was followed by 6 five-minute washes in PBS-T while on the rotating shaker at room temperature. The secondary antibody (1:500) was then incubated for two hours at room temperature, and the slides were again rinsed with 6 five-minute washes with PBS-T on the rotating shaker. The slides were viewed on a confocal microscope at 543 nm for excitation and 625 nm for emission.
RESULTS

Figure 2: A) OVE26 Mouse Hippocampal Mosaic Slide 18A and B) DG at 40X
Figure 3: A) FVB Mouse Hippocampal Mosaic Slide 30 at 20X B) DG at 40X
Figure 4: Diabetic DG depicting hyperchromatic neurons
Figure 5: Normal DG depicting hyperchromatic neurons
Images were taken with a light microscope at 200X magnification of OVE26 and FVB brain sections, then merged in MosaicJ by overlapping landmarks in the photos (Figures 2 and 3). Neurons were counted in the DG, CA1, CA2, and CA3 regions using 200 μm fields of view generated through Inkscape. Then an average was taken and the results were graphed, revealing that in all four areas, diabetic mice led with the highest number of hyperchromatic neurons, and the differences were statistically significant in the dentate gyrus and CA1 region (Figure 6). Then a view of the dentate gyrus in each was procured at 400X, in order to determine whether there were differences in the degree of staining, in other words whether diabetic neurons absorb more cresyl violet than their normal counterparts (Figure 2B and 3B). The comparison revealed that diabetic neurons were hyperchromatic and stained more darkly. The arrows in Figure 2 and
Figure 4 depict hyperchromatic neurons in the diabetic brain, which were consistently more highly stained in comparison to the background tissue than the neurons depicted in Figures 3 and 5 for the normal mouse.

Figure 7: Fluoro-Jade C Staining of diabetic dentate gyrus at 40X as a Mosaic
Figure 8: Fluoro-Jade C stain of normal dentate gyrus at A) 40X as a mosaic and B) 1000X
Figure 9: Fluoro-Jade C diabetic DG at higher magnification
Figure 10: Fluoro-Jade C normal DG at higher magnification
Figure 11: Trial 2 for Fluoro-Jade C diabetic DG as a 40x mosaic

Figure 12: Fluoro-Jade diabetic stained cell at 100X from box in Figure 11
Figure 13: Trial 2 for Fluoro-Jade C normal DG as a 40x mosaic

Figure 14: Number of degenerating neurons in DG using Fluoro-Jade

Number of Degenerating Neurons in the Dentate Gyrus of Diabetic and Non-Diabetic Mice in Fluoro-Jade Staining

Figure 14: Number of degenerating neurons in DG using Fluoro-Jade
The Fluoro-Jade C stains were viewed under a confocal microscope at 488 nm at 40X magnification, with a focus on the dentate gyrus. Mosaics were made for both the diabetic and normal mice, both depicting the presence of degenerate neurons via the presence of hotspots consisting of fluorescent green pigment (Figures 7 and 8A). Examples of degenerate neurons in both tissue types are labeled with orange arrows in Figures 9, 10, 11, and 13. When counts of the mosaics were performed, the number of degenerate neurons in the diabetic dentate gyrus exceeded that of the wild type (Figure 14). In addition to degenerating neurons, which are normally more oblong and irregularly shaped, some of the glial cells in the tissue also appear to be degenerating, and are characterized as very round green intensities (Figure 8B and 12).

The first trial of Fluoro-Jade staining produced a greenish border around the dentate gyrus of the diabetic sections, and a more faint border around the normal dentate gyrus, labeled with yellow arrows in Figures 9 and 10. At first, it was thought to be background staining due to insufficient rinsing or too little tissue clearance with Histoclear. However a second trial in which the number of rinses was doubled, and the time exposed to Hisoclear increased from 3 minutes to five minutes showed less background staining, but the same green border around the dentate gyrus (Figures 11 and 13). The diabetic sections consistently showed more pigment in this area than the normal hippocampal sections, leading to the possibility that there is increased neurodegeneration in the ependymal cells as well as the DG neurons of the diabetic mouse in comparison to the normal.
Figure 15: Mushashi-1 immunohistochemistry for wild type DG slide 37 at 20X
Figure 16: Musashi-1 immunohistochemistry of diabetic DG at A) 20X and B) 40X
Immunohistochemistry using Musashi-1 antibodies was used to detect for differences in neurogenesis. Figure 15A shows a part of the dentate gyrus for the wild type mouse at 20X, 15B is at 40X magnification, and C1-C3 are consecutive 0.5 um slices through the section in panel B. Figure 16A is an image of the diabetic dentate gyrus with a close-up of one panel at 40X. Ideally there should have been a higher number of fluorescent hot spots for the wild type mouse, indicating decreased neurogenesis in the diabetic model. However, in both tissues there was a high degree of non-specific binding making these results inconclusive. Even the pattern of fluorescence that was present did not suggest that the antibody bound specifically, since Musashi-1 should be found cytoplasmically, but the antibody instead outlined the neurons from this trial. This is depicted by the green arrows in Figures 15 and 16, where the borders of neurons are clearly seen but very few antibodies penetrate the interior in comparison to the rest of the tissue. There may be a pattern in the binding, but it is difficult to interpret without a secondary stain.
DISCUSSION

Analysis of Results

Nissl staining is performed with basic dyes, such as the cresyl violet used in these experiments, and binds to the negatively charged nucleic acids present in the nucleus of the cells, as well as the endoplasmic reticulum. Because the cells in brain tissue all contain these components, the stain does not differentiate between glia and neurons, but the neurons retain more of the stain because of their high rate of protein synthesis, and are also distinguishable by the irregular shape of their dendritic spines. When these neurons sustain any type of damage and begin to undergo degeneration, the stain increases in density and the cells are considered to be “hyperchromatic.” The results of the Nissl stains performed in these experiments indicate a greater number of hyperchromatic cells in the DG, CA1, CA2, and CA3 regions of the hippocampus for diabetic mice in comparison to the wild type control. By extension this means that the hippocampus of a diabetic mouse sustains greater levels of neurodegeneration than the normal, and the expected clinical manifestation would be cognitive deficit based on evidence from the literature.

To confirm that the hyperchromatic neurons observed were in fact degenerating, and not merely histological artifacts, a second set of experiments was conducted using Fluoro-Jade C. The precise mechanism of this stain is unknown, however, the present theory is that dying neurons release strongly basic molecules that attract the acidic Fluoro-Jade to it. This basic “degeneration molecule” is a byproduct of the process of neurodegeneration and is inaccessible to Fluoro-Jade unless the cell membrane is disrupted, therefore making it a prime indicator for neuronal cell death. The data collected using confocal microscopy and this staining technique
clearly demonstrated an increased level of neurodegeneration in the diabetic mouse model in comparison to the wild type control. Moreover, there was also a larger number of fluorescent glial and ependymal cells present in the diabetic brain sections, revealing that diabetes decreases the presence of these cells in the dentate gyrus as well.

Musashi-1 was used to quantify the number of progenitor stem cells in the hippocampus of the diabetic and normal mice, in order to determine whether diabetes disrupts neurogenesis in the dentate gyrus. The target of the antibody, Musashi-1, is a protein that binds RNA and is expressed predominantly in the progenitor cells, and undergoes down regulation once the cell has fully differentiated. With regards to the Musashi-1 experiment, the results obtained were inconclusive. Despite the fact that binding of the antibody did occur in both tissues, most of it appeared to be non-specific, especially since the protein being sought out is cytosolic, whereas the data obtained showed a great deal of membranous binding, as depicted by the way the antibodies often outlined the neurons in the tissue as opposed to penetrating them. This is most likely due to secondary antibody becoming trapped between the cells, rather than a true antibody-antigen interaction.

In the future, we would like to perform a greater number of trials with Fluoro-Jade C to add more weight to the conclusion we obtained, which is that diabetes does incur greater neurodegeneration in the hippocampus. Moreover, the Musashi-1 immunohistochemistry protocol needs to be optimized in order to reduce non-specific binding and increase the penetration of the antibodies into the brain tissue. This could be accomplished by using 8 μm slices instead of 12 μm sections, and by blocking the tissue with BSA overnight as opposed to only blocking for two hours. The concentration of BSA was also determined to be only 2%,
when typically a 10% blocking solution is utilized, so a change in this regard would greatly reduce nonspecific binding. Another possible change to the protocol to make interpretation of the data more efficient would be incorporating a double stain with β-tubulin III, which would produce an inline of the neurons and would help us determine where these Musashi-1 antibodies are binding.

Moreover, we would like to attempt this immunohistochemical procedure with sections of the olfactory bulb, to determine whether neurogenesis is inhibited in this area in diabetic mice. This would be of significant importance because there are many neurodegenerative diseases that have been linked to reductions in the sense of smell.20 The remainder of this thesis is dedicated to exploring the clinical manifestations of increased neurodegeneration and decreased neurogenesis in the hippocampus, the potential mechanisms behind neuronal cell death and cognitive decline, and how these findings may influence current and future treatments of diabetes mellitus.

**Cognitive Impairment**

*Clinical Manifestations*

As treatments for insulin resistance improve and as patients continue to live longer with diabetes mellitus, the effects of this disease have shown to be clinically more widespread than previously thought, with effects reaching as far as the skeletal system and the CNS.21 In type 2 diabetics especially, direct correlations were made between elevated A1c levels and poor performance on tests evaluating neurocognition, showing an inverse relationship between glycemic control and cognitive performance.21 With regards to type I diabetics, moderate
declines in processes related to problem solving, mental and motor speed, learning, and memory were noted as well.\textsuperscript{7}

Multiple studies have been performed evaluating the cognitive performance of diabetics by relating the scores they receive on various tests to their blood sugar levels. When comparing type I diabetics with an HbA1C level of less than 7.4\% to those with a level greater than 8.8\%, the general trend shown was that the greater the glycemic control, the better the person's performance in academic achievement, verbal IQ scores, psychomotor efficiency, and memory.\textsuperscript{8} A hyperglycemic state was also associated with loss of focus, decreased speed in information processing, and a larger number of errors in mental subtraction.\textsuperscript{8}

Hypoglycemia too has been thought to be involved in the clinical manifestations of neurocognitive decline, especially with regards to recurrent hypoglycemia or severe hypoglycemic episodes requiring third party assistance. When a diabetic patient was presently hypoglycemic, their performance in terms of prospective, delayed, immediate visual, and working memory was impaired, along with their visual-motor skills and visual-spatial skills.\textsuperscript{8} This occurred regardless of whether the patient was aware or unaware of their low blood sugar.\textsuperscript{8} It is the effects of recurrent hypoglycemia that interest researchers the most however, because its long-term impact on patients' cognition in a euglycemic state is what produces mixed results.\textsuperscript{8} Currently, such persons have been observed to have lower IQ scores, verbal skills, memory, and attention, although even those who had a history of severe episodes did not show greater impairment than diabetics who did not have that history upon performance of an eighteen-year follow-up.\textsuperscript{8} The risks of having a hypoglycemic blood sugar level are often increased the greater the glycemic control, especially if the patient is undergoing intensive insulin management in
order to minimize their risks for vascular disease. It would therefore be of significant concern if hypoglycemia was a culprit of severe cognitive decline, and providers and patients had to balance the benefits of maintaining blood sugar with the risks of cognitive dysfunction. However the current evidence is not really supportive of this view, as a study done on children who had frequent hypoglycemic episodes showed that they had higher verbal and memory scores, not to mention academic achievement, than diabetic children with less glycemic control. This would allow researchers to hazard the opinion that overall, the benefits of more stringent glycemic control compensate for any neurocognitive damage instigated by hypoglycemia.

Despite the decrease in cognitive capacities in hypo- and hyperglycemic states, patients suffering from diabetes mellitus are still at a cognitive disadvantage even in euglycemia. If compared to their respective controls, type I diabetics consistently score less in overall cognition, speed of information processing, visual and sustained attention, visual perception, mental flexibility, and crystallized intelligence. On average, those with type 2 DM exhibit decreased working memory, immediate and delayed recall, verbal fluency, executive function (also considered to be frontal lobe function), processing speed, attention, and verbal retention and fluency. Whether as a result of hypoinsulinemia or a reduction in the body’s ability to respond to insulin, the distributional pattern of glucose metabolism in diabetic patients as they work on memory tests is altered, showing more widespread activity in brain regions adjacent to those normally activated during such tasks. Although it has yet to be confirmed whether these observations are the result of cerebral defects, diabetic patients also present with a lack of balance, slower walking, and a greater potential for falling.
As patients with diabetes progress to having complications such as angiopathy, changes in functionality manifest by way of decreases in the connectivity and synchronization of neural networks between different parts of the brain. Functional MRI revealed that type I diabetic patients with microangiopathy suffered a reduction in functional connectivity in five separate neural networks. These include the secondary visual network, ventral attention network, sensorimotor network, language-processing network, and the left frontoparietal network. However, researchers were startled to find that while type I diabetics without microangiopathy suffered from a decrease in functional synchronization in three of these networks, they actually had greater connectivity in the sensorimotor and secondary visual networks than even nondiabetics. Moreover, this has also been observed in patients with other pathological states of the brain such as cognitive impairment and multiple sclerosis. Because the acquisition of microangiopathy is considered to be characteristic of a later stage of diabetes, researchers have come to the conclusion that as diabetes begins to incur minor brain damage, the brain undergoes “functional reorganization” in a compensatory effort to stem the tide of dysfunction, thereby increasing the functionality of certain areas. However, as seen in other diseases such as multiple sclerosis, synchronization in brain activity eventually declines in all five networks.

Also from a functional perspective, the electrophysiological changes in diabetic patients also correlate to the progression of the disease as response potentials to certain stimuli actually reveal a decrease in nerve conduction velocity. The P100 wave for example is a potential that is visually evoked and thought to originate in the visual cortex. The longer the patient has had diabetes and the higher their HbA1C level, the greater the latency of that potential. Flash electroretinopathy confirms this based on the fact that even before any ophthalmoscopic
indications of retinopathy are present, there are reduced potentials sent out from the retina.\textsuperscript{8} The nerve response to acoustic stimuli is abnormal as well, and the auditory-evoked potentials from the brainstem in diabetic patients are characteristic of “acoustic pathway impairment.”\textsuperscript{8} Moreover, the event-related P300 wave associated with memory processes and used particularly whenever a target stimulus must be identified from the midst of standard stimuli has been shown to have greater latency, and could be related to defects in attention and short-term memory in type 2 diabetics.\textsuperscript{7, 8} Though normally seen in typical aging, the decrease in conduction velocity of the P300 wave is much more pronounced in diabetics, and central somatosensory-evoked potentials are also delayed.\textsuperscript{7, 8} To assess electrical activity in the cerebrum, EEGs were also performed on diabetics, and in patients with type 2 DM there was “slowing in the EEG frequency band analysis over the central cortex and reduction of alpha activity over the parietal area.”\textsuperscript{8} When compared to outcomes of neurocognitive tests, these results were correlated to reduced visual retention and could only be accounted for by the presence of diabetes because hyperglycemic nondiabetics did not express these findings.\textsuperscript{8} Furthermore, because diabetes often manifests as accelerated aging, decreases in hippocampal synaptic plasticity have been observed and implicated as being one of the major causes of the disadvantages that affect diabetics in terms of both memory and learning.\textsuperscript{7} According to Saladin, synaptic plasticity describes the ability of the brain to transmit potentials from one neuron to another to create new connections, and the ease with which this transmission occurs down a familiar neural circuit is related to how memories are retained and new tasks are learned, or synaptic potentiation.\textsuperscript{23} Afferent activity at a high frequency induces what is called long-term potentiation, while long-term depression occurs when there is a prolonged period in which a low-frequency stimulus is detected, thereby causing
a reduction in the strength of the synapse.\textsuperscript{7} Aging, or accelerated aging in the case of diabetes, results in an increase in the threshold needed to induce an LTP and a decreased threshold for an LTD.\textsuperscript{7}

One of the many concerns that has arisen for patients with diabetes is the increased prevalence of both Alzheimer’s and vascular dementia in this subset of the population, even when other cofactors and comorbidities are taken into account.\textsuperscript{21} In diabetics, the risk of acquiring dementia is actually doubled in comparison to non-diabetics, and a theory currently exists insinuating that Alzheimer’s and insulin resistance may have the same or at least a similar pathogenesis.\textsuperscript{7} Secondary to these debilitating cognitive states in the later years, elderly diabetics were also found to be twice as likely to suffer from depression, which also affects their ability to perform daily abilities and function cognitively.\textsuperscript{8} Epidemiological studies have shown that 11.3\% were cognitively impaired, 14.2\% had clinical depression, and 17.5\% had “moderate to severe deficits in activities of daily living.”\textsuperscript{8}

So far the signs and symptoms of decline noted were observed in older diabetics from mid-life on, which might suggest that age is a necessary component for seeing the detrimental effects of DM on the brain. However these cognitive deficits can be seen as early as childhood and become more obvious the earlier the onset of the disease. From the age of two, children diagnosed with DM show less positive incremental changes in learning with regards to block design, speed of processing, general intelligence and vocabulary.\textsuperscript{8} By the time six years have transpired since the time of diagnosis, they begin to manifest with debilitations in long-term memory, executive function, processing speed, attention, and IQ.\textsuperscript{8} In those diagnosed at less than
four years of age, the impairment in processing speed, executive skills, and attention was significantly greater than in those who acquired diabetes after the age of four.\(^8\)

Gender also elicits a difference in cognitive effects, as a greater performance gap exists between diabetic men and their controls in tests of strength grip, oscillation, and somatosensory ability in comparison to the deficit observed between female diabetics and their respective controls.\(^8\) In addition, worse glycemic control correlates to a verbal intelligence decline in boys ages seven through sixteen, an effect that is absent in their female counterparts.\(^8\)

**Modifications in Macrostructure**

Upon examination, the brains of diabetic patients have revealed that these clinical manifestations of cognitive decline are linked to changes in the macrostructure of the brain, and more specifically, atrophy. Moreover, the extent of the atrophy is linked to the age at which onset of the disease occurred, the prevalence of hypoglycemic episodes in the patient’s life, as well as whether he or she was known to suffer from chronic hyperglycemia.\(^21\) Because these factors vary from patient to patient, it explains precisely why the epidemiological studies done on diabetics have mixed results regarding the localization and extent of these structural changes on the brain.\(^7, 22\)

Young adults diagnosed with type I diabetes in childhood, for example, exhibit reduced white matter volume and changes in its microstructure, as well as reduced gray matter density associated with memory and language processing.\(^21\) Children with severe hypoglycemic episodes too show decreases in posterior parietal white matter, and in the gray matter of the cuneus and precuneus gyri.\(^8\) Voxel-based morphometric studies indicate that the location of GM density loss varies based on diabetic conditions, as those suffering from chronic hyperglycemia show reduced
density in areas responsible for attention, memory, and language, while those with a history of multiple hypoglycemic episodes have less GM in the cerebellar posterior lobe. The reduction in density in type I diabetics was found predominantly in the thalamus, temporal lobe, and frontal lobe. Strangely, diabetic children with a history of severe hypoglycemia exhibit a higher GM density in the right prefrontal cortex, an area thought to correlate to self-recognition.

Studies have also discovered localized atrophy in the amygdala and hippocampus, regions that also show degeneracy in Alzheimer’s disease. In other cases, certain areas of the brain in type I diabetics were associated with disorganization of typical white matter tracts that showed more preservation the greater the long-term blood sugar control. White matter tract aberrations were also associated with areas of greater myelin degeneration, and may be linked to the declines in cognitive performance.

If diabetes mellitus leads to the complication angiopathy, diffuse abnormalities can occur in the brain in patients younger than the age of forty, characterized by degenerative modifications that are so marked that a dual pathogenesis has been implicated. Researchers have concluded that the cerebral atrophy characterized by “widened sulci and/or enlarged ventricles” on MRI and CT must be due to the combined effects of diabetic abnormalities in neural tissue and ischemia induced by the angiopathy. Magnetic resonance imaging has also revealed the presence of white hyperintensities that may be linked to the autopsy finding of ischemic lesions. Strongly correlated to the aging process, leukariosis in the white matter and basal ganglia have been a consistent presence in the brain tissue of both type I and type 2 diabetics.

From a volumetric standpoint, significant changes have been observed in diabetic patients that would indicate quite clearly that the aging process is not only accelerated on a cognitive, but
anatomical level as well. Magnetic resonance imaging revealed that diabetic patients have on average 2.5 cm$^3$ of abnormal tissue, consisting of 5% grey, 8% deep gray, and 81% white matter.\textsuperscript{25} The longer the duration of DM and the higher the fasting plasma glucose level, the lower the normal tissue volume and the larger the ischemic lesions.\textsuperscript{25} The lesions themselves are believed to be the result of “nonnecrotic demyelination” originating from insufficient blood flow, and have been linked to balance, gait, and cognition.\textsuperscript{25} In the long-term, a difference of 10 years in diabetic onset made a difference of 4.28 cm$^3$ in total gray matter and could not be accounted for by the normal aging process, which typically only results in a loss of 2.4 mL.\textsuperscript{25, 26} As a result, the brain of a diabetic patient looks roughly two years older than that of a nondiabetic for every ten years of disease duration.\textsuperscript{25}

One of the more unexplained phenomena with regards to macrostructural changes in the brain involves the effect of recurrent hypoglycemic episodes in children with diabetes.\textsuperscript{21} Contrary to the data collected on adults with type I diabetes, in whom hippocampal volumes remained consistent despite chronic hypoglycemia, children who had an early onset of DM and also had chronic hypoglycemia had increased hippocampal volumes.\textsuperscript{21} The theory at present is that the brain in a state of development will respond to hypoglycemic conditions in a different manner than an adult brain.\textsuperscript{21}

\textit{Potential Mechanisms of Pathophysiology}

The pathophysiology of diabetes mellitus continues to remain elusive, making it difficult to target the causative agents of cognitive dysfunction and therefore deliver a treatment.\textsuperscript{8} However multiple theories have been proposed, and it is possible that a combination of these is responsible for the accelerated brain aging found in diabetics.
Because diabetes affects the cardiovascular system by changing the nature of blood vessels and their ability to perfuse certain tissues, these modifications have been implicated in the cerebrovascular changes responsible for cognitive deficits. In addition to intracranial vessel atherosclerosis, which is partly responsible for the two to six-fold increase in the likelihood of thrombolytic stroke in diabetic patients, other microvascular abnormalities exist such as thickening of the basement membrane in capillaries.\textsuperscript{7,8} The microvessels undergo endothelial degeneration, the walls of larger vessels accumulate more collagen and become inflexible, and there is an overall decrease in perivascular nerve density.\textsuperscript{7} Hyaline atherosclerosis occurs in the smaller vessels (with diameters less than one nanometer), and involves replacement of the smooth muscle with fibrous tissue that thickens the intima.\textsuperscript{7}

Changes in the blood flow of the brain consequently contribute to cerebral insult. For example cerebral blood flow levels in type 2 diabetics at rest and in response to hypercapnia are both markedly lower than those in their respective controls, and it has been theorized that prior to detectable structural changes in the brain, the pathology begins at the level of hemodynamics as the cerebral microcirculation begins to undergo modification.\textsuperscript{22} Any type of aging, whether it be normal or the accelerated form seen in diabetes, results in a decrease in the ability of the cerebral blood vessels to autoregulate their response to perfusion pressure changes.\textsuperscript{7} Vasomotor tone regulation is diminished, and could be linked to the decreased density of VIP and serotonin-containing nerves found in STZ-injected rats.\textsuperscript{7,22} Decreases in vasodilation mediated by nitric oxide and increased vasoconstriction in the arterioles, along with hypertrophy of the smooth muscle found in precapillary vessels have also been observed.\textsuperscript{7,22} Patients with diabetes also seem to have reduced levels of C-peptide, which when normalized increased cognitive function
in humans and decreased hippocampal apoptosis in the rat model. This is due to C-peptide’s actions in the endothelium, which involve its binding to a G-protein coupled receptor and beginning a cascade of events that not only trigger the expression of nitric oxide synthase (involved in vasodilation), but activate several transcription factors involved in mechanisms that are anti-oxidative and anti-inflammatory. Diabetic patients also seem to have greater stimulation of the thromboxane A2 receptor, which may be responsible for the decreased capacity of cerebral vessels to dilate as well.

As regions of the brain receive less blood, cell death occurs and the cell loss results in an additional decline in cerebral blood volume because the metabolic demands of the brain have been consequently altered. This phenomenon is observed in normal aging at a less drastic rate than in diabetic patients, and is also accompanied by morphological changes in neurons and glia as the state of chronic hypoperfusion continues. In rats, the hippocampal CA1 neurons, which are especially susceptible to the effects of ischemia, display higher levels of astrocyte components and a reduction in microtubule-associated protein 2 in the dendrites. Lower microtubule-associated protein 2 is actually an indication of early ischemic damage, and because astrocytes are responsible for replacing dead tissue via the process of sclerosis or scarring, an increase in astrocyte levels is also an indicator of cerebral ischemia. As a result of vascular disease that has developed due to the progression of DM, these patients often exhibit structural changes such as demyelination of the spinal cord and cranial nerves, pseudocalcinos, nerve fibrosis, and brain degeneration throughout.

Many theories exist to explain how hyperglycemia may be causing the detrimental structural and cognitive changes in both type I and type 2 diabetics. The first is that
hyperglycemia increases the flux going through the polyol pathway, a biosynthetic progression involving two enzymes that convert glucose into sorbitol and fructose. Sorbitol has been correlated to an increase in diabetic lesions in the CNS, so if more substrate is present there will be greater conversion to sorbitol. This substance has also been located in the peripheral nerves of STZ-treated rats. Upon treatment with an aldose reductase inhibitor, the levels of sorbitol decreased drastically and normal cognitive function was restored. Another possibility is that excess glucose is shunted to the hexoamine pathway for the production of cerebral chitin, a molecule that accumulates in Alzheimer’s patients and may also be the cause of abnormal cognition in diabetics.

Hyperglycemia can also result in accelerated AGEing, which refers to advanced glycated end-products that are a form of non-enzymatic protein glycation caused by the high concentration of glucose in the blood. This is an irreversible reaction that occurs as a function of time and the concentration of reduced sugars resulting in a linkage with the free amino groups of proteins. AGEs are found in normal aging, but concentrations are considerably higher in those with DM or Alzheimer’s, and their production has been linked to an increase in ROS in cells. AGEing also causes modification of the endothelial basement membrane, contributing to vascular dysfunction and the decreased ability of blood vessels to respond to nitric oxide for vasodilation. Thus AGEing may contribute to ischemia in the brain, which in turn could lead to neuronal cell death. These substances have also been linked to higher levels of oxidative stress in the diabetic brain, as observed by increased levels of lipid peroxidation products. The number of oxidized, dysfunctional proteins in the neurons is increased because there are higher levels of reactive oxygen species being produced as the result of glucose autoxidation. Moreover,
enzymes like SOD and glutathione catalase which function as ROS scavengers are also irreversibly glycated and consequently have reduced functionality as the body’s natural antioxidants.\(^5\) Certain markers linked to these biochemical changes have been found to be up-regulated in the hippocampus, such as nuclear factor κB transcription factors and the S-100 protein.\(^8\) The former is a proinflammatory gene marker that is activated in the presence of AGEs, while the latter is indicative of brain injury and binds to receptors for AGEs.\(^8\)

In concert, hyperglycemia and ischemia have proven to be a dangerous combination, and the elevation of blood glucose has been correlated to less efficient clinical recovery from cerebrovascular events.\(^8\) The underlying mechanism is thought to be glutamate accumulation, which occurs during hyperglycemia as the excitatory neurotransmitter causes damage to the neurons when ischemic conditions are present.\(^8\) Another theory is that hyperglycemia exacerbates ischemic damage by providing more substrate for conversion into lactate; this lactate then results in cellular acidosis which potentiates the brain injury.\(^8\)

On the other side of the spectrum, hypoglycemia too has been implicated as a pathogenic mechanism for the neurological damage seen in diabetes. Normally seen as a result of tight glycemic control, the areas most sensitive to hypoglycemic damage are the hippocampus, basal ganglia, and cortex according to autopsy reports.\(^8\) The effects seem to be isolated to neurons exclusively, while sparing oligodendrocytes and astrocytes, and present as multifocal or widespread necrosis of the cerebrum.\(^8\) The ganglion cells also exhibit chromatolysis.\(^8\) Although once thought to be a passive process in which neurons simply died of starvation, in reality hypoglycemia may actually induce a cascade of events that result in overactivation of the excitatory NMDA receptor.\(^8,29\) This overactivation is by the massive release of aspartate, which
targets the dendrites causing calcium fluxes that in turn cause membrane dissolution and necrosis. This would therefore make cell death the result of endogenous excitotoxins, which in turn would explain the seizures present in severe clinical cases of hypoglycemia. Unlike ischemia, which normally affects the brainstem and cerebellum, these regions are spared in hypoglycemia and instead necrosis is typically detected in the dentate gyrus of the hippocampus. There is therefore therapeutic potential in an NMDA receptor antagonist that would shield type I diabetic children from possible hypoglycemic damage.

Because intracellular concentrations of Ca\(^{2+}\), as well as the signal transduction cascades induced by them, play a role in the pathway leading to neuronal cell death in the normal aging process, an imbalance in Ca\(^{2+}\) is a possible mechanism for the cellular changes observed in the CNS of diabetic patients. According to the literature, a drastic change in the calcium concentration over a short duration produces the same amount of damage as an incremental damage over a long period of time, and disruptions in calcium homeostasis are linked tightly to ischemia, cell death, and the production of reactive oxygen species. Although there are few details on precisely how this imbalance occurs, it does seem to influence the changes in LTD and LTP observed in diabetic patients.

Moreover these changes in cognition may be accounted for by decreases in hippocampal synaptic plasticity, as well as fluctuations in neurotransmitter levels or their respective receptors. According to studies done on STZ-injected rats, the deficit in plasticity is mainly postsynaptic and the induction of diabetes in these rats was quickly followed by increased sensitivity to adenosine, an inhibitory neuromodulator. Believed to be caused by reduced nucleoside uptake, it is thought that increased levels of adenosine play a role in long-term potentiation deficits, since
LTP is modulated by adenosine.\textsuperscript{7} Synaptic transmission is dependent upon effective binding of glutamate to NMDA and AMPA receptors, so it is interesting to note that in diabetic rodent models, the density of NMDA receptors found in the hippocampus is significantly reduced.\textsuperscript{7,8} Moreover, glutamate has a decreased affinity for AMPA receptors because the GluR1 subunit has reduced expression.\textsuperscript{7} The result is an impairment of LTP, which is a cause of learning deficits and can be ameliorated by insulin treatment.\textsuperscript{8} Other observations include decreases in ACh levels, which led to amnesia and hyperactivity in STZ rats in relation to the control, decreased dopamine activity, and lower serotonin degradation.\textsuperscript{8} The conclusion arrived at by researchers is that fluctuating blood sugar levels seen in patients with poor glycemic control have a detrimental effect on the function of multiple neurotransmitters in the brain.\textsuperscript{8}

Perhaps the biggest lead with regards to why diabetes mellitus increases and exacerbates cognitive decline in patients comes from the very cause of diabetes mellitus itself: insulin resistance. Although the effects of insulin on metabolism in peripheral tissues is relatively well understood, little is known about the effects of insulin on brain metabolism as the brain was originally thought to be insulin independent in this regard.\textsuperscript{8,31} Despite the fact that insulin receptors have been found on the endothelium of the blood brain barrier, hypothalamus, cortex, cerebellum, amygdala, olfactory bulb, and hippocampus, and studies have shown that insulin does indeed penetrate the CSF and spread globally throughout the CNS, data from PET scans indicate that increasing insulin levels does not increase the uptake of glucose in the brain.\textsuperscript{8,31} But if this is the case and insulin resistance plays no role in the pathogenesis of DM, why is it that those with Alzheimer’s disease have greater insulin resistance and are at a higher risk of developing DM, and why is that those with DM are 1.2 to 1.7 times more likely to acquire
Alzheimer’s disease? The studies performed on these correlations actually suggest the same or a similar pathogenesis between the development of these two diseases, and the root lies in insulin resistance. Those with Alzheimer’s seem to actually secrete more insulin in response to a glycemic load, and this “desensitization of neuronal insulin receptors” is what is believed to contribute to the development of sporadic Alzheimer’s disease in diabetics.

Other observations have been noted to exist in both diabetics and those with Alzheimer’s that may lead scientists and clinicians closer to the cause of cognitive impairment in these patients. First is the increased prevalence of inflammatory markers such as IL-6, C-reactive protein, and α-1-antichymotrypsin in any patient with insulin resistance. The second observation was disturbance of the hypothalamic-pituitary adrenal axis leading to excess levels of cortisol, which correlated to poorer memory performance and damaging structural changes in the hippocampus. The last observation was that DM patients had increased formation of senile plaques commonly found in Alzheimer’s patients, which are the result of cleavage of amyloid precursor protein in neurons into β-amyloid. Insulin-degrading enzyme is typically responsible for the breakdown of APP and β-amyloid, and is up-regulated by the presence of insulin. However in an insulin resistant environment, higher glucose levels result in AGEing, and the enzyme instead binds to RAGEs (Receptor for Advanced Glycated End Products); the plaques are allowed to accumulate, thus bringing about glial and neuronal dysfunction.

Cardiovascular Neuropathy and the CNS

According to the Toronto Consensus Panel on Diabetic Neuropathy, sudden cardiac death is two to four times more likely in type 2 diabetics than any other subset of the population.

What is even more surprising is that this alarming statistic is not the product of accumulated
comorbidities that usually present with diabetes, but rather SCD is more prevalent because of cardiovascular autonomic neuropathy, a term assigned to “the impairment of cardiovascular autonomic control in the setting of diabetes after the exclusion of other causes.” Present in about 20% of the diabetic population, its prevalence can increase, depending on the duration of diabetes and age, to 65%. CAN is characterized by damage to the autonomic nerves that provide innervation to the blood vessels and heart. Because the ANS is primarily responsible for regulating heart rate variability and cardiac performance, damage to these nerves manifests as silent myocardial infarction, intraoperative cardiovascular liability, exercise intolerance, orthostatic hypotension, and resting tachycardia. All of these classic signs are linked to neuropathy that begins peripherally and works its way proximally. Moreover, because neuropathy affects longer nerves first, the vagus nerve is affected almost immediately and parasympathetic activity is severely down-regulated, as the tenth cranial nerve is normally responsible for 75% of parasympathetic function.

Underlying all these clinical manifestations is a distinct inability of the body to interpret sensory stimuli and respond appropriately. With regards to exercise intolerance, for example, autonomic dysfunction minimizes the normal rise in blood pressure and heart rate that would normally occur, thereby reducing cardiac output and the person’s ability to supply their more metabolically active tissues with oxygen. The tachycardia observed is from impairment of the vagus nerve, which results in sympathetic overactivation characterized by abnormal norepinephrine metabolism and signaling. QTi prolongation is also present, which is quantified as the delay in ventricular repolarization, and could be the reason why type I diabetics
suffer from arrhythmias and are more prone to the ‘dead in bed’ syndrome.\textsuperscript{32} Such cardiac rhythm disturbances are thought to be the result of electrolytic myocardial changes, as well as imbalances in sympathetic innervation.\textsuperscript{32} Orthostatic hypotension is thought to be the result of a reduction in the norepinephrine response that is proper for a relative drop in blood pressure, and would indicate a defect in the “centrally mediated” baroreflex.\textsuperscript{33} Reduction in the ventilator drive induced by hypoxia has also been observed in diabetic patients with CAN.\textsuperscript{33} Even in sleep, blood pressure variations based on circadian rhythms are improperly regulated, resulting in nocturnal hypertension.\textsuperscript{32, 34}

Although neuropathy is often a complication of diabetes mellitus and plays a role in much of the cardiovascular dysfunction observed in the diabetic population, the decreased effectiveness of certain cardiac and respiratory reflexes would suggest that diabetes might have a detrimental effect on heart rate variability via central influences as well. A study conducted on STZ-injected rats revealed that they had decreased sensitivity to neuropeptide Y, which led them to conclude that chronic diabetes results in a decrease in the sensitivity of NTS neurons to certain neurotransmitters, and as a result sensory stimuli.\textsuperscript{35} In addition, diabetes may also influence the neurotransmitter metabolism.\textsuperscript{2} One circulating hypothesis is that cardiovascular autonomic neuropathy is instigated by the “dysregulation of neuropeptides, such as calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP)” and is characterized by prolongation of the interval of ventricular depolarization and repolarization.\textsuperscript{36} The cause seems to be irregular plasma levels of CGRP, ANP, VIP, NPY, and SP, whose vasomodulatory effects are what maintain cardiovascular homeostasis.\textsuperscript{36} Thus there exists a potential mechanism stemming from the central nervous system by which diabetes mellitus might influence cardiovascular function.
Effects of Diabetes Mellitus on Neurogenesis

Neurogenesis is defined as the process by which progenitor cells of the brain differentiate and becoming integrated on a functional level in the adult brain.\(^{37}\) After development of the mammalian brain \textit{in utero}, this plasticity becomes limited to a few defined regions, such as the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles, while the rest of the cerebral cortex is limited to neurogenic-to-astrogenic transformations.\(^{38,39}\) In the subgranular zone of the DG, for example, progenitor cells proliferate and then travel to the granular cell layer.\(^{38}\) The stimulus for a plastic event usually stems from the environment, or a multiplicity of factors involving stress, pathological situations, aging, hormones, neurotransmitters, growth factors, neurodegeneration, or inflammation.\(^{37}\) Neural progenitor proliferation is consequently induced by a large multitude of transcription factors that are able to “sense” these changes and appropriate the proper physiological response, and often the production of these new hippocampal neurons is associated with memory formation and learning.\(^{37,38,39}\) With regards to differentiation, it is surprising to note that most newly generated neurons actually do not survive even in a normal physiological state, making the integration of those that do survive of even greater importance.\(^{37}\) Moreover, because the addition of new neurons in response to external and internal stimuli is purposed to optimize the communication between the CA3 pyramidal subfield and the dentate gyrus, any changes to the process of neurogenesis itself could influence and potentially interfere with the functions of this brain region.\(^{38}\)

In diabetes, the state of the brain is not favorable towards the normal process of neurogenesis, and according to the literature, hippocampal alterations occur long before diabetes
clinically manifests, as early as the pre-diabetic state. In NOD mice, for example, there was a 50% decrease in the rate of cell survival in the dentate gyrus, an overall low level of proliferation, and hippocampal astrogliosis was also observed. These pathological signs are normally descriptors of aging and stress-induced depression, but unfortunately are not the only features of premature decline in diabetics. In addition to early astrogliosis and low DG neuron count, increased levels of microglia indicative of ongoing inflammation are often present, in addition to greater expression of immune response genes, cytokines, and cytokine receptors. This inflammation is detrimental for neurogenesis in the hippocampus and has also been implicated in the progression of Alzheimer’s disease and depression.

Alterations in the structure of the diabetic hippocampus have also been correlated to the presence of the neuromarker apolipoprotein-E, which is normally a sign of neuronal dysfunction. Typically, this protein enters neurons when the cell is undergoing repair following some form of insult or injury, and is expressed by ependymal cells, astrocytes, and oligodendrocytes. Apo-E is thought to facilitate the removal of cellular debris because when the gene encoding it is mutated, the products of degeneration remain in the hippocampal cells. Moreover, the worse the hippocampal injury, the greater the intensity of Apo-E expression in neurons that are degenerating, and most especially in the pyramidal neurons of CA1 and CA3. Considered to have a neurotrophic role involved in learning and neuroplasticity, Apo-E expression has been linked to an attempt by the body to counter the progression of neurodegeneration. In addition, there is abnormal expression of the genes coding for proteins that would normally induce neuronal activation, such as Jun+ in the DG, CA1, and CA3 regions.
One of the main reasons why diabetes is detrimental to the brain state, and more specifically hippocampal neurogenesis, is because diabetes is a metabolic stress. Although stress would normally induce neuroplasticity in order to invoke adaptation to the stimuli, prolonged exposure actually instead produces an interminable burden resulting in allostatic overload.\textsuperscript{41} Consequently, there is an increase in the expression of hypothalamic hormones, which in turn lead to overactivation of the hypothalamo-pituitary-adrenal axis (increasing cortisol levels) and an elevation in the sensitivity of the brain to stress.\textsuperscript{38, 40} Moreover because the hippocampus is rich in glucocorticoid receptors, and because glucocorticoids regulate hippocampal neurogenesis negatively, the result is a decreased number of new neurons in the dentate gyrus.\textsuperscript{37, 38} Other effects of this metabolic stressor include increased expression of apoptosis markers in the hippocampus, as well as reductions in the expression of synaptic proteins and dendritic spines.\textsuperscript{41} Because dendrites actually comprise 95\% of the total neuronal volume, this would account for why diabetics have reduced hippocampal volumes.\textsuperscript{41}

Current studies have made an association not only between diabetes and neurodegenerative diseases such as Alzheimer’s, but depression as well.\textsuperscript{37} Epidemiological studies reveal that depression occurs at a rate of 12\% in the type I diabetic population versus 3.2\% in the non-diabetic population, and 19.1\% in type II diabetics versus the general populace.\textsuperscript{41} This reveals an interesting relationship between the comorbidities, which is reciprocal in the sense that diabetes increases the chance of suffering from depression and depression increases the likelihood of diabetes.\textsuperscript{41} Although poor self-care and lack of medical adherence may be at the root of this issue, insulin resistance in the brain is a more likely culprit as the link between the acquisition of depression and diabetes.\textsuperscript{41} As a result of hyperinsulinemia,
there are alterations in the levels of dopamine and serotonin in the CNS, as well as neurotrophins. Moreover, studies performed on animal models of depression and diabetes showed defective and reduced neurogenesis in the dentate gyrus, with decreases in hippocampal cell proliferation as large as 56% in comparison to the 27% decrease in diabetic rodents without depression. Neuronal differentiation also suffered a decline in depressed diabetic rats by a difference of 17%, and cell survival was diminished by 44% in comparison to the diabetic model, which only suffered a 15% decrease in survival. Researchers therefore made the claim that depression occurs as a result of a decline in hippocampal neurogenesis below a certain threshold. Because diabetes interferes with neurogenesis in the brain, a subset of the diabetic population will reach and pass that threshold, resulting in depressive behavior. In support of this conclusion, treatment with antidepressant medications actually restores neurogenesis in the hippocampus to some degree.

A newer area of study involving the effects of cognition and behavior, and to which the effects of diabetes would be interesting to explore, is related to another area of neurogenesis, the olfactory bulb. Although more crucial to the behavior of animals such as mice, in the mammalian brain in general, progenitor neuronal cells are known to travel from the subventricular zone to the olfactory bulb via the rostral migratory stream. Once there, these stem cells become inhibitory interneurons through the process of differentiation. In a study involving a mutated \( \text{erk}5 \) gene, a sequence which codes for the signaling protein EPK5 that helps to regulate neurogenesis in the SVZ-RMS-OB axis, researchers observed that the mice had reduced short-term memory for certain odors, insensitivity with regards to odor detection, and deficits in olfactory-dependent behaviors. This finding has great significance in that many
neurodegenerative diseases, injuries, and even ordinary aging are linked to impairment in olfaction. It would therefore be considerable if a strategy was developed by which the decline in olfactory function could be halted and potentially mitigated in order to restore olfactory-linked functions in behavior and memory.

**Implications for Current and Future Treatments**

Although current treatments have managed to extend the length and quality of the lives of diabetic patients by reducing the incidence of complications, it has not been sufficient to wholly prevent its toll on their peripheral nerves, hearts, eyes, vasculature, and kidneys. Some studies have even suggested that current treatments may be the very cause of cognitive dysfunction and the increased prevalence of dementia in older diabetic patients.

The first concern is that tighter glycemic control increases the prevalence and severity of hypoglycemic episodes, which have been previously implicated as the cause of decreased cognitive ability in diabetics. Occurring as often as 30 times per year, these events are characterized by malaise, hunger, anxiety, diaphoresis, and tremors, and the more often they occur the more likely the patient is to acquire hypoglycemia unawareness. Blunting of the counterregulatory response to hypoglycemia can in turn lead to neuroglycopenia characterized by confusion and seizures, and persistent hypoglycemia is associated with a torrent of sympathetic activity via the release of catecholamines. The resulting hemodynamic changes are thought to precipitate events of cerebral ischemia, and it is that reduced blood flow that could be the very mechanism for neurodegeneration on a regional and global level in the diabetic brain. There are, however, two sides to this concept. Though diabetic patients from epidemiological studies in an ambulatory setting did have a higher risk of cerebrovascular events if they had a
prior history of hypoglycemia, those that chose to participate in an intensive insulin therapy group and were consequently more prone to hypoglycemia did not.\textsuperscript{42} Therefore, it may be concluded that the cardioprotective benefits of these more intensive therapies outweigh the effects of hypoglycemia.\textsuperscript{32, 42}

Other studies have posed that insulin itself, and not hypoglycemia, is the culprit of “age-related memory decline and diabetic encephalopathy.”\textsuperscript{7} Though once thought incapable of crossing the blood brain barrier (with the brain itself considered an insulin-independent organ) this hormone has been found in abundance in the hypothalamus and olfactory bulb, and its receptors have been found in the hippocampus and limbic system as well.\textsuperscript{7} Researchers postulate that insulin enables a gradual amassment of oxidized proteins by competitively inhibiting the role of insulin-degrading enzyme in protein turnover.\textsuperscript{7} Moreover, insulin actually inactivates a family of transcription factors that would normally allow the expression of genes responsible for defending against oxidative stress.\textsuperscript{7} This has been correlated to the observation that of the population suffering from type 2 diabetes, those undergoing insulin therapy have the greatest risk of developing dementia.\textsuperscript{7}

Furthermore, although most studies indicate that insulin is beneficial in the CNS because it increases the activity of insulin-degrading enzyme, which is responsible for the breakdown of senile amyloid plaques, others have suggested that insulin decreases intracellular levels of β-amyloid by facilitating its secretion.\textsuperscript{8} This would raise levels of extracellular β-amyloid, which is allegedly responsible for memory loss and the development of Alzheimer’s disease.\textsuperscript{8} Such conflicting results foster a greater sense of urgency for research in this field, so that the causative agents of cognitive decline do not turn out to be rooted in present-day treatments.
According to the literature, some of the findings related to macrostructural brain changes and their related pathological mechanisms suggest possible avenues in and of themselves for ameliorating the progression and severity of cognitive decline. The study that discovered that brain glucose metabolism in type 2 diabetics is more widespread regionally than in their nondiabetic counterparts, for example, found that increasing metabolism in the normally activated regions resulted in better performance on memory tests. Moreover, a reduction in the modifiable vascular risk factors thought to be associated with the development of Alzheimer’s and VaD would decrease the likelihood of such pathological states manifesting in older diabetics, especially if treated prior to the development of clinically significant cerebrovascular disease. These forms of secondary prevention would also reduce the risk of strokes and cardiovascular disease, and improve recovery rates in diabetics, a subset of the population in which risks are already higher than normal and rehabilitation outcomes less than satisfactory.

Such an emphasis has been placed on increasing the foundational understanding of diabetes mellitus and its neurophysiologic and structural effects on the brain because with regards to quality of life and functional ability in the elderly, cognition plays an important role. With its decline comes an actual decreased response to treatments for various medical conditions; therefore better comprehension of the progression, pathophysiology, and risk factors that accentuate these cognitive defects will allow healthcare providers and patients to take advantage of earlier windows of opportunity where medical interventions will prove most effective.
APPENDIX A: ABREVIATIONS

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apo-E: Apolipoprotein E
CA1: Cornu Ammonis area 1
CA2: Cornu Ammonis area 2
CA3: Cornu Ammonis area 3
CAN: Cardiovascular Autonomic Neuropathy
CBF: Cerebral Blood Flow
CNS: Central Nervous System
CSF: Cerebrospinal Fluid
CT: Computed Tomography
DG: Dentate Gyrus
DM: Diabetes Mellitus
EEG: Electroencephalogram
FVB: Friend Virus B (Non-diabetic transgenic mouse model)
GM: Gray matter
HbA1c: Glycated hemoglobin
LTD: Long Term Depression
LTP: Long Term Potentiation
MI: Myocardial Infarction
MRI: Magnetic Resonance Imaging
NMDA: N-methyl-D-aspartate
OB: Olfactory Bulb
OVE26: Transgenic type I diabetic mouse model
PET: Positron Emission Tomography
RAGE: Receptor for advanced glycated end products
RMS: Rostral Migratory Stream
ROS: Reactive Oxygen Species
SCD: Sudden Cardiac Death
SOD: Superoxide Dismutase
STZ: Streptozotocin
SVZ: Subventricular Zone
WM: White matter
APPENDIX B: SOLUTIONS FOR IMMUNOHISTOCHEMISTRY

**BSA Stock, 0.04 g/mL (50 mL)**
- 2 g BSA (Bovine Serum Albumin) powder
- Bring to 50 mL with PBS in 50 mL conical tube
- Mix well by vortexing

**Triton X 100 (TX-100) Stock (50 μL/mL, 50 mL)**
- 2.5 mL TX-100
- Bring to 50 mL with PBS in 50 mL conical
- Mix well by vortexing

**Sodium Azide (NaNH₃) Stock, 8 mg/mL (10 mL)**
- Bring 80 mg (0.08g) sodium azide to 10 mL PBS in 15 mL conical tube.
- Mix well by vortexing

**10X PBST, 30 μL/mL TX-100 (50 mL)**
- 1.5 mL TX-100 to 50 mL PBS
- Mix completely by vortexing

**PBS-T, 3 μL/mL TX-100 (50 mL)**
- 5 mL 10 PBS-T
- Bring to 50 mL in PBS

**Blocking Solution (15 mL)**
- 3.750 mL PBS Buffer
- 7.500 mL BSA Stock
- 0.750 mL Normal Donkey Sera
- 1.500 mL NaNH₃ Stock

**Primary Antibody, 1ug/mL (1.5 mL)**
- 0.480 mL PBS Buffer
- 0.750 mL BSA Stock
- 0.090 mL TX-100 Stock
- 0.030 mL Normal Donkey Sera
- 0.150 mL NaNH₃ Stock
- 0.015 mL Primary Antibody [Abcam Rabbit Polyclonal anti-musashi1 antibody (ab21628, Lot# GR 72252-1), 100ug/mL]

**Secondary Antibody (1:500)**
- 1.5 mL PBS-T
- 3 μL Secondary Antibody [Alexafluor Donkey Anti-Rabbit (a21207, Lot# 421207), 2 μg/mL]
REFERENCES


