An Investigation of the Role of Insulin Deficiency and Loss of PI3K-Akt Signaling in the Pathogenesis of the Diabetic Brain

by

Fatemeh Derakhshan

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Abstract

Type 1 diabetes mellitus (DM) is associated with cognitive dysfunction, cerebral atrophy and white matter abnormalities composing diabetic leukoencephalopathy (DLE). Insulin deficiency contributes to these deficits and is amenable to replacement with intervention using intranasal insulin (I-I) delivery. An important insulin-mediated signalling pathway is propagated through phosphatidylinositol 3-kinase (PI3K) and Akt. We hypothesized that blockade of the PI3K-Akt pathway would prevent I-I’s beneficial effects in mice with DLE, and that this blockade would contribute to development of similar dysfunction in non-diabetic mice. Transgenic mice overexpressing cerebral Akt were expected to be protected from cognitive and white matter changes associated with DM. We interrogated the PI3K-Akt signalling pathway in a mouse model of Streptozotocin-induced type 1 DM over 7 months of life. Diabetic and non-diabetic mice received daily I-I (or intranasal-saline (I-S) for controls) concurrently with intranasal delivery of a PI3K inhibitor (Wortmannin) or an Akt inhibitor (API2). Mice were tested weekly for cognitive function, using a battery of behavioural tests, and endpoint magnetic resonance imaging (MRI). DM mice receiving I-I were protected from cognitive decline, while those mice receiving I-I along with either Wortmannin or API2 were subject to cognitive decline. Interestingly, non-DM mice receiving Wortmannin also developed significant cognitive dysfunction. Akt overexpressing transgenic diabetic mice were protected from cognitive decline. These results suggesting the importance of the PI3K-Akt pathway in DLE in the mouse model of DM.
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AC</td>
<td>Anterior commissure</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end-products</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AKTO</td>
<td>Akt overexpressing</td>
</tr>
<tr>
<td>API-2</td>
<td>Akt/Protein Kinase B Signaling Inhibitor-2</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood–Brain Barrier</td>
</tr>
<tr>
<td>CAP</td>
<td>Cbl-associated protein</td>
</tr>
<tr>
<td>Cbl</td>
<td>Casitas b-lineage lymphoma</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus collosumn</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPu</td>
<td>Caudate/putamen</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>DLE</td>
<td>Diabetic leukoencephalopathy</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
</tr>
<tr>
<td>FA</td>
<td>Fractional anisotropy</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>IC</td>
<td>Onternal capsule</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like Growth Factor</td>
</tr>
<tr>
<td>I-I</td>
<td>Intranasal insulin</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>I-S</td>
<td>Intranasal Saline</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>M1</td>
<td>Primary motor cortex</td>
</tr>
<tr>
<td>MANOVA</td>
<td>Multivariate analysis of variance</td>
</tr>
<tr>
<td>MAPK/ERK</td>
<td>Ras–mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTR</td>
<td>Magnetization transfer ratio</td>
</tr>
<tr>
<td>PC</td>
<td>Posterior commissure</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Regions of interest</td>
</tr>
<tr>
<td>S1</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TE</td>
<td>Echo Time</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine kinases</td>
</tr>
<tr>
<td>V1</td>
<td>Primary visual cortex</td>
</tr>
<tr>
<td>WMA</td>
<td>White matter abnormalities</td>
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Chapter One: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder of improper glucose utilization that can occur in multiple species. DM has a prevalence of about 8-10% of the Canadian human population [1, 2], and its increasing prevalence throughout the world has become an important public health problem. DM is classified into two main types: type 1 diabetes, and type 2 diabetes [3, 4]. The present project focuses on type 1 diabetes which is typically caused by an autoimmune destruction of insulin-producing beta cells of the pancreas. DM patients present with multiple systemic complications such as neuropathy, nephropathy, retinopathy, heart disease, and vascular disease [3, 5]. As patients with DM continue to live longer, the complications of DM will become more evident. An overlooked complication is the development of diabetic leukoencephalopathy - DM-mediated changes in the brain, including cognitive impairment [6-8]. DM patients experience a slowing of information processing, reduced psychomotor efficiency, attention deficits, and impaired mental flexibility with neuropsychological testing [7, 8]. Likewise, using established behavioural tests, non-human diabetes models, specifically spontaneous or experimentally induced diabetic rodents, have demonstrated learning and memory deficits, poor task comprehension, and lack of problem solving abilities [6, 9, 10].

The murine model of streptozotocin (STZ)-induced type 1 DM brain is well characterized to have pancreatic beta cell loss, limited insulin production, and abrupt onset of untreated, yet sustainable, type 1 DM [10]. Previous studies have shown cognitive dysfunction, brain atrophy (>20% loss of brain mass and volume), and brain structural changes in STZ-induced diabetic mice [9-12]. Similar changes have also been detected in murine models of type 2 diabetes [12].
Determination of the cause of DM-mediated cerebral changes has been challenging. While some studies have targeted direct ‘neurotoxic’ effects of hyperglycaemia, including increased oxidative stress, and enhanced formation of advanced glycation end-products (AGEs)[13, 14], other studies focus on vascular changes, including alterations in cerebral blood flow and angiopathy [14]. Recent findings which demonstrate deficiency of insulin, its receptor and associated signalling machinery in both rat and mouse DM brains [9, 15] highlight the role of insulin in the pathogenesis of DM brain. Moreover, recent studies have demonstrated that direct insulin replacement to the brain alleviates such changes in the experimental type 1 DM brain [9, 16-18]. Therefore, a potential mechanism explaining diabetic changes may be the impairment of insulin signalling.

Currently, the effects of diabetes upon cognition are only beginning to be understood and no preventive therapies are available. Our laboratory has used an alternative method of insulin delivery to the brain in a mouse model of DM, intranasal insulin (I-I); this has improved behavioral and morphological abnormalities within the DM mouse brain [19]. I-I achieves higher cerebral concentrations, lower mortality, and less systemic delivery than does subcutaneous insulin delivery, which is associated with hypoglycemia when similar amounts of insulin are provided [18, 19]. It is believed that insulin’s effects in the brain occur through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which is responsible for most of the metabolic actions of the insulin signalling machinery [20-22] [Figure 1.1]. Our prior work has shown that PI3K, Akt, pAkt, pCREB, and pGSK3β, which are all critical signals in the insulin-PI3K-Akt pathway, are all diminished in the experimental DM brain, but are partially restored when I-I treatment is conducted over many months[9]. Building on these findings, the focus of
my research project was to determine the importance of the insulin-mediated PI3K-Akt signalling pathway in the pathogenesis of DM brain changes.

The main hypothesis of this study was that the changes seen in the DM brain are mainly due to reduced insulin signalling through the PI3K-Akt pathway, and that direct support of the PI3K-Akt pathway can prevent cognitive dysfunction, cerebral atrophy and white matter abnormalities (WMA) in the diabetic mouse brain.

I studied both blockade and enhancement of the PI3K-Akt pathway in a mouse model that had STZ-induced-diabetes. My project examined the impact of modulation of the PI3K-Akt pathway upon both cognition and in vivo brain imaging. We studied type 1 diabetic mice (receiving STZ injections at 1 month of age), which received I-I (or intranasal-saline for controls) with concurrent PI3K inhibitor or Akt inhibitor for 7 months. We also studied non-diabetic mice (receiving citrate injections), which also received PI3K inhibitor or Akt inhibitor for 7 months. Mice were tested for brain structure changes using magnetic resonance imaging (MRI) and cognitive function. We anticipated DM mice receiving PI3K inhibitor or Akt inhibitor would lose the beneficial effects of receiving I-I. Meanwhile, non-diabetic mice receiving PI3K inhibitor or Akt inhibitor were expected to develop similar neurodegenerative changes as seen in diabetic mice without intervention. This work was complemented by studies of transgenic mice overexpressing cerebral Akt, with similar interventions and endpoints. That assisted in our understanding of the role of Akt in the brain exposed to diabetes. This study is instrumental in determining whether DM-mediated changes in the brain can be remedied in humans with DM by using novel forms of therapy targeted downstream of insulin’s effect.
Figure 1.1: Major insulin signalling pathways

The insulin signalling pathways regulate a diverse range of biologic responses, including glucose transport, protein synthesis, mitogenesis, and cell survival via 3 major pathways. Pharmacological inhibitors, Wortmannin and API-2, were applied in this study to block PI3K and Akt activity, respectively.
1.2 BACKGROUND

1.2.1 Diabetes Mellitus

Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycemia. The two broad categories of DM are called type 1 and type 2. Both types of diabetes are preceded by a phase of abnormal glucose homeostasis as the pathogenic processes progress [1, 23]. Type 1 DM is the result of total or sub-total insulin deficiency. It is characterized by a specific destruction of the pancreatic β-cells, commonly associated with immune-mediated damage, which finally progress to absolute or near complete insulinopenia [3]. While the exact cause of Type 1 DM is not known, it is believed that the etiology of type 1 DM involves a combination of genetic and environmental factors leading to an autoimmune defect. The treatment of type 1 DM includes insulin replacement and rigid blood glucose level monitoring with the ultimate goal of normal glucose maintenance [3]. However, despite adequate or even strict type 1 DM management, DM continues to lead to major complications, such as diabetic leukoencephalopathy, which will be discussed in detail in a subsequent section [24-26]. For our research, we investigated a model of type 1 DM, but our laboratory has noted similar neurodegenerative features in models of type 2 DM as well [11].

Type 2 DM is a heterogeneous group of disorders described by variable degrees of insulin resistance, impaired insulin release in response to glucose, or increased hepatic glucose production [3, 4]. Formerly termed adult-onset DM, as it is generally diagnosed in adults, type 2 DM is now becoming more common in children and adolescents in concurrence with the rising rates in childhood obesity [27]. Type 2 DM also leads to systemic complications such as cardiovascular disease and diabetic leukoencephalopathy [3, 7, 27-29].
The metabolic dysregulation associated with DM causes secondary pathophysiologic changes in multiple organ systems that impose a tremendous burden on the individual with DM and on the health care system. This is occurring while the prevalence of DM has been exploding, exceeding prognostications for 2030 and 2050 already as of 2010 [30].

As our population ages, the various complications of DM are becoming more evident. While DM is best known for its peripheral complications, characterized by such end-organ damage-as neuropathy, nephropathy, and retinopathy, it also leads to slowly progressing changes in the brain, often referred to as “diabetic leukoencephalopathy” (DLE) [13, 31-33]. DLE includes cognitive changes, cerebral atrophy, white matter abnormalities (WMAs), and identifiable structural changes [34]. Therefore, a greater understanding of the potential etiologies of this condition is sorely needed in the next decades.

1.2.2 Human Diabetic Leukoencephalopathy

Changes in the diabetic brain can be summarized by the term diabetic leukoencephalopathy (DLE), which is defined as DM-mediated changes in cerebral function and structure that encompass functional impairment of cognition, cerebral signal conduction, neurotransmission and synaptic plasticity, as well as underlying structural pathology [9, 10, 13, 19, 31, 34-38]. The present project focused on the cognitive impairment component of DLE, as well as WMAs and cerebral atrophy, which can be identified through non-invasive in vivo MRI studies.

1.2.2.1 Diabetes and Cognitive Impairment

Cognition refers to a set of vastly complex processes, including, but not limited to: memory, language, problem solving, attention and thinking, which apply plans and strategies to sensations and perceptions. Both neuropsychological testing in human DM patients and behavioral testing
in non-human DM models have shown DM-associated cognitive decline; moreover, studies examining the structure of the diabetic brain in humans and non-humans have demonstrated related cerebral abnormalities, particularly in the hippocampus, a structure in the medial temporal lobe, which has attracted particular attention because of its well studied role in the learning and memory storage processes [6, 7, 34, 39, 40]. Furthermore, DM is associated with dementia and numerous neurodegenerative disorders, in particular Alzheimer's disease (AD) [28, 29, 32, 41, 42].

The most consistent cognitive deficits described in DM patients are slowing of information processing, reduced psychomotor efficiency, attention deficits, declining visuospatial memory, and impaired mental flexibility [6, 43, 44]. Additionally, other domains affected include general intelligence, executive function, memory, learning, problem solving, motor speed and strength, and vocabulary [45-48]. Although the changes within the DM brain resemble those of exaggerated aging, they are not limited to the elderly; diabetic brain changes have been described in both children [46, 49, 50] and adults, as well as in both type 1 and type 2 DM [7, 27-29, 39, 41, 51, 52]. It does not appear as though hypoglycaemic events are important in the development of cognitive decline or impairment in patients with type 1 DM [24, 53, 54].

*Type 2 Diabetes and Cognitive Impairment*

The results of neuropsychological studies in type 2 diabetic patients suggest moderate degrees of cognitive impairment, predominantly in tasks involving verbal memory or information processing speed [55, 56]. Although, the risk of cognitive impairment in Type 2 DM is well established, the underlying mechanisms remain largely unexplained. Type 2 DM typically develops in the context of “metabolic syndrome”, which describes a cluster of vascular and
metabolic risk factors, including: hypertension, dyslipidemia, and obesity [57]. The metabolic syndrome itself, with or without hyperglycemia, is associated with atherosclerotic cardiovascular disease and ischaemic stroke, as well as with cognitive decline and dementia [58-61].

**Type 1 Diabetes in Children and Cognitive Impairment**

A few studies have looked at neuropsychological test performance and school achievement in children with DM. Although there is some controversy as to how DM affects children’s cognitive function [62-65], the observation that children with early onset DM (e.g., before the age of 6) are at increased risk for slowing of intellectual development is quite consistent [49, 50, 62-65]. Most studies report that children with type 1 DM perform worse than control subjects on measures of intelligence, attention, processing speed, long-term memory, and executive skills [63, 64]. Performance is worse the earlier the onset of the disease; the difference might be up to 10–20 IQ points.

**Type 1 Diabetes in Adults and Cognitive Impairment**

A meta-analysis of studies on cognition in adult patients with type 1 DM showed that cognitive performance in patients with type 1 DM is characterized by mild-to-moderate slowing of mental speed and a diminished mental flexibility [8, 26]. In adults, DM is associated with poor cognitive performance in both older (> 58 years) and younger (< 58 years) age groups [26]. In addition, there are clear indications that the impairments are progressive over time [66].

The potential mechanisms for these changes are not yet understood. Previous data suggest that at least part of the cognitive changes observed in type 1 diabetic patients are due to complications of chronic hyperglycemia [26, 67], despite the fact that a relationship between impaired cognition and increased glycated hemoglobin (HbA1c) levels, which is the indicator of long-term blood glucose control, has not been clearly established [26]. In this context, the effect
of hypoglycemia also needs to be addressed. The occurrence of episodes of severe hypoglycemia is an unwanted side effect of intensified insulin therapy [24]. However, a large prospective survey on the consequences of severe hypoglycemic episodes on cognition in patients receiving intensified insulin therapy does not show significant negative effects [68].

Some studies point out the effects of insulin and insulin receptors on cognitive deficits in diabetic patients [69-74]. Studies have shown that insulin plays a modulator role in certain behaviours, including the tasks of learning and memory [73-75]. The intriguing fact that the degree of cognitive dysfunction and brain atrophy are greater in humans with type 2 DM as compared with type 1 DM [76], while patients with type 1 DM usually have greater overall complications [3], can also be suggestive of the role of insulin in the diabetic brain. As type 1 DM patients commonly are treated with insulin, and this possibly can prevent neurodegeneration to some degree. The possible role of insulin in the DM brain will be discussed below.

I examined the impact of DM upon white matter and cerebral atrophy in a mouse model of type 1 DM, already established to have white matter hyperintensity, cerebral atrophy and cognitive decline [9, 13]. Thus, this can be considered analogous to the human condition.

**Diabetes, Mild Cognitive Impairment and Alzheimer’s Disease**

Numerous biochemical [77, 78] imaging-related [79], and histopathological findings [80] support a causal link between DM and cognitive impairments. With the global increasing prevalence of DM, the evidence that DM is a risk factor for dementia is of significant public health importance. Numerous studies have demonstrated that patients with DM have an increased risk of developing AD when compared with individuals without DM [26, 32].
Likewise, 80% of AD patients have glucose intolerance or DM [81]. There are some central associations between AD and DM - insulin resistance and dysregulation of the degradation of neurotoxic amyloid and insulin in particular. Such links have led to AD being called “Type 3 diabetes” [82]. Reduced glucose tolerance, insulin resistance and hyperinsulinemia exert a negative influence on memory, with associations to hippocampal atrophy [79, 83, 84]. DM is associated with neuropathological markers of AD [85]. Although initially, it could be assumed that changes in the diabetic brain are related to microvascular and macrovascular changes seen elsewhere in the body, the pathogenic mechanisms behind DLE remain multifactorial, complex, and unclear.

Along with the strong association of DM and risk of dementia, it is recently shown that DM increases the risk of conversion from mild cognitive impairment to dementia [86]. Mild cognitive impairment (MCI) is defined as a worsening of memory, attention, and cognitive function that exceeds what would be expected for the individual’s age and level of education, yet does not cause impairment of daily activities [87, 88]. MCI may be a precursor of dementia; the rate of transition from MCI to dementia is estimated to be about 10% to 15% per year [89]. Recent evidence suggests that impaired energy metabolism, oxidative stress, insulin resistance, and insulin deficiency in the brain, which are all seen in DM, should be incorporated into MCI pathophysiology [67, 86, 90, 91]. In patients with mild cognitive impairment fasting plasma insulin and levels of Aβ42, the amyloid beta isoform which is the main component of amyloid plaque in the brains of patients with Alzheimer's disease, are linearly correlated [91]. Interestingly, to the extent that WMA is linked to DLE, initial white matter changes among individuals with MCI may predict their cognitive status overtime [92].
1.2.2.2 White Matter Abnormalities in the Diabetic Brain

The Rotterdam Study puts forward some of the strongest evidence for a link between WMA and cognitive decline: the risk of incident dementia was modestly elevated in the presence of WMA [93-96]. Other population-based studies also show higher risk of cognitive decline in patients with high WMA burden at baseline [97, 98]. WMA is introduced as a potential pathological marker of DM brain cognitive decline, which can frequently be detected in the human DM brain using T2-weighted MRI [51, 93]. Moreover, WMA are a risk factor for stroke, and abnormalities in gait and balance [66, 99]. The pathological features of WMA vary and include axonal loss, decreased myelin content, loss of ependymal cell layer and reactive gliosis (also called granular ependymitis), infarction and accompanying arteriosclerotic vessel changes [6]. WMA theoretically lead to disconnection of axonal connections between the subcortical areas and the cortex [96].

1.2.2.3 Cerebral Atrophy in the Diabetic Brain

Several studies suggest that some degree of cerebral atrophy occurs in both type 1 and 2 DM patients [9, 28, 51, 79, 100, 101]. Schmidt et al. showed that cerebral atrophy, which is known as another pathological manifestation of the DM brain, is more prevalent by 10% in the DM brains as compared to the non-DM brains, and this worsens with poor diabetic control [100, 102-104]. Both cortical and subcortical atrophy occurs over time in the DM brain [28, 51, 102, 105, 106]. Macroscopic neuropathological studies have also revealed moderate to severe atrophy in DM brains. Medial temporal lobe atrophy, which is one of the early manifestations of Alzheimer’s disease (AD), seems to be an important anatomical feature of the DM brain [104]. Determination of the cause of DM-mediated cerebral atrophy has been challenging – While neuronal loss could
not be confirmed in different studies, there is consistent widespread synaptic loss [14, 38, 107-109] witnessed in each model of type 1 and 2 DM. This is analogous to AD, where synaptic loss precedes other forms of pathology [15] including neuronal loss. The presence of insulin receptors at central synapses suggests that insulin plays a critical role in the prevention of synaptic loss in the DM brain [14, 37, 72, 75, 110, 111]. Thus, synaptic loss may relate to blunting of the neuronal response to insulin, or absence of insulin, during DM; Synaptic loss due to insulin signalling deficiency may further lead to the subsequent neuronal loss [10, 14, 37, 112].

1.2.2.4 Brain Imaging in Diabetes

Studies on the structural brain changes were largely dependent on neuropathology before introduction of powerful neuroimaging techniques, such as computed tomography, and even more so MRI [34]. The non-invasive nature of MRI has opened unique opportunities for in vivo investigation of the changes in both human and experimental brains. Neuroimaging now is used for cognition and dementia research as well as daily clinical practice [106].

Previous studies used various imaging techniques to show changes in diabetic brain [11, 106]. MR images in particular were used to show WMA and brain atrophy in both human and diabetic murine models. In humans, a number of studies have assessed atrophy or white matter lesions in diabetic patients. Taken together, these studies suggest that white matter lesions and brain atrophy are more common in diabetic patients than in controls [34]. In the present study, three sets of MR scans, T2, MTR, and DTI were obtained at the endpoint (7 month of age). Here a brief overview on the principles and the applications of these imaging sequences is provided.

Since the hydrogen associated with water is the source of signal in the MR images, contrast in T2 images is based on local differences in the number of hydrogen nuclei per unit of tissue volume. T2 is the transverse relaxation time of the hydrogen protons, following manipulation of
their magnetic fields with a pulse of electromagnetic energy at a specific radiofrequency (RF). T2 value reflects the local rate of such “de-phasing” within a population of nuclei. The transverse magnetization after RF pulse decreases in an exponential manner characterized by T2 \[M_{xy}=M_0 \exp(-t/T2)\]. In T2 weighted images, MR images are acquired at one time-point (echo [TE]) after the application of each RF pulse. In brain, the more structured the tissue, the more quickly the de-phasing and hence the shorter the T2. Demyelination, gliosis, axonal loss and increased amounts of free water all are shown to produce high signal on T2-weighted images [9, 31, 113]. Brain atrophy could also be detected in these images, using volumetric measurements [9].

Biological tissues, like brain, are complex heterogeneous structures where protons may have free movement (e.g. in water, long T2), or limited motion as a consequence of their bonds to macromolecular structures (short T2). Restricted motion of protons in the ‘bound’ pool results in too fast T2 relaxation to allow its detection by conventional MRI methods. This may make conventional MRIs insensitive to early brain injury that can be detected histologically [114]. The magnetization transfer (MT) imaging sequence use an off-resonance radiofrequency pulse to saturate the restricted hydrogen pool, which results in an exchange of magnetization between the free and restricted hydrogen protons and causes changes in MR signal intensity depending on the rate and degree of this exchange. In clinical practice, the most frequently applied MT image is the MT ratio (MTR), which is obtained from a ratio of the image signal intensity acquired with and without the application of the off-resonance saturation pulse [113, 114]. MTR is often used as an index for assessing tissue integrity, white matter in particular. MTR reduction is evident in patients with multiple sclerosis within white matter lesions and regions of demyelination. MT imaging also detects tissue changes associated with stroke where, reductions in MTR have been
reported after cerebral ischemia or infarction [113, 114]. Diabetic human and rodents have been
demonstrated to have low MTR in different brain regions [11, 115].

Diffusion tensor imaging (DTI) allows measurement of the degree and directionality of
water proton diffusion within cerebral tissue [116-118]. Because diffusion is constrained
by cellular elements such as plasma membranes and myelin, the degree and direction of
diffusion is dependent on the microarchitecture of the tissue being measured. In cerebral
white matter, water proton diffusion in the direction perpendicular to the orientation of the
white matter fibres due to constraint of axonal localization. Thus, water protons are more
likely to diffuse along the direction of the white matter tracts. MRI DTI measures the
degree of diffusion in multiple directions, allowing an assessment of the overall extent of
diffusion in any direction (the mean diffusivity), the direction of the greatest amount of
diffusion (which is considered parallel to the orientation of white matter tracts in that MRI
voxel), and a mathematical measure of the overall directionality of diffusion termed
fractional anisotropy (FA) which ranges from 0 to 1 [117]. FA can range between values of
0 to 1, with an isotropic diffusion (such as in cerebral spinal fluid) having an FA of 0, while a
linear diffusion (such as water in a tubular structure) will have an FA of near 1 [Figure 1.2].
A loss of FA can be due to an increase in the water diffusion in the direction perpendicular to the
course of the WM fibers [116]; this can be interpreted as the degeneration of the axonal myelin
sheath (demyelination) and/or replacement of axonal fibers with other cells.

The white matter tracts consist of densely packed axons (neuronal projections) in
addition to various types of neuroglia and other small populations of cells. Water
molecules are distributed between these cell types and the extracellular space [116].
Moreover, the white matter consists of tracts that are running along various directions and
water molecules diffuse parallel to, rather than across, these fiber tracts. DTI is uniquely suited to assess white matter microstructure [116-119]. In humans, DTI has been studied in patients with stroke, brain tumour, multiple sclerosis and Alzheimer’s disease [11, 31, 116-120]. In patients with type 1 DM, DTI shows reduced FA in white matter tracts, with deficits correlated with impaired neurocognitive performance [118].

**Figure 1.2: Description of fractional anisotropy.**

DTI measures extracellular water diffusion. Isotropic diffusion happens when water can freely diffuse in a spherically symmetric manner (left). In the second row the circles exemplify the cross section of axons. When the axons are far apart FA indicates more isotropic diffusion. As the axons become more numerous, acquire myelin, and become tightly packed the diffusion of water becomes more anisotropic (right). Picture modified from Nagy et al (2005).

1.2.2.5 Oligodendrocytes in the Diabetic Brain

Neuropathological and imaging experiments in both human and diabetic mouse have shown DM related WMA [9, 51, 93, 98, 121]. These studies lead to a focus upon oligodendrocytes, the myelinating cells of the central nervous system. Several studies have reported decreased myelin content and downregulation of myelin markers [11, 122, 123]. Additionally, rigorous three-dimensional counting has demonstrated oligodendrocyte cell loss in DM mice and rats [9, 13].
In the present study we investigated the effect of Akt overexpression on DLE, using transgenic mice that overexpressed constitutively active Akt in oligodendrocytes. It was shown previously that oligodendrocytes in these mice continued actively myelinating throughout life, which therefore enhances CNS myelination [236].

1.2.3. Animal Models in Diabetic Encephalopathy

Animal models have been used extensively in DM research. Most experiments are performed on rodents [10], although some studies are still carried out on larger animals like rabbits and dogs [124-126]. Several toxins, including streptozotocin (STZ) and alloxan, can be used to induce hyperglycemia in rats and mice through pancreatic toxicity [10, 127]. Moreover, selective inbreeding has produced several strains of animal that are considered reasonable models of Type 1 DM, Type 2 DM and related phenotypes such as obesity and insulin resistance [128-130]. However, notably, these examples are only a model of DM and cannot precisely imitate the disease condition as it occurs in humans [127]. Despite this limitation, rodent models of DM are currently the best available non-human model of human type 1 DM. Besides, using rodents is cost effective and allows direct access to tissues, long-term study with a relatively short murine lifespan, breeding control and gene alteration, and the testing of novel therapies [127-129].

Type 1 DM in humans is characterized by autoimmune induced obliteration of the pancreatic beta cells. Although the damage may occur silently over many years, it may progress to absolute insulinopenia [3, 4]. There are spontaneous and experimentally induced forms of type 1 DM rodent models. Spontaneous diabetic rodent models are genetically susceptible to developing DM, while methods to induce experimental diabetes typically involve damage of the pancreas,
either surgically or chemically through the administration of toxins [128, 130]. The models of type 1 DM have a broad spectrum of pathophysiology, but the general feature is hyperglycemia. Of the experimentally induced type 1 DM models, streptozotocin (STZ) is the most commonly used β-cell cytotoxic agent. STZ is a nitrosourea derivative isolated from *Streptomyces Achromogenes* with broad-spectrum antibiotic and anti-neoplastic activity. It is a powerful alkylating agent that can interfere with glucose transport, and glucokinase function, as well as inducing multiple DNA strand breaks [130, 131]. It is taken up into the cell via the GLUT-2 glucose transporter, which is present at high levels in the insulin-producing β-cells of the islets of Langerhans [132, 133]. Since β-cells have low nicotinamide adenine dinucleotide content, they are particularly sensitive to STZ [131]. The GLUT-2 glucose transporter is not found at the blood-brain barrier [132, 134, 135], and as such the brain is excluded from any significant direct effects of STZ. Besides, no degenerating neurons were found in the mouse cortices 24 hours after STZ injection, according to Sweetnam et al study, which also suggest that STZ does not have a direct toxic effect on the brain [136].

There are a number of reliable protocols for establishing STZ diabetes in mice [133]. Multiple low doses of STZ over consecutive days induce incomplete β-cell destruction, leading to small amounts of residual insulin persisting. Therefore, STZ-diabetic rodents can survive without insulin treatment and rarely develop ketoacidosis due to low endogenous insulin levels. STZ-diabetic rodents have low levels of insulin along with hyperglycemia, both comparable to type 1 DM in humans. Comparable to humans, STZ-diabetic mice develop common diabetic complications including neuropathy, nephropathy, retinopathy, and others [128, 130].

Non-human models of DM also have cognitive impairments. Using behavioural cognitive tests, STZ induced diabetic mice exhibit learning and memory deficits, poor task comprehension
and procedural memory, and lack of problem solving skills [9, 13]. Interestingly, the performance of STZ-induced diabetic rodents on relatively simple behavioural tasks with a strong emotional component, such as passive avoidance paradigms, is preserved or even improved [10] whereas performance on more complex tests, such as a water maze or object recognition task, is disturbed [9, 10]. Our lab’s previous studies detected declines in cognitive function in visuospatial and procedural tasks in Type 1 diabetic mice [9]. The development of performance deficits on these tasks is dependent on DM duration and the severity of hyperglycaemia, and the deficits can be prevented with intensive insulin treatment [10]. The behavioural cognitive test battery used in this study has previously been applied in other studies examining cognition in diabetic mice.

According to Magarinos and McEwen’s study, STZ-injected rats show retraction and simplification of apical dendrites of hippocampal CA3 pyramidal neurons, only nine days after injection; STZ-induced DM also causes morphological changes in the pre-synaptic mossy fiber terminals that form excitatory synaptic contacts with the proximal CA3 apical dendrites [108]. It has also been shown that in the STZ-models of type 1 DM there is a downregulation of insulin receptor expression in the brain [13, 19].

1.2.4 Pathophysiology of the Diabetic Brain

The pathophysiology of the diabetic brain is not yet understood; however, a number of potentially causative pathological and pathophysiological changes occur in DLE. Some studies, for example, focused on the effect of pathways activated by hyperglycemia including the: AGE-RAGE pathway (advanced glycation end products binding to its receptor, RAGE) [12, 13, 137, 138], polyol/sorbitol pathway [33], and oxidative stress pathways [139-141]. It has been
proposed that all these pathways may converge to cause detrimental diabetic brain changes and eventually cognitive impairment. Likewise, the loss of insulin and insulin signalling machinery has been shown to play an important role in both DLE and AD [9, 19, 42, 142, 143]. Recent studies, which have begun to explain diabetic brain abnormalities, point to a direct link between insulin and impaired cognitive performance [144, 145].

The changes in insulin level, its downstream signalling pathway and its degrading enzyme appear to promote important pathological changes in the diabetic brain [111]. The potential role of insulin degrading enzyme (IDE) in the diabetic brain is mentioned in a few previous articles [42, 44, 78, 111, 144, 146, 147]. IDE is a protease that can degrade amyloid beta protein; thus, it probably represents an important link between AD and DM. A loss of IDE was reported in the murine diabetic brain as compared to the non-diabetic brain [78]. Future studies are needed to clarify the role of IDE in diabetic leukoencephalopathy.

Deficiency of insulin, its receptor and the downstream signalling machinery has been demonstrated in both human and experimental DM brains [73, 75, 110, 111, 148, 149]. Insulin is not just important for glycemic control; insulin and its receptor are also important for numerous other cellular mechanisms [73, 111]. Unlike in the periphery, the neuronal insulin receptor is not involved in glucose metabolism, but instead impacts upon neuronal health and synaptic activity [37, 73, 150]. Insulin receptors are present in high concentration on central neurons, synapses, and glia, particularly in the cerebral cortex, olfactory bulb, hippocampus, amygdala and septum [110, 111]. In the hippocampus, activation of insulin receptors leads to enhanced learning and memory [75, 151]. In fact, there is a learning-specific increase in the expression of insulin receptors and insulin-signalling pathways in the hippocampus [72, 75].
Intracerebroventricular injection of insulin enhances memory during passive-avoidance tasks [151], and intranasal insulin treatment avoids cognitive changes in STZ-injected mice [9]. In humans, intravenous or intranasal insulin delivery can also facilitate memory which was suggested to be the result of improved consolidation of information [112, 143, 152-154]. Further, our recent lab findings, arising from studying the diabetic mouse model, have revealed a newly emerging role of insulin; our previous experiments have demonstrated that long-term intranasal insulin delivery protects diabetic mice from brain atrophy and impaired cognitive function without impact upon serum glycemia or hemoglobin A1C (important for long term glycemia determination) levels. These data indicates that insulin is an important neurotrophic factor in the management of DM-mediated brain disease [9]. However, the exact mechanism by which insulin protects the diabetic brain is not known and may not involve its typically studied role in glycemic regulation.

1.2.4.1 Insulin Profile in the Brain

Insulin, a hormone produced by the pancreatic B cells, is mainly known for its peripheral effects upon glucose, fat, and protein metabolism. Early studies showed that the ability of certain tissues, such as skeletal muscle, to utilize glucose is mainly dependant on the insulin signal [150], while the brain was considered to have insulin independent glucose uptake [155]. Consequently, the general scientific interest in the central role of insulin subsided. Over time, it has become recognized that the neonatal brain can produce insulin [156], although this is not detected at later ages. Later, the discovery of insulin receptors’ (IRs) wide expression in both rodent and human brains [148, 157-159], combined with the fact that insulin crosses the blood–brain barrier (BBB) via a receptor mediated transcytosis [157], has challenged prior concepts of
the brain being insulin insensitive. Thus, insulin’s effects upon the brain have had a revival of scientific interest.

The presence of insulin and its specific receptors in different brain regions has now been well documented. In order for insulin to exert its actions at IRs deep within the brain, insulin may cross the BBB by a saturable transport system [18, 155, 160]. However, the rate of insulin transport across the BBB can be altered in many physiologic and pathologic situations. For instance, the BBB transport of insulin is enhanced in the neonatal period [92, 161], impaired in obesity [162, 163] and AD [164], distorted by starvation [164, 165] depressed with dexamethasone therapy [166] and terminated during hibernation [167]. Thus, it is likely that the insulin transporter function is regulated by unknown factors associated to the central actions of insulin.

The action of insulin within the brain once it has reached its IRs, which are present in neurons as well as in glial cells [164], is not still completely understood. The localization of IRs in different brain areas [168] suggests that insulin affects a variety of brain functions, including glucoregulation, circuit development and plasticity, food intake and body weight, fertility and reproduction, learning, memory, and attention [150, 164].

1.2.4.2 Insulin Receptor and Its Expression Pattern in the Brain

The insulin signalling pathways are activated by binding of insulin to IR, which belongs to a family of receptor tyrosine kinases (TK) that includes the Insulin-like Growth Factor (IGF) receptor and the Insulin Receptor-Related (IRR) Receptor [142, 169, 170]. The major properties of brain insulin receptors are the same as with peripheral insulin receptors. IR is a tetrameric protein, consisting of two α and two β subunits that function as allosteric enzymes [164]; binding
of insulin to the α subunit results in activation and auto-phosphorylation of the TK portion of the β subunit [21, 164]. There are, however, some differences between the neuronal and peripheral insulin receptors. Both the α and β subunits of the neuronal IRs have a slightly lower molecular weight than peripheral and glial receptors do [164, 171]. Moreover, unlike the peripheral receptors, the number of neuronal IRs does not downregulate in response to high concentrations of insulin [164, 171]. In the nervous system, the IRs are distributed widely but still irregularly, with the highest density in the hypothalamus, olfactory bulbs and hippocampus [70, 149]. The enrichment of IRs within the synaptic densities suggests a possible link between the density of receptors and the activity of neurons and neuronal plasticity [37, 73]. In certain regions of the CNS, the number of IRs depends on the stage of the brain development. For example it is shown that the number of receptors in the thalamus is upregulated during neurogenesis, with downregulation seen in adult animals [172], implying a critical role for insulin in early embryonic development and synaptic formation.

The IR signalling cascade is similar in the CNS and other peripheral target tissues. The phosphorylation of IR is followed by the phosphorylation of the specialized adapter protein, insulin receptor substrate (IRS), on tyrosine residues, which induce the activation of downstream pathways and regulation of a diverse range of biologic responses, including glucose transport, protein synthesis, mitogenesis, and cell survival [164, 173]. The family of IRS proteins consists of six similarly structured proteins (IRS1–6) with different tissue distribution and functions in the conduction of the hormonal signal [164, 174]. IRS1 and IRS2 are broadly distributed in the body, whereas IRS3 is mainly limited to the adipocytes, and IRS4 is expressed primarily in embryonic tissues or cell lines [175]. IRS5 and IRS6 seem to have restricted tissue expression.
and role in signalling [164]. IRS1 is extensively expressed in neurons from several areas of the brain and spinal cord, including the hippocampus, many hypothalamic and thalamic nuclei, the basal ganglia, the cerebellar cortex, and the brainstem nuclei [174, 176]. The sustained elevation of insulin levels is shown to reduce the expression of IRS1 in cultured neurons as well as in the peripheral tissues of mice, which suggests that IRS1 may play a crucial role in the development of neuronal insulin resistance [177].

### 1.2.4.3 PI3K-Akt Pathway

The insulin signalling pathways, activated by binding of insulin to its receptor, regulate a diverse range of biologic responses, including glucose transport, protein synthesis, mitogenesis, and cell survival. The insulin signalling network involves three major pathways: 1) the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, which is responsible for most insulin metabolic action [20]; 2) the Ras–mitogen-activated protein kinase (MAPK) pathway, which in turn regulates cell growth and proliferation through activation of ERK/MEK, Raf, CDC42, and JNK 1-3 [22]; 3) The casitas b-lineage lymphoma (Cbl) / Cbl-associated protein (CAP) pathways which can mediate glucose transport in a PI3K-independent manner [22] [Figure 1]. Although these pathways share several signalling components that affect one another in complex manners, each of these pathways has their own unique characteristics.

PI3K activation results in the generation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which is able to activate other signalling molecules as well as regulators of the cytoskeleton at the cell membrane. One of the best known downstream mediators of PI3K signalling is Akt (also called protein kinase B) [178]. Akt has three mammalian isoforms: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). Akt1 shares 81% and 83% amino acid identity with Akt2 and Akt3,
respectively. Although the three isoforms show broad tissue distribution, Akt1 is the most widely expressed isoform [179, 180]. These three isoforms have similar biochemical characteristics and phosphorylation of two sites is required for full activation of each isoform [179, 181]. Translocation of Akt to the plasma membrane through its pleckstrin homology domain is likely required for its activity [179]. Both the phosphorylation of Akt through the upstream activating kinases and the recruitment of Akt to the plasma membrane are dependent upon the products of PI3K [179, 182].

Insulin signalling through the PI3K-Akt pathway leads to inactivation of glycogen synthase kinase 3 (GSK-3). Akt mediated phosphorylation of isoforms of GSK-3 (serine9 of GSK-3beta; serine21 of GSK-3alpha) inhibits GSK-3 activity [183, 184]. Clear evidence shows GSK3 activation occurring in the degenerative diseases such as AD and DM [20, 185, 186], leading to impaired insulin signalling, abnormal cell function, and premature cell death [20]. Collectively, insulin-mediated GSK-3 inhibition may have beneficial effects in preventing tau protein hyperphosphorylation, reducing Aβ protein accumulation and promoting protein synthesis [186].

Another critical transcription factor in the insulin-PI3K-Akt pathway is CREB (cAMP Response Element-Binding Protein), which is activated by phosphorylation at Ser\textsuperscript{133} by the PI3K-Akt pathway [183, 187-189]. CREB is a transcriptional factor that is important for cellular proliferation, differentiation, adaptive responses and processes involving learning and memory [187-189]. Localized within the nucleus, transcription factors such as CREB are essential for stimulus–transcription coupling: the conduction of events that happen at cell membranes into changes in gene expression [187, 190]. Therefore, by regulating the expression of many types of neuronal proteins, CREB can ultimately affect the function of individual neurons and entire
neuronal circuits. It is important to note that, in addition to PI3K pathway, numerous other intracellular signalling pathways are also interacting with CREB in order to transmit information initiated by membrane receptor-mediated actions to the cell nucleus. Among them, the effects of signalling pathways involving adenylyl cyclase (AC) and cAMP [191], Ca$^{2+}$ [191, 192] and MAPK [193] on CREB and CREB-regulated gene transcription have been well studied. CREB recognizes and binds to the cAMP-response element (CRE), the 8 base pair TGACGTCA stretch of DNA. CRE sites are found within the regulatory region of numerous genes. In this way, CREB is critical in many important functions in the nervous system, including neurogenesis and neuronal survival, neuroprotection, as well as axonal outgrowth and regeneration, circadian rhythms, addiction, synaptic plasticity, and memory formation [183, 187-190, 194, 195]. CREB is believed to be involved in memory processes [189]. The mechanisms that empower CREB to be able to facilitate memory are not completely understood – they could involve processes such as the induction of long-term potentiation or depression of synaptic strength [196, 197], the growth and formation of new synaptic connections [198], or protein synthesis-dependent processes involved in the retrieval and reconsolidation of memory [199]. Efforts are still underway to identify the specific target genes of CREB that mediate its facilitation of learning and memory within the various brain regions and organisms studied.

The role of PI3K pathway activation on synaptic plasticity is also emerging. The ability to store and recall memories is dependent upon synaptic plasticity. Thus, long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission, that is the strengthening or reduction of synaptic efficacy, are believed to be the cellular mechanism of learning and memory [200]. PI3K signalling has been involved in the induction and maintenance of both LTP [201,
and specific forms of LTD [203, 204]; it has been suggested that the PI3K pathway involvement in LTP is dependent on its strength of induction [205, 206]. Therefore, via activation of PI3K, insulin has the uncommon capacity to increase as well as decrease synaptic efficacy.

Our lab’s previous studies show down-regulation of PI3K-Akt throughout the diabetic mice brain [9]. The downstream regulators of the pathway, PI3K, Akt, pAkt, pCREB, and pGSK3β are all diminished in the experimental DM brain, while a near return to normal ranges occurs with Intranasal Insulin delivery [9]. However, our preliminary data indicate that the downstream regulators of the MAPK-ERK pathway, the main alternative insulin-mediated pathway do not develop chronic changes in the DM brain.

*Inhibitors Targeting the PI3K-Akt Pathway:* Wortmannin is a fungal metabolite and a potent inhibitor of PI3K. It has been shown to be capable of inhibiting the formation of PI3K in intact cells. In vitro, 50% inhibitory concentration for inhibition of PI3K by Wortmannin is 2 to 4 nM [207, 208]. It has been shown that Wortmannin is a non-competitive, irreversible inhibitor of PI3K in both a time- and concentration-dependent manner. Wortmannin has previously been reported to be an inhibitor of myosin light chain kinase; but with an inhibitory concentration of 0.2 µM [208]. Wortmannin was found not to be an inhibitor of phosphatidylinositol-4-kinase, protein kinase C, or protein tyrosine kinase; however, it is capable of off-target inhibition of MAPK [207, 208].

Akt/protein kinase B signaling inhibitor-2 (API-2) is a potent and selective inhibitor of the Akt signalling pathway in cells [209]. API-2 suppresses the kinase activity and phosphorylation level of Akt, preventing downstream phosphorylation of GSK3β. It does not inhibit known
upstream activators of Akt. It does not inhibit PKC, PKA, ERK1/2, p38, STAT3, or JNK signalling pathways [209, 210].

1.2.5 Intranasal Delivery to the Central Nervous System:

Intranasal delivery is a noninvasive and convenient method that rapidly targets insulin or other peptides and therapeutics [9, 19, 153, 211-214] to the central nervous system (CNS), bypassing the Blood Brain Barrier and minimizing systemic exposure. Direct intranasal delivery of therapeutics to the brain was first proposed in 1989 by William H. Frey II [215, 216]. Since then, numerous studies have reported that intranasal therapeutics are in fact delivered to the CNS and can potentially treat neurological diseases and disorders.

Intranasal insulin delivery was initially developed as a non-invasive alternative to subcutaneous insulin injections; however, further investigations established that in order to enter the systemic circulation, I-I needs the addition of enzyme inhibitors, mucoadhesives, and absorption enhancers to increase systemic bioavailability which is limited by barriers present in the nasal passages [215, 217]. The high and frequent dosing regimens needed to control the glucose level, in addition to nasal irritation from the required additives, resulted in limited clinical achievement with intranasal insulin for DM management [212, 215, 218]. Several decades later, use of the intranasal method was proposed for direct delivery of insulin to the brain, along nerves connecting the nasal passages to the brain, as a treatment for AD [150, 153, 212, 213, 219]. Intranasal insulin improves memory in patients suffering from AD; it improves attention, memory, and cognitive function within 21 days of commencing intranasal treatment [214, 220, 221]. In these studies, intranasal insulin did not alter blood insulin or glucose levels.
Further, previous experiments in our lab showed that a low dose of I-I alleviated behavioral, morphological, and molecular abnormalities in the experimental DM brain, without modifying systemic glucose level [9, 19]. I-I achieves higher cerebral concentrations, lower mortality due to the risks of systemic hypoglycemia, and less systemic delivery than does subcutaneous delivery. In contrast, subcutaneous insulin delivery using the same dosing and duration of exposure failed to provide the same level of neuroprotection [9].

Our preliminary study also revealed that mouse cortex and hippocampus exposed to intranasal Wortmannin or API-2 show a reduction in protein levels of pAkt, pCREB, and pGSK3β [Figure 1.3], and in levels of pCREB DNA binding upon electrophoretic mobility shift assays [Image and data courtesy of Dr. Cory Toth.]. These data demonstrate penetration of the mouse brain by these small inhibitor molecules with appropriate pharmacological blockade of PI3K-Akt. It is also important to note that the amount of drugs absorbed, the exact uptake mechanism, and the precise localization within the brain for its penetration is not specifically known and may differ between species [217]. While the exact mechanisms underlying intranasal drug delivery to the CNS are not entirely understood, an accumulating body of evidence demonstrates that pathways involving nerves connecting the nasal passages to the brain, especially the Olfactory and Trigeminal nerve pathways, are most important [217, 222]. In addition, pathways involving the vasculature, and cerebrospinal fluid might be associated with transferring the molecules from the nasal cavity to the CNS, although their role is limited for large hydrophilic molecules such as insulin [215, 217].
1.2.5.1 Olfactory Nerve Pathways

The dendrites of olfactory bipolar receptor neurons extend into the mucous layer of the olfactory epithelium within the nose, and the axons extend centrally, passing through the subarachnoid space containing CSF in order to synapse with mitral cells in the olfactory bulbs. From here, neural projections extend diffusely to the olfactory tract, anterior olfactory nucleus, piriform cortex, amygdala, and hypothalamus [223]. The nasal drug delivery to the CNS is thought to involve either an intraneuronal or extraneuronal pathway [215, 224, 225]. Intranasal administration of fluorescent tracers to rats resulted in its transcellular absorption across the olfactory epithelium and transfer to the olfactory bulb, within only several minutes [217]. Intraneuronal transport process within the nerve tract is slow, taking several hours [226, 227], and cannot explain the rapid intranasal delivery of drugs to the brain which can be identified within minutes.

A unique process occurs at the level of the olfactory receptor neurons, in that they regenerate every month due to direct contact with toxins externally – this leads to a “leaky” BBB due to their constant turnover. Meanwhile, Schwann cell-like cells termed olfactory ensheathing cells surround the axons of olfactory receptor neurons, creating continuous, fluid-filled perineurial channels which remain open, permitting travel along their length as well [225, 228, 229]. The extracellular mechanisms may permit transport to occur over only several minutes.

1.2.5.2 Trigeminal Nerve Pathways

Another important pathway connecting the nose to the CNS is the trigeminal nerve, which innervates both the respiratory and olfactory areas of the nasal passages. For the trigeminal nerve, branches from the ophthalmic division (V1) innervate the dorsal nasal mucosa and the anterior nose, while branches of the maxillary division (V2) innervate the lateral walls of the
nasal mucosa. The trigeminal nerve enters the brain from the respiratory epithelium of the nasal passages at two locations: (1) at the pons; and (2) through the cribriform plate near the olfactory bulbs, permitting entry into both caudal and rostral brain with intranasal administration [217, 228].

Small portions of the trigeminal nerve also terminate in the olfactory bulbs. Intranasal drug delivery to the brain along trigeminal pathways was first demonstrated by Thorne et al.; using $^{125}$I-IGF-I, Thorne et al. observed high concentrations of radioactivity in the trigeminal nerve branches, trigeminal ganglion, cervical spinal cord, medulla, and pons [230]. Intranasal studies with other drugs, including interferon-β1b [231, 232], hypocretin-1 [233, 234], and peptoids [235], also identified high levels of radioactivity in the trigeminal nerve.

The intranasal route of administration has traditionally been utilized to deliver drugs to the systemic circulation through absorption into capillary blood vessels within the nasal mucosa, a highly vascular structure. The vasculature in the respiratory region of the nasal mucosa permits the small molecule passage, although delivery from the nasal mucosa to blood is more limited for large hydrophilic molecules such as insulin.
Figure 1.3: Intranasal Wortmannin and API-2 were capable of inhibiting PI3K and Akt in our preliminary experiments.

Montage of Western blots performed in preliminary experiments using non-diabetic brain samples. Samples of proteins extracted from hippocampi of mouse brains receiving intranasal delivery of Wortmannin (1 μg/d) (A), and API-2 (50 μg/d) (B) are demonstrated. Quantification of protein levels are presented underneath each individual montage. Wortmannin intervention downregulated PI3K, Akt, pAkt, and suppressed phosphorylation of GSK3β and CREB (A) delivery of API-2 suppressed Akt and pAkt levels and also suppressed phosphorylation of GSK3β and CREB(B). Image and Data courtesy of Dr. Cory Toth.
Chapter Two: HYPOTHESIS AND OBJECTIVE

Type 1 DM is associated with cognitive dysfunction, cerebral atrophy and white matter abnormalities composing DLE. Insulin deficiency contributes to these deficits and is amenable to replacement interventions using I-I delivery. An important insulin-mediated signalling pathway is mediated through phosphatidylinositol 3-kinase (PI3K) and Akt [9]. Therefore, we designed this project to specifically analyze the role of the insulin-PI3K- Akt signalling pathway in DM neurodegeneration, and to determine if diabetic brain changes can be attributed to dysfunctional PI3K-Akt signalling. The Insulin-PI3K-Akt signalling pathway in the DM brain was modulated using small molecules provided through intranasal delivery, chosen so as to avoid potential hypoglycemia and unwanted systemic effects. We also used newly available transgenic mouse models, Akt over-expressing transgenic mice, to assist in analysis of the PI3K-Akt pathway.

2.1 MAIN RESEARCH HYPOTHESIS:

The cognitive and structural changes seen in DLE are mainly due to impaired insulin-mediated signalling through the PI3K-Akt pathway, and direct support of the PI3K-Akt pathway will prevent cognitive dysfunction, cerebral atrophy and WMA in the diabetic mouse brain.

2.2 SPECIFIC HYPOTHESES:

2.2.1 Specific Hypothesis 1:

Diabetic mice receiving a PI3K inhibitor or Akt inhibitor will lose the beneficial effects of receiving I-I.

To study the effect of pharmacological inhibition of PI3K-Akt in the type 1 diabetic mouse brain receiving insulin, I monitored changes in cognition function along with endpoint MRI studies in type 1 DM mice receiving I-I and intranasal Wortmannin (PI3K inhibitor) or I-I and
intranasal API-2 (Akt inhibitor). Wortmannin or API-2 was expected to nullify the beneficial effects of I-I in DM mice. Type 1 diabetic mice receiving I-I or intranasal saline (I-S) along with intranasal placebo were studied [Figure 2.1].

2.2.2 *Specific Hypothesis 2:* 

*Non-diabetic mice receiving a PI3K inhibitor or Akt inhibitor will demonstrate similar functional and structural deficits as diabetic mice that do not receive intervention.*

To evaluate pharmacological inhibition of PI3K-Akt in the non-diabetic mouse brain for changes indicative of the diabetic brain, I examined those Non-DM CD1 mice (receiving citrate injections) which were also receiving PI3K inhibitor or Akt inhibitor for 7 months. Since it was hypothesized that an impaired PI3K-Akt signalling pathway plays a critical role in the pathogenesis of the DM-brain, blocking the pathway in non-diabetic mice was expected to cause cognitive decline and reduced fractional anisotropy in white matter tracts, reminiscent of the diabetic brain [Figure 2.1].

2.2.3 *Specific Hypothesis 3:* 

*Akt over-expressing transgenic mice will be protected from cognitive dysfunction and WMA associated with DM and there will be no difference between Akt overexpressing diabetic mice receiving intranasal insulin or intranasal saline in terms of their cognitive function and magnetic resonance imaging (MRI) results.*

To assess Akt over-expressing transgenic mice for neuroprotection in DM, I sequentially monitored changes in cognition, along with endpoint MRI in type 1 DM and non-DM transgenic mice, receiving I-I or I-S.
A flowchart of the overall study design is presented in Figure 2.1.

**Figure 2.1: Flowchart of the study design**

Colors have been selected to represent different study cohorts. I-I: Intranasal Insulin, I-S: Intranasal Saline.
Chapter Three: PLAN AND METHODS

3.1 DM and Non-DM Mice

Wildtype CD1 (Charles River, PQ) with an initial weight of 20-30g and Akt over-expression transgenic mice (see below) were injected over three days with STZ to induce DM, or were injected with the citrate vehicle for non-diabetics (Flowchart, Timeline). At 1 month of age, we injected mice destined for diabetes with STZ (Sigma, St. Louis, MO); after fasting for eight hours, the diabetic experimental groups were injected intraperitoneally with STZ dissolved in the citrate buffer solution (pH=4.8) (Sigma-Aldrich Co., St.Louis, Missouri) for each of three consecutive days with once daily doses of 60 mg/kg, 50 mg/kg, and then 40 mg/kg to induce DM. Mice in non-DM groups were treated with a similar volume citrate buffer solution introduced intraperitoneally for creation of placebo-treated non-diabetic cohorts. Considering that generally about 5% of STZ mice fail to develop DM and would therefore not develop DM pathology after STZ injection [9, 19], only the mice, whose hyperglycaemic state were confirmed, 1 week after STZ injection, were enrolled in the study. Tail venous blood was used to measure glucose levels one week after injections. Diabetes was confirmed at levels ≥16mmol/L [9]. Monthly glucose levels and endpoint glycosylated haemoglobin (HbA1C) values (indicator of glucose concentrations over a prolonged period of time) were checked.

To account for missing data for mice that died after three months of study but before the seven month endpoint, the data from any animal that experiences mortality after the 12-week point of the cognitive studies were carried through using the last obtainable data point.

3.2 Akt Overexpressing Transgenic Mice

Transgenic mice expressing constitutively active Akt (HAAkt308D473D, Akt-DD), a kind gift from Dr. Macklin to Dr. Toth, were driven by the proteolipid protein (PLP) promoter [236].
PLP is a gene that is distinctively expressed throughout the oligodendrocyte lineage [236-238]. The PLP gene is located on the X chromosome and is composed of seven exons with a large first intron. The coding sequence of the gene is highly conserved with 100% identity among the mouse, rat, and human sequences at the amino acid level and >95% identity at the nucleotide level [237]. The PLP gene promoter is very cell-specific. It is most highly expressed in maturing oligodendrocytes in the developing postnatal brain, but it is also expressed in oligodendrocytes progenitors [236, 238, 239], in Schwann cells [237, 239] and in cells in other tissues like heart [240].

Using a promoter construct that contains all the information required for spatiotemporal expression similar to PLP, Akt1 overexpressing transgenic mice (Plp-Akt-DD) were generated. An Akt cDNA was inserted into the Ascl/PacI sites of the modified PLP promoter cassette; it was then injected to generate transgenics in SJL/SWR F1 mice. According to previous studies, Akt is overexpressed from an early age (e.g. postnatal day 14 (P14), and P21) in these mice with escalating amounts as the animals mature, and Akt expression is significantly enriched in oligodendrocytes [236].

3.3 Interventions

A sample size for intervention groups was calculated based upon differences in brain atrophy observed in DM and non-DM mice to date. An $\alpha$ of 0.05 and $\beta$ of 0.5 using observed mean and standard deviation provides a minimal sample size of n=6 within each intervention group. According to our preliminary data and previous studies [9], the mortality rate in STZ-induced DM mice approaches 50% after seven months of DM; thus, we decided to study 9 to 10 mice in each diabetic group initially and 6 to 10 mice in each non-diabetic one. A second cohort was
used to obtain additional mouse data for mouse cohorts with higher levels of mortality in some cases.

Mice received I-I (0.87U/d) or intranasal saline (I-S) in combination with one of intranasal Wortmannin (PI3K inhibitor, 1 μg/day), intranasal API-2 (Akt inhibitor, 50 μg/day), or intranasal placebo (DMSO 0.1 %) (See Flowchart 1). Intranasal delivery of insulin, saline, Wortmannin, API-2 or Placebo occurred daily with 6 drops of 5μl each at 6 consecutive minute intervals with mouse held prone and neck extended. Un-anesthetized mice are hoisted by the scruff of their necks and held gently, but firmly in the palm of the hand so that the mouse cannot move. Each mouse was given 6 nose drops of the selected drugs spaced one minutes apart, with the therapy placed on alternating nostrils for the animal, using a 20-μL pipettor.

Intervention doses were selected based upon success in our preliminary studies. No other management of diabetes occurred throughout the mouse lifetime in our experiments. Complementary sensorimotor testing was used to rule out the presence of any significant motor deficit in the mice performing cognitive testing; mice were disqualified from further cognitive studies should any significant motor deficit be detected. The rotarod test was used to assess motor coordination using the latency of the mouse to fall from the rotating rod at accelerating speed as an indicator. The test protocol involved an accelerating protocol from 4 to 40 rpm over a 5 min period that was ended automatically when the mouse fell off the rotor or when the protocol was completed. Latency to fall was recorded for each mouse and the ability of the animal to run and rotate without falling was considered. Data less than two standard deviations away from the mean were considered as severe motor deficit. The rotarod test was done twice monthly.
3.4 Behavioural Testing

All mice were tested weekly for a total of 7 months, using a battery of behavioural cognitive tests that assessed the consequences of long-term DM. The set of cognitive tests for assessment of visuospatial and procedural memory in mice included: Morris water maze, radial arm, holeboard, and object recognition. The tests were performed to examine general overall cognition abilities. Such a battery of tests is often required for added validity. The tests chosen have been used previously to investigate learning and memory in mice [9, 241-245]; each test use different skills to account for the potential confounding factors of diabetic complications, for example visual acuity and motor-sensory skills. While the holeboard and radial arm tests evaluate spatial information processing and memory, the Morris water maze evaluates procedural memory and aversive motivation [242, 244, 245]. In contrast, the object recognition test evaluates novelty seeking and exploratory behaviour [9, 246].

We performed weekly testing, after two weeks of training and confirmation of the diabetic state, until 8 months of age. Every mouse was trained in each test paradigm for two weeks before initiation of test recording, and prior to DM initiation. Behavioural testing equipment remained in a fixed place and position. Tests performed in a single designated environment with identical visual cues presented on each occasion. For all the tests, except the Morris water maze, mice fasted for twelve hours prior to testing in order to induce food search motivation.

The holeboard test is composed of a rectangular open field (60 X 90 cm) made of cardboard walls of 60 cm height and an opaque black floor, with eight holes (2.5 cm diameter) which are placed in two lines of four, equidistant from each other and from the walls. In this test, the mouse started in the same corner of the box each time, while the food reward (Cheerio) was always
placed in the same hole [Figure 3.1]. The mouse was expected to go to the hole containing the food reward. The mouse was timed to see how long it took to find the hole containing the food reward (latency). The maximum threshold permitted for the test was 12 minutes [9, 242].

The radial arm test is based on the natural foraging tendencies of mice and is used to evaluate spatial information processing and long-term memory. The test consists of a central platform with 8 radiating arms (each 76 cm long and 12 cm wide) with adjacent arms separated by 45°, which serve as runways. Completion time was recorded in the journey to find a food reward (Cheerio), which was located in a single runway 180° from the initial constant starting point. The mouse was to find the arm containing the food reward and was timed to see how long it took them to find the correct arm (latency) [Figure 3.1]. The maximum threshold permitted for the test is 12 minutes. Similar to the holeboard test, this test involves the hippocampal complex for initial spatial information processing and learning, the dorsal striatum for procedure comprehension, and the neocortex for long-term memory storage [9, 243]. The main differences between the holeboard and radial arm tests are their setup and the underlying inherent behavior considered in each test. The holeboard test is based on exploratory behavior while the radial arm test is based on prolonged foraging behavior [247].

The Morris water maze is often used to assess hippocampal-based learning and memory in diabetic mice. A mouse-adapted Morris water maze task was used to evaluate spatial information processing, procedural memory, and aversive motivation. A coloured circular pool (88 cm in diameter and 20 cm in height) was filled with water at 28°C. The clear 10 cm radius platform was hidden 1cm below the surface of the water. The mouse was always placed in the pool in the same position, opposite from the hidden platform. In this test, the mouse was required to swim to
the hidden platform, which is located in a fixed-position for all testing over the entire study period [Figure 3.1]. The latency was defined as the time spent by the mouse to find the platform. Brain regions involved include the hippocampal complex for initial spatial information processing and learning, the dorsal striatum for procedure comprehension, the amygdala for escape behaviour, and the neocortex for long-term memory storage. The maximum threshold permitted for the test is 3 minutes [9, 244].

The **object recognition** test is based on novel object preference and is used to evaluate novelty seeking and exploratory behaviour in rodents. The main brain regions involved are the perirhinal cortex, located in the parahippocampus, which is important for visual perception and visual memory, and the hippocampal complex key for short-term memory processing. The task was performed in an open wooden box (60 x 60 x 60 cm) containing unique objects built from Lego blocks. The test consists of two trials. In the first sample trial (T1) the mouse was placed in a box containing two identical objects, for three minutes. The times spent at each object and the number of visits to each object was recorded. A visit was considered to occur when a mouse directs its nose to the object at a distance or when it touched the object. Then, the mouse was returned to its home cage and, after thirty minutes, the mouse would be put back into the box for the second 3-min choice trial (T2), where one of the objects presented in T1 was replaced by a new, completely different object. Again, the times spent at each object and the number of times each object were visited, were recorded. Mice were required to spend more time at the novel object. Objects and the box environment were cleaned between trials and between mice, to prevent the possibility of scent traces forming an olfactory cue [246].
Testers were blinded to the nature of experimental groups of the mice and their treatments in order to prevent bias. Testing was performed blind with respect to the groups, which were categorized alphabetically, and to the treatments, using pre-enumerated solutions. Treatments were prepared initially, before running the experiments, aliquoted to the similar tubes, and blinded immediately by Dr. Cory Toth.

3.5 Magnetic Resonance Imaging (MRI)

The endpoint MRI analysis was performed on four mice from each cohort. MRI scanning carried out at the Experimental Imaging Centre at the University of Calgary. The mice were anaesthetized by mask with isoflurane before MR scan and their respiration rate and temperature were monitored during the procedure. MRI was done using quadrature volume coil and a Bruker 9.4 Tesla 21 cm bore MR imaging system. Three different sets of MR scans were performed, using sequences to obtain T2, DTI and magnetization transfer (MT) imaging for each mouse. T2-weighted images were obtained from multi-spin-echo images with a repetition time (TR) of 1200 ms and an echo time (TE) of 12.5 ms. The field of view was 2 x 2 cm², with an acquisition matrix of 256 x 256 and a slice thickness of 0.75 mm. In addition, T2-weighted MR images were used to calculate overall brain volume. Volumetric brain measurements were calculated as a summation of cross-sectional areas for each slice multiplied by the thickness of the MR slices.

For DTI, a repetition time of 6.5 s, an echo time of 35 ms, and 6 signal averages were used to acquire a series of diffusion-weighted images at $b = 1000-1200 \text{ s/mm}^2$ in 30 different directions including the acquisition of five Ao images ($b=0$). DTI images were acquired for 0.5 mm thick slices, using a 2x2 cm² field of view and a 128x128 matrix. Color map images are generated by combining the images of primary eigenvector (indicating direction of diffusion) and FA into red
(caudal-to-rostral)-green (medial-to-lateral)-blue (dorsal-to-ventral) images. Thus, colors are coded according to the direction of diffusion.

Magnetization Transfer (MT) images are used to determine microstructural abnormalities, including white matter abnormalities, which are difficult to identify using more conventional MRI [21, 22]. MT were acquired within five 0.75 mm thick slices in the mid-cerebrum using proton density-weighted spin echo images (TR = 5000 ms, TE = 15 ms) with magnetization transfer saturation off and on (40 G pulses with a power of 4 μT and a frequency offset of 1500 Hz) [20]. For analysis of the images, MT ratio (MTR) was calculated as $\text{MTR} = \left(\frac{M_o - M_s}{M_o}\right) \times 100\%$, where $M_s$ and $M_o$ were the signal intensities obtained with and without MT saturation, respectively.

T2 values and MTR were analysed using locally available Marevisi software (Marevisi, IBD), by an observer blinded to the treatment group. Likewise, analysis of DTI for FA and tractography occurred with previously used shareware software (MedINRIA, INRIA-Asclepios Research Team, v1.8.0). Measurements were made within brain regions of interest (ROI) bilaterally for each of the control and DM animals. Manual ROI selection was used to mask out skull and outer-brain areas and to determine areas such as whole brain, hippocampal regions (CA1-CA3), primary somatosensory cortex (S1), primary motor cortex (M1), primary visual cortex (V1), caudate/putamen (CPu), corpus collosum (CC), internal capsule (IC), Anterior commissure (AC), posterior commissure (PC), pons, and cerebellum. Representing regions are known to be abnormal within diabetic brains, as well as cortical and subcortical regions important in memory and cognition [9, 11, 93, 248, 249]. MRI studies on the mentioned ROI were performed previously in our lab and revealed development of white matter changes in some
of these regions over time. ROI templates were created according to the mouse brain atlas of Paxinos and Franklin [250], then drawn manually for each image to obtain an optimal reference-object fit. Figure 3.2 shows two representative slices with exemplified ROI. Results between groups were compared using ANOVA testing.

3.6 Sacrifice of Animals

Endpoint sacrifice of mice occurred immediately after final MRI imaging, followed by cardiocentesis, with blood obtained sent for HbA1C levels (Calgary Laboratory Services). Brains were harvested, and then weighed. The left hemisphere of the brain was surgically dissected into regions of interest for preservation for future molecular studies, while the right hemisphere remained intact for future structural and immunohistochemical studies, as performed previously [9]. Additional tissues were obtained for other associated studies of DM neuropathy, maximizing animal potential.

3.7 Analysis

I analyzed cognitive data from each mouse groups with single factor ANOVA (ANOVA) testing between cohorts for each weekly time point. Multivariate analysis of variance (MANOVA) was also performed for comparison. For ANOVA to be valid, the following statistical assumptions have to be met: normality, homogeneity, fixed effects, data independence, and adequate sample size. For all the cognitive test measures, repeated measures ANOVAs were also performed. All cognitive assessments were analyzed independently. For experimental group characteristics, monthly glucose levels were analyzed with a one-way ANOVA; HbA1C similarly was analyzed after 8 months. MRI results between the groups were compared using
ANOVA testing. For all cognitive tests, additional area under the curve (AUC) measurements was performed to show the significant differences between the groups.

Relevant comparisons were performed between the groups (see results). The dependent variables for the ANOVAs were the measures from each test. The independent variables were the disease state, the treatments, and time.

3.8 Timeline

The procedure timeline for the study occurred over a course of seven months for each mouse cohort. The experimental groups were formed at approximately 6 weeks of age. Beginning one week after DM induction in STZ mice and subsequent fasting glucose confirmation, we began weekly cognitive testing and monthly glycemia testing until 7 months of DM occurred. Non-DM animals were studied at equivalent time points.
Figure 3.1: Cognitive behavioural test diagrams.

The mouse was timed to see how long it took to find the platform in Morris water maze (blue) or food reward in holeboard (green), and radial arm test (purple).
Figure 3.2: Representative MRI slices with exemplified ROI.

Representations of brain ROI measured bilaterally in coronal T2 slices at Bregma 0.980 mm (A) and -2.80 mm(B). Some additional anatomical landmarks are indicated on the scan (V3: 3rd ventricle, LV: lateral ventricle).
Chapter Four: RESULTS

4.1 Experimental Group Characteristics

Three experimental cohorts were examined, according to the pre-planned timeline; the first and second experimental cohort were to study the effect of PI3K and Akt pharmacological inhibition, respectively, and the third experimental cohort involved Akt overexpressing transgenic mice. Mice were divided into the fifteen experimental groups at one month of age and were examined until the seven month endpoint (n = 6-15). By the seven month endpoint, 5.08% of the non-diabetic mice and 30.61% of the diabetic mice did not survive, similar to past studies. Due to higher than expected mortality rates in some of the experimental groups, we were required to assess a second cohort in order to maintain an N value of more than 6 in each individual cohort. This led to a data set with a minimum of six mice in each intervention group. Only male wildtype CD1 animals were enrolled in the study and the Akt over-expressing transgenic mouse groups contained approximately even ratios of males to females. Mice that died before four months of age (three months of diabetes) were excluded, because the study was investigating the long-term effects of diabetes. Mice that died after that time point were included and for the missing behavioural data the last data point was carried forward for imputation. [See Figure 4.1 for table of initial and final experimental group numbers]. Data less than two standard deviations away from the mean of the group were not considered. Accordingly, the last observation was carried forward for 5.7% of the total 157 mice. As it was mentioned previously, the mice were receiving daily intranasal treatments of PI3K inhibitor (Wortmannin, 1 μg/day)/Akt inhibitor (API-2, 50 μg/day)/ Placebo and Intranasal Insulin (I-I, 0.87U/day) / Intranasal Saline (I-S) over seven months of study.
4.1.1 Glucose Levels

Of the mice injected with STZ, those failing to become diabetic were not enrolled in the study. We examined monthly glucose levels of diabetic groups, and those mice that despite the initial hyperglycaemia did not show the diabetic state, as defined above, were excluded from the study.

The endpoint glucose levels and HbA1C values were measured at the seven month endpoint and the diabetic groups had significantly higher glucose and HbA1C levels compared to the non-diabetic mice (ANOVA with Bonferroni correction; p<0.008 for wildtype mice, and P<0.01 for Akt overexpressing transgenic mice). The only effect upon HbA1C value was the occurrence of the diabetes disease state, and treatments did not affect HbA1C level [Figure 4.2 A and B].

4.1.2 Body weight

We started our study with a total of 113 CD1 wild-type mice with initial weight of 29.70 ± 4.88 g (mean ± SEM), and 44 Akt over-expressing transgenic mice with initial weight of 18.47 ± 1.24 g (mean ± SEM). Diabetic mice were smaller than non-diabetic mice throughout life and had lower body weight at the end of study (seven months of age) [Figure 4.3]. Unlike our laboratory's previous study which showed diabetic mice receiving intranasal insulin were maintaining weights better than control diabetic mice [9], in the present study different treatments did not affect diabetic and non-diabetic mouse weight.
Figure 4.1: Table of final experimental group numbers.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Initial number</th>
<th>Final number</th>
<th>Mortality Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic: I-I + Placebo</td>
<td>9</td>
<td>6</td>
<td>33.33%</td>
</tr>
<tr>
<td>Diabetic: I-I + Wortmannin</td>
<td>12</td>
<td>7</td>
<td>41.66%</td>
</tr>
<tr>
<td>Diabetic: I-S + Placebo</td>
<td>12</td>
<td>6</td>
<td>50%</td>
</tr>
<tr>
<td>Diabetic: I-S + Wortmannin</td>
<td>12</td>
<td>7</td>
<td>41.66%</td>
</tr>
<tr>
<td>Non-Diabetic: I-S + Wortmannin</td>
<td>8</td>
<td>7</td>
<td>12.5%</td>
</tr>
<tr>
<td>Non-Diabetic: I-S + Placebo</td>
<td>15</td>
<td>15</td>
<td>0%</td>
</tr>
<tr>
<td>Diabetic: I-I + API-2</td>
<td>10</td>
<td>8</td>
<td>20%</td>
</tr>
<tr>
<td>Diabetic: I-S + API-2</td>
<td>9</td>
<td>6</td>
<td>33.33%</td>
</tr>
<tr>
<td>Diabetic: I-S + Placebo</td>
<td>10</td>
<td>8</td>
<td>20%</td>
</tr>
<tr>
<td>Non-Diabetic: I-S + API-2</td>
<td>8</td>
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<td>0%</td>
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<tr>
<td>Non-Diabetic: I-S + Placebo</td>
<td>8</td>
<td>8</td>
<td>0%</td>
</tr>
<tr>
<td>Diabetic, Akt over-expressing transgenic mice: I-I</td>
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<td>11</td>
<td>8.33%</td>
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<tr>
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</tr>
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<td>Non-Diabetic, Akt over-expressing transgenic mice: I-I</td>
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<td>9</td>
<td>10%</td>
</tr>
<tr>
<td>Non-Diabetic, Akt over-expressing transgenic mice: I-S</td>
<td>10</td>
<td>9</td>
<td>10%</td>
</tr>
</tbody>
</table>
Figure 4.2: Endpoint HbA1C Level

The diabetic wild type [A] and Akt overexpressing (AKTO) transgenic mice [B] have significantly higher HbA1C levels compared to the non-diabetic groups, regardless of drug treatment. (ANOVA with Bonferroni correction; main effect of disease state, p<0.008 [A] and p<0.01 [B]). Error bars represent mean ± SEM.
Figure 4.3: Endpoint Body Weight

CD1 wildtype [A] and Akt overexpressing (AKTO) transgenic [B] diabetic and non-diabetic mice’s final weight. * Indicates significance with comparison of non-diabetic control mice to diabetic control mice using ANOVA testing with Bonferroni post hoc Turkey’s comparisons (p<0.008 [A] and p<0.01 [B]).
4.2 Results of PI3K Pharmacological Modulation

To examine the effect of pharmacological inhibition of PI3K in the type 1 diabetic mouse brain receiving insulin, relevant comparisons were performed between the groups. The comparisons were done according to the pre-planned scheme [Figure 4.2.A]. The groups which differ only in only one factor were compared, accordingly.

Cognitive testing began from the first week of diabetes and was completed after 26 weeks of diabetes. There were no significant differences between the groups at baseline (ANOVA with Bonferroni correction; p<0.008). For each mouse cohort, cognitive testing demonstrated initial learning ability, regardless of diabetes presence. Learning processes for each of the tasks appeared to be similar between diabetic and non-diabetic mice over the first weeks. All cognitive data was based upon a minimum of six mice in each cohort group at all time-points. Data from the mice with severe motor dysfunction or illness were excluded from the time-point.
Figure 4.2.A: Comparison Chart of First Experimental Cohort

Pre-planned comparisons between the groups were performed as follows. The groups, which are highlighted, were compared for differences in the cognitive and MRI results. Six pairs of comparison were made. The cells in grey color are the mirror of the same pairs in table. The numbers show the order of results being shown.

<table>
<thead>
<tr>
<th>Diabetic Mice</th>
<th>Non-Diabetic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wortmannin + I-S</td>
</tr>
<tr>
<td>Diabetic Mice</td>
<td></td>
</tr>
<tr>
<td>Wortmannin + I-S</td>
<td>Y</td>
</tr>
<tr>
<td>Wortmannin + I-I</td>
<td></td>
</tr>
<tr>
<td>Placebo + I-S</td>
<td></td>
</tr>
<tr>
<td>Placebo + I-I</td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic Mice</td>
<td></td>
</tr>
<tr>
<td>Wortmannin + I-S</td>
<td></td>
</tr>
<tr>
<td>Placebo + I-S</td>
<td></td>
</tr>
</tbody>
</table>

4.2.I Cognitive Behavioural Data and MRI Results for Diabetic Control Mice Receiving I-S and Placebo and Their Non-Diabetic Control Littermates receiving I-S and Placebo.

Cognitive decline for wildtype diabetic mice was demonstrated after 2 months of diabetes, with longer escape latencies in water maze test [4.2.I.A] compared to control non-diabetic mice.

Similarly, radial arm [4.2.I.B] and holeboard testing [4.2.I.C] demonstrated significant loss of cognitive performances of the wildtype diabetic mice, as evident by the longer latencies to find the food rewards, compared to control non-diabetic mice. The object recognition task [4.2.I.D] demonstrated less novelty seeking behaviour in terms of time spent at a novel object in the second portion of the experiment for wildtype control diabetic mice compared to control non-
diabetic mice. Results for cognitive behavioural tasks are also presented as the average of each of 5 consecutive weeks [4.2.1 A1-D1].

For A-D, AUC measurements were performed and demonstrated significantly impaired performances for wildtype diabetic mice compared to wildtype non diabetic mice. For all cognitive tests ANOVA tests were performed for significance purposes with an asterisk [*] indicating significant P<0.0083 after Bonferroni correction. Data from the mice with severe motor dysfunction or illness were not included in the final reported results. The rotarod test was done biweekly to exclude mice with severe motor dysfunction from the study. There were no significant differences between diabetic control mice and non-diabetic control mice in rotarod test performance, until the final weeks of our study [4.2.1. E].

Brain atrophy and development of white matter changes were demonstrated in the diabetic brain over the course of diabetes. Higher T2 values in diabetic brain were identified indicating diffuse areas of hyper-intensities throughout whole brain slice and in different brain regions [4.2.I.F]. Diabetic mice demonstrated elevated T2 map values across grey and white matter regions of the brain. Lower MTR in diabetic brain suggests lower tissue integrity in diabetic mouse brain [4.2.I. G]. Volumetric measurements of the entire brain demonstrated significant loss of brain volume in diabetic control groups (receiving I-S and Placebo) at the endpoint [4.2.I.H]. Lower FA values [4.2.I.I] and fiber numbers [4.2.I.J] in Corpus Callosum (CC) and Internal Capsule(IC) were seen in diabetic mouse brain, suggesting poor myelination or axonal loss in these areas of the brain due to diabetes. The data clearly demonstrated poor cognitive performance in the diabetic animals, contributing with the existence of WMA showed in the diabetic brain.
Non-diabetic wildtype mice treated with placebo (nonDiab:I-S+Placebo) performed better than diabetic wildtype mice treated with Placebo (Diab:I-S+Placebo) (p<0.0083). The graphs demonstrate performance of nonDiab:I-S+Placebo, and Diab:I-S+Placebo, in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests. For A1-D1 twenty six weeks were categorized into five-week periods of time. The fifth period is considered weeks 21 through 26 (21-26). AUC measurements were performed and demonstrated significantly better performances for nonDiab:I-S+Placebo in comparison to Diab:I-S+Placebo in all four cognitive behavioural tests [A-D, p<0.0083]. Volumetric measurements of the entire brain demonstrated brain atrophy in Diab: I-S+Placebo in comparison to control non-diabetic group [F]. In different brain regions, T2 map values were significantly elevated [G] and MTR values were significantly depressed [H] for Diab:I-S+Placebo in comparison to nonDiab:I-S+Placebo. FA values [I] and fiber numbers [J] in Diab and nonDiab mouse brains of first experimental cohort is shown. Error bars represent mean ± SEM.
Figure 4.2.1

Watermaze Latency

- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

* p<0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time

Radial Arm Latency

- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

* p<0.0083

Time (s)

Weeks
Figure 4.2.I

Radial-Arm Latency
- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

* p<0.0083

HoleBoard Latency
- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

Time (s)

Weeks

* p<0.0083
**Figure 4.2.I**

**HoleBoard Latency**
- nonDiab: I-S + Placebo  n= 15
- Diab: I-S + Placebo  n= 6-12

* p<0.0083

**Object Recognition Time (Novel-Known)**
- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

* p<0.0083
Figure 4.2.I

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

* p< 0.0083

Rotarod Latency

- Diab: I-S + Placebo  n= 6-12
- nonDiab: I-S + Placebo  n= 15

* p< 0.0083
Figure 4.2.I

MR T2 Values

- Diab: L-S + Placebo  n= 4
- nonDiab: L-S + Placebo  n= 4

* P<0.0083

T2 Value (ms)

- Whole Brain
- S1
- Caudate/Putamen
- M1
- Anterior Commissure
- Corpus Callosum
- Internal Capsule
- CA1
- CA2
- CA3
- Posterior Commissure
- Pons
- Cerebellum
Figure 4.2.I

**MR MT Values**
- Diab: I-S + Placebo \( n = 4 \)
- nonDiab: I-S + Placebo \( n = 4 \)

\[ * p < 0.0083 \]

<table>
<thead>
<tr>
<th>Region</th>
<th>Diab</th>
<th>nonDiab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td></td>
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</tr>
<tr>
<td>CA1</td>
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<tr>
<td>CA2</td>
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<tr>
<td>CA3</td>
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<td>Cortex</td>
<td></td>
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<tr>
<td>Posterior Commissure</td>
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<tr>
<td>Caudate/Futamen</td>
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<tr>
<td>Anterior Commissure</td>
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**MR Volumes**

<table>
<thead>
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<th>Region</th>
<th>Diab</th>
<th>nonDiab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ * p < 0.0083 \]
Figure 4.2.1

**Fractional Anisotropy**

- **FA Value**
  - CC: 0.5 ± 0.05
  - Whole Brain: 0.45 ± 0.05
  - CA1: 0.48 ± 0.05
  - CA2: 0.42 ± 0.05
  - IC: 0.6 ± 0.05

- **Significance**:
  - *P < 0.0083

**Total Tract Fibers**

- **Corpus Callosum**
  - Diab: I-I + Placebo: 800 ± 50
  - Diab: I-I + Wortmannin: 900 ± 50
  - Diab: I-S + Placebo: 750 ± 50
  - Diab: I-S + Wortmannin: 1000 ± 50
  - nonDiab: I-S + Wortmannin: 1200 ± 50
  - nonDiab: I-S + Placebo: 1100 ± 50

- **Internal Capsule**
  - Diab: I-I + Placebo: 200 ± 50
  - Diab: I-I + Wortmannin: 300 ± 50
  - Diab: I-S + Placebo: 250 ± 50
  - Diab: I-S + Wortmannin: 350 ± 50
  - nonDiab: I-S + Wortmannin: 300 ± 50
  - nonDiab: I-S + Placebo: 250 ± 50

- **Significance**:
  - *P < 0.0083

**Legend**
- Diab: I-I + Placebo: n=6
- Diab: I-I + Wortmannin: n=4
- Diab: I-S + Placebo: n=4
- Diab: I-S + Wortmannin: n=4
- nonDiab: I-S + Wortmannin: n=4
- nonDiab: I-S + Placebo: n=4
4.2.II  Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and their diabetic control littermates receiving I-S and Placebo.

To show the effects of I-I on the cognitive function and WMA, which was detected in the diabetic animals, the cognitive performance and MRI imaging results of the diabetic animals treated with I-I was compared with their control diabetic littermates receiving I-S and Placebo. Diabetic Mice receiving I-I outperformed wildtype control diabetic mice in all four cognitive tests. While diabetic mice receiving I-S and Placebo showed persistent cognitive decline beginning at the initial stages of diabetes, diabetic mice receiving I-I and Placebo were requiring less time to find the hidden platform and food rewards in water maze [4.2.II.A], and radial arm [4.2.II.B]/ holeboard [4.2.II.C] tests, respectively. In the object recognition task [4.2.II.D], diabetic mice receiving I-I and Placebo passed more time with the novel object in comparison to diabetic mice receiving I-S and Placebo. For A-D, AUC measurements demonstrated significantly better performances for wildtype diabetic mice receiving I-I compared to wildtype diabetic mice receiving I-S. Results for cognitive behavioural tasks are also presented as the average of each of 5 consecutive weeks. I-I treatment could improve the motor function of the diabetic mice at final stages of the study, as demonstrated with the rotarod test [4.2.II.E].

While control diabetic mice demonstrated brain atrophy and development of white matter changes over the course of diabetes, diabetic mice receiving I-I were protected from these changes. Volumetric measurements of brain demonstrated whole brain atrophy in diabetic mice, with protection demonstrated in diabetic mice treated with I-I [4.2.II. F].

Lower MR T2 values [4.2.II. G] and higher MTR [4.2.II. H] in diabetic mice treated with I-I in comparison to control diabetic mice could be seen in different areas of the brain. In addition, higher FA values [4.2.I. I] and increased fiber tract numbers [4.2.I. J] of Corpus Callosum (CC)
were seen in diabetic mouse brain treated with I-I, suggesting a protective role for I-I in the white matter changes occurring in the diabetic mouse brain, along with its beneficiary effects on mouse cognitive function.

**Figure 4.2.II: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Their Diabetic Control Littermates Receiving I-S and Placebo.**

Diabetic wildtype mice treated with I-I and Placebo (Diab:I-I+Placebo) performed better than Diab mice treated with I-S and Placebo (Diab:I-S+Placebo) (p<0.0083). The graphs demonstrate performance (mean±SEM) of Diab:I-I+Placebo, and Diab:I-S+Placebo, in watermaze [A], radial arm [B], holeboard [C], object recognition [D], and rotarod [E] tests. For A1-D1 twenty six weeks were categorized into five-week periods of time. The fifth period is considered weeks 21 through 26 (21-26). AUC measurements were performed and demonstrated significantly better performances for Diab:I-I+Placebo in comparison to Diab:I-S+Placebo in all four cognitive behavioural tests [A-D, p<0.0083]. Volumetric measurements of the entire brain are shown [F]. In different brain regions, T2 map values were significantly decreased [G] and MTR values were significantly increased [H] for Diab:I-I+Placebo.
Figure 4.2.II

Watermaze Latency
- Diab: I-S + Placebo  n= 6-12
- Diab: I-I + Placebo  n= 6-9

* p< 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Radial Arm Latency
- Diab: I-S + Placebo  n= 6-12
- Diab: I-I + Placebo  n= 6-9

* p< 0.0083

Time (s)

Weeks
Figure 4.2.II

Radial-Arm Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-I + Placebo  n= 6-9

* p<0.0083

HoleBoard Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-I + Placebo  n= 6-9

* p<0.0083
Figure 4.2.II

Hole Board Latency

- Diab: l-S + Placebo  n = 6-12
- Diab: l-l + Placebo  n = 6-9

* P < 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Object Recognition Time (Novel-Known)

- Diab: l-S + Placebo  n = 6-12
- Diab: l-l + Placebo  n = 6-9

* p < 0.0083

Time (s)

Weeks
Figure 4.2.II

Object Recognition Time (Novel-Known)

* p<0.0083

- Diab: L-S + Placebo n=6-12
- Diab: L-L + Placebo n=6-9

Rotarod Latency

* p<0.0083

- Diab: L-S + Placebo n=6-12
- Diab: L-L + Placebo n=6-9
4.2.III Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Diabetic Mice Receiving I-I and Wortmannin.

Over seven months, a group of diabetic mice (n=7-12) were provided with daily intranasal delivery of the PI3K inhibitor Wortmannin and I-I, to study the result of PI3K inhibition on I-I protective effects on cognitive function and WMA. Diabetic mice receiving I-I and placebo performed significantly better than mice receiving intranasal Wortmannin concurrently with I-I, in all four cognitive behavioural tests [4.2.III. A-D].

Although volumetric measurements of the entire brain showed no significant difference between diabetic mice treated with I-I and placebo and diabetic mice treated with I-I and Wortmannin [4.2.III. H], T2 map values in different brain regions were significantly higher [4.2.3.F] and MTR were significantly lower [4.2.III.G] for diabetic mice treated with I-I and
Wortmannin concurrently in comparison to diabetic mice treated with I-I and placebo. While I-I treated diabetic mice were partially protected from the elevation of T2 values, Wortmannin treated diabetic mice continued to demonstrate elevations in T2 values across grey and white matter regions of the brain, despite concurrent I-I treatment. T2 values were elevated within CC, IC, CA1 and CA2 regions of the hippocampus and PC. Fractional anisotropy [4.2.I.I] of CC and IC, and fiber tract numbers [4.2.I.J] of CC were significantly decreased in diabetic brains treated with I-I along with Wortmannin in comparison to mice treated with I-I and placebo.

**Figure 4.2.III:** Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Diabetic Mice Receiving I-I and Wortmannin.

Diabetic mice treated with I-I performed better than diabetic mice treated with I-I and Wortmannin. (p<0.0083) The graphs demonstrate performance (mean±SE) of diabetic wildtype mice treated with I-I and Placebo (Diab:I-I+Placebo), and diabetic wildtype mice treated with I-I and Wortmannin (Diab:I-I+Wortmannin), in watermaze [A], radial arm [B], holeboard [C], object recognition [D], and rotarod [E] tests. Categorization into five-week periods [A1-D1] and AUC measures [A-D] also was different between the groups statistically. AUC measurement was not significantly different between the groups for the rotarod test [E]. In different brain regions, T2 map values were significantly increased [F] and MTR values were significantly decreased [G] for Diab:I-I+Wortmannin compared to Diab:I-I+Placebo. The difference between the overall brain volumes of the two groups was not statistically significant [H].
Figure 4.2.III

Radial Arm Latency

- Diab: I-I + Placebo  n= 6-9
- Diab: I-I + Wortmannin n= 7-12

* p < 0.0083

Time (s)

Weeks

Radial-Arm Latency

- Diab: I-I + Placebo  n= 6-9
- Diab: I-I + Wortmannin n= 7-12

* p < 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.2.III

HoleBoard Latency

- Diab: l-l + Placebo  n= 6-9
- Diab: l-l + Wortmannin  n= 7-12

* p< 0.0083

Time (s)

Weeks

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.2.III

Object Recognition Time (Novel-Known)

- Diab: I-I + Placebo $n=6-9$
- Diab: I-I + Wortmannin $n=7-12$

* $p<0.0083$

Weeks

Object Recognition Time (Novel-Known)

- Diab: I-I + Placebo $n=6-9$
- Diab: I-I + Wortmannin $n=7-12$

* $p<0.0083$

Weeks

- Week 1 to 5
- Week 6 to 10
- Week 11 to 15
- Week 16 to 20
- Week 21 to 26
Figure 4.2.III

Rotarod Latency

- Diab: I-I + Placebo  \( n = 6-9 \)
- Diab: I-I + Wortmannin  \( n = 7-12 \)

![Rotarod Latency Graph]

* \( p < 0.0083 \)

Figure 4.2.III

MR T2 Values

- Diab: I-I + Placebo  \( n = 6 \)
- Diab: I-I + Wortmannin  \( n = 4 \)

* \( p < 0.0083 \)

![MR T2 Values Graph]
Figure 4.2.III

**MR MT Values**

- **Diab: I-I + Placebo**  n= 6
- **Diab: I-I + Wortmannin**  n= 4

* p< 0.0083

**MR Volumes**

- **Diab: I-I + Placebo**  n= 6
- **Diab: I-I + Wortmannin**  n= 4
4.2.IV Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-S and Wortmannin and Their Control Diabetic Littermates Receiving I-S and Placebo.

To study whether PI3K inhibition in diabetic animals can be of any further detrimental effect (and to rule out the occurrence of any systemic toxic effects of Wortmannin that can affect the cognitive function of treated mice?) we compared the results of behavioural and MRI tests of diabetic mice receiving I-S and Wortmannin with their control diabetic littermates receiving I-S and placebo. The difference between control diabetic wildtype mice and Wortmannin treated diabetic mouse were not significant in the four cognitive tests performed (MANOVA, p<0.0083). Additional assessments using the AUC for each intervention cohort also showed no significant differences (Figure 4.2.IV [A-D]). There were also no significant differences in MR T2 [F], MTR [G] FA values [4.2.I. I], or fiber numbers [4.2.I. J] measured between the two groups. Thus, overall, there was no significant difference between diabetic wildtype Wortmannin treated mice and placebo treated diabetic group, demonstrating that PI3K inhibition was not of further detriment to cognitive performance and MR detectable brain changes of diabetic mice.

Figure 4.2.IV: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-S and Wortmannin and Diabetic Mice Receiving I-S and Placebo.

Outcome measures were similar in diabetic mice treated with I-S and Wortmannin (Diab:I-S+Wortmannin) and control diabetic mice treated with I-S and Placebo (Diab:I-S+Placebo) (p<0.0083). There were no differences in the performance of diabetic (Diab:I-S+Wortmannin), and (Diab:I-S+Placebo), in watermaze [A], radial arm [B], holeboard [C], object recognition [D], and Rotarod [E] tests. Categorization into five-week periods [A1-D1] and AUC measures were also similar between the groups. Similarly, the difference between the T2 map values [F], MTR values [G], and overall brain volumes [H] of two groups were not different statistically.
Figure 4.2.IV

WaterMaze Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin  n= 7-12

* p < 0.0083

Time (s)

Weeks

A

Watermaze Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin  n= 7-12

* p < 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time

A1
Figure 4.2.IV

Radial Arm Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin  n= 7-12

* p<0.0083

Weeks

Radial-Arm Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin  n= 7-12

* p<0.0083

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.2.IV

Holeboard Latency

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin  n= 7-12

*p<0.0083

Time (s)

Weeks

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.2.IV

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin n= 7-12

* p<0.0083

Time (s) vs. Weeks

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 6-12
- Diab: I-S + Wortmannin n= 7-12

Time (s) vs. Time

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.2.IV

**Rotation Latency**

- **Diab: I-S + Placebo** $n=6-12$
- **Diab: I-S + Wortmannin** $n=7-12$

**MR T2 Values**

- **Diab: I-S + Placebo** $n=4$
- **Diab: I-S + Wortmannin** $n=4$
Figure 4.2.IV

MR MT Values

- Diab: I-S + Placebo  n= 4
- Diab: I-S + Wortmannin  n= 4

MR Volumes

- Diab: I-S + Placebo  n= 4
- Diab: I-S + Wortmannin  n= 4
4.2.V Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Wortmannin and Diabetic Mice Receiving I-S and Wortmannin.

To study the effect of PI3K inhibition on the I-I effects we compared the cognitive function and MRI results of the diabetic animals receiving I-I and Wortmannin concurrently, with the diabetic ones receiving Wortmannin and I-S. The performance of diabetic mice receiving PI3K inhibitor along with I-I did not significantly differ from that of mice treated with I-S and Wortmannin, as demonstrated in watermaze [4.2.V.A], holeboard [4.2.V.C] and object recognition [4.2.V.D] tests (ANOVA, p<0.0083). Diabetic mice receiving I-S and Wortmannin took more time to perform the radial arm task than diabetic mice receiving I-I and Wortmannin, during the final weeks of study [4.2.V.B and B1]. (ANOVA, P < 0.0083)

Differences between these two groups in terms of their whole brain volume [4.2.V.F], T2 values [4.2.V.G], MTR [4.2.V.H], fractional anisotropy [4.2.I.I] and fiber tract numbers [4.2.I.J] generally did not achieve statistical significance.

The results suggest that PI3K inhibition could effectively nullify the beneficial effects of I-I in DM brain.

Figure 4.2.V: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Wortmannin and Diabetic Mice Receiving I-S and Wortmannin.

There was no difference between diabetic mice treated with I-S and Wortmannin (Diab:I-S+Wortmannin), and diabetic mice treated with I-I and Wortmannin (Diab:I-I+Wortmannin), in watermaze [A], holeboard [C], object recognition [D], and Rotarod [E] tests. Diab:I-I+Wortmannin performed better than Diab:I-S+Wortmannin, in radial arm test over the last half of the study [B]. Categorization into five-week periods [A1-D1] and AUC measures were also similar between the groups [A-D, p<0.0083]. The difference between the T2 map values [F], MTR values [G], and overall brain volumes [H] were not different statistically.
Figure 4.2.V

WaterMaze Latency

- Diab: I-S + Wortmannin  n= 7-12
- Diab: I-I + Wortmannin  n= 7-12

* p< 0.0083

Weeks

Watermaze Latency

- Diab: I-S + Wortmannin  n= 7-12
- Diab: I-I + Wortmannin  n= 7-12

* p< 0.0083

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.2.V

Radial Arm Latency

- Diab: I-S + Wortmannin  n= 7-12
- Diab: I-I + Wortmannin  n= 7-12

* p< 0.0083

Time (s)

Weeks

Radial-Arm Latency

- Diab: I-S + Wortmannin  n= 7-12
- Diab: I-I + Wortmannin  n= 7-12

* p< 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.2.V

Object Recognition Time (Novel-Known)

- Diab: I-S + Wortmannin  n= 7-12
- Diab: I-I + Wortmannin  n= 7-12

* p < 0.0083

Time (s)

Weeks
Figure 4.2.V

Rotarod Latency

* $p < 0.0083$

- Diab: I-S + Wortmannin $n = 7-12$
- Diab: I-I + Wortmannin $n = 7-12$

Figure 4.2.F

MR T2 Values

* $P < 0.0083$

- Diab: I-S + Wortmannin $n = 4$
- Diab: I-I + Wortmannin $n = 4$
Figure 4.2.V

**MR MT Values**

- *Diab: I-S + Wortmannin*  
  - n = 4
- *Diab: I-L + Wortmannin*  
  - n = 4

**MR Volumes**

- *Diab: I-S + Wortmannin*  
  - n = 4
- *Diab: I-L + Wortmannin*  
  - n = 4
4.2.VI Cognitive Behavioural Data and MRI Results for non-Diabetic Mice Receiving I-S and Wortmannin their non-diabetic control littermates receiving I-S and Placebo.

To study the effect of PI3K inhibition in non-diabetic mice and to test our hypothesis which indicates that PI3K inhibition in the non-diabetic animals would lead to cognitive dysfunction and WMA, as seen in diabetic animals, we compared non diabetic mice receiving Wortmannin with their control non-diabetic littermates. In non-diabetic mice, Wortmannin treatments had some effects upon cognition and MRI results. A cognitive decline for wildtype non-diabetic mice receiving intranasal Wortmannin was demonstrated after 2 weeks of treatment, with longer escape latencies in water maze test [4.2.VI.A] compared to control non-diabetic mice. Similarly, radial arm [4.2.VI.B] and holeboard testing [4.2.VI.C] demonstrated significant loss of cognitive performances of the wildtype non-diabetic mice receiving the PI3K inhibitor, as evident by the longer latencies to endpoint, compared to control non-diabetic mice. However, the performance of non-diabetic mice receiving Wortmannin and control non-diabetic mice in object recognition task [4.2.VI.D] was similar (ANOVA, P < 0.0083).

Higher MR T2 values [4.2.VI.F] and lower MTR [4.2.VI.G] in non-diabetic mice treated with Wortmannin in comparison to control diabetic mice were observed in various brain areas. In addition, lower FA values [4.2.I. I] and decreased fiber tract numbers [4.2.I. J] of CC were seen in non-diabetic mouse brain treated with Wortmannin. Whole brain volume was lower in non-diabetic mice treated with Wortmannin in comparison to their control non-diabetic littermates receiving I-S and Placebo [4.2.VI.H]. These results are consistant with the cognitive behavioural findings, showing that PI3K inhibition in non-diabetic mice were causing similar cognitive and structural changes as could be seen in diabetic animals.
Figure 4.2.VI: Cognitive Behavioural Data and MRI Results for non-Diabetic Mice Receiving I-S and Wortmannin their Non-diabetic Control Littermates Receiving I-S and Placebo.

Non-diabetic mice treated with Placebo (nonDiab:I-S+Placebo) performed better than non-diabetic mice treated with Wortmannin (nonDiab:I-S+Wortmannin), in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests (p<0.0083). Categorization into five-week periods was shown in A1-D1. AUC measures were significant between the groups, for holeboard and radial arm tests [B and C, p<0.0083]. In different brain regions, T2 map values were significantly elevated [F] and MTR values were significantly depressed [G] for nonDiab:I-S+Wortmannin. Volumetric measurements of the entire brain demonstrated brain atrophy in nonDiab:I-S+Wortmannin [H].
Figure 4.2.VI

Watermaze Latency

- ▲ nonDiab: I-S + Placebo n=15
- ◆ nonDiab: I-S + Wortmannin n=7-8

* p<0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Radial Arm Latency

- ▲ nonDiab: I-S + Placebo n=15
- ◆ nonDiab: I-S + Wortmannin n=7-8

* p<0.0083

Time (s)

Weeks

A1

B
Figure 4.2.VI

**Radial-Arm Latency**

- nonDiab: I-S + Placebo, n = 15
- nonDiab: I-S + Wortmannin, n = 7-8

**HoleBoard Latency**

- nonDiab: I-S + Placebo, n = 15
- nonDiab: I-S + Wortmannin, n = 7-8

* p < 0.0083
Figure 4.2 VI

Object Recognition Time (Novel-Known)

- nonDiab: I-S + Placebo  n = 15
- nonDiab: I-S + Wortmannin n = 7-8

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Rotarod Latency

- nonDiab: I-S + Placebo  n = 15
- nonDiab: I-S + Wortmannin n = 7-8

Time (s)

1-2  3-4  5-6  7-8  9-10  11-12  13-14  15-16  17-18  19-20  21-22  23-24  25-26

*p < 0.0083
Figure 4.2.VI

MR T2 Values

- nonDiab: I-S + Wortmannin  n=4
- nonDiab: I-S + Placebo  n=4

* P<0.0083

T2 Value (ms)

Whole Brain  SI  Caudate/putamen  MI  Anterior Commissure  Corpus Callosum  Internal Capsule  CA1  CA2  CA3  VI  Posterior Commissure  Pons  Cerebellum

Figure 4.2.G

MR MT Values

- nonDiab: I-S + Wortmannin  n=4
- nonDiab: I-S + Placebo  n=4

* p<0.0083

MTR (p.u.)

Whole Brain  CA1  CA2  CA3  Corpus Callosum  Cortex  Posterior Commissure  Caudate/putamen  Internal Capsule  Anterior Commissure
4.3 Results of Akt Pharmacological Modulation

To examine the effect of pharmacological inhibition of Akt in the diabetic mouse brain receiving insulin, a second cohort of mice was tested and relevant comparisons performed between the groups at the end of the study. The comparisons were done according to the pre-planned scheme [Figure 4.3.A]. The groups which differ only in only one factor were compared, accordingly.

Mice were distributed randomly among the groups and there were no significant differences between the groups at baseline and after the training period. (ANOVA with Bonferroni correction; p<0.008) Similar to the first cohort of animals, the initial learning ability was seen in all experimental groups, regardless of diabetes disease state. Learning processes for each of the tasks appeared to be similar between diabetic and non-diabetic mice over the first weeks. All cognitive data was based upon a minimum of six mice in each cohort group at all time-points. Data from the mice with severe motor dysfunction, according to rotarod test results, or illness were excluded from the time-point.
Figure 4.3.A: Comparison Chart of Second Experimental Cohort

Pre-planned comparisons between the groups were performed as follows. The groups, which are highlighted, compared for differences in the cognitive and MRI results. Six pairs of comparison were made. The cells in grey color are the mirror of the same pairs in table. Numbers show the order of results being shown.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Mice</th>
<th></th>
<th>Non- Diabetic mice</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>API-2 + I-S</td>
<td>V</td>
<td>API-2 + I-S</td>
<td></td>
</tr>
<tr>
<td>Diabetic Mice</td>
<td>API-2 + I-I</td>
<td>IV</td>
<td>Placebo + I-S</td>
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</tr>
<tr>
<td></td>
<td>Placebo + I-S</td>
<td></td>
<td>Placebo + I-I</td>
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<tr>
<td></td>
<td>Placebo + I-I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic mice</td>
<td>API-2 + I-S</td>
<td></td>
<td></td>
<td>VI</td>
</tr>
<tr>
<td></td>
<td>Placebo + I-S</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3.I Cognitive Behavioural Data and MRI Results for Diabetic Control Mice Receiving I-S and Placebo and Their Non-Diabetic Control Littermates Receiving I-S and Placebo.

The cognitive and MRI results of the second cohort of control diabetic and non-diabetic mice receiving I-S and Placebo were similar to the first cohort of animals receiving the same treatments [see 4.2.I]. The results demonstrated significantly impaired performances for control wildtype diabetic mice compared to control wildtype non-diabetic mice, receiving I-S and Placebo. Brain atrophy and development of white matter changes were confirmed in the diabetic brain over the course of diabetes [4.3.I A-H]. Lower FA values [4.3.III. I] and fiber numbers...
[4.3. III. J] in Corpus Callosum (CC) and Internal Capsule (IC) were recognized in diabetic mouse brain. The results, thus, confirmed the findings from the first cohort of animals [4.2. I].

**Figure 4.3. I:** Cognitive Behavioural Data and MRI Results for Diabetic Control Mice Receiving I-S and Placebo and Their Non-Diabetic Control Littermates receiving I-S and Placebo. Non-diabetic mice treated with placebo (nonDiab:I-S+Placebo) performed better than diabetic mice treated with Placebo (Diab:I-S+Placebo) in the second cohort of the study (p<0.0083).
Figure 4.3.1

HoleBoard Latency

- Diab: I-S + Placebo  n= 8-10
- nonDiab: I-S + Placebo  n= 8

* p<0.0083

Weeks

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.3.1

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 8-10
- nonDiab: I-S + Placebo  n= 8

* p< 0.0083

Time (s)

Weeks

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.3. I

Rotarod Latency

- Diab: I-S + Placebo  n= 8-10
- nonDiab: I-S + Placebo  n= 8

MR T2 Values

- * P<0.0083

T2 Value (ms)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Diab: I-S + Placebo</th>
<th>nonDiab: I-S + Placebo</th>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior Commissure</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Corpus Callosum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal Capsule</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CA1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>CA2</td>
<td>*</td>
<td>*</td>
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<td>CA3</td>
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<tr>
<td>Posterior Commissure</td>
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</tr>
<tr>
<td>Pons</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

105
Figure 4.3. I

MR MT Values

- Diab: I-S + Placebo  n= 4
- nonDiab: I-S + Placebo  n= 4

* p<0.0083

- Whole Brain
- CA1
- CA2
- CA3
- Corpus Callosum
- Cortex
- Posterior Commissure
- Caudate/Putamen
- Internal Capsule
- Anterior Commissure

MR Volumes

- Diab: I-S + Placebo  n= 4
- nonDiab: I-S + Placebo  n= 4

*
4.3.II Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Their Diabetic Control Littermates Receiving I-S and Placebo.

Diabetic Mice receiving I-I outperformed the second cohort of wildtype control diabetic mice enrolled in our study, similar to the first part of the results [see 4.2.II]. Diabetic mice receiving I-S and Placebo took longer latencies to find the hidden platform in water maze test and food rewards in radial arm or holeboard tests. In object recognition task, diabetic mice receiving I-I and Placebo passed more time with the novel object in comparison to diabetic mice receiving I-S and Placebo. Under the Curve measurements demonstrated significantly better performances for wildtype diabetic mice receiving I-I compared to wildtype diabetic mice receiving I-S in holeboard and radial arm tests. Whole brain volume was higher in diabetic mice treated with I-I in comparison to their control diabetic littermates receiving I-S and Placebo. I-I treated diabetic mice were partially protected from heightened T2 map values and diminished MTR in different areas of the diabetic brain [4.3.II A-H]

FA values [4.3.III.I] and fiber tract numbers [4.3.III.J] of CC were significantly increased in diabetic mouse brain treated with I-I. These results confirmed the beneficiary effect of I-I on cognitive function and its protective effects on brain structural changes, which were shown in first cohort of our study [4.2.II].
In different brain regions, T2 map values were significantly decreased [H] and MT values were significantly increased [I] for Diab: I-I + Placebo.
Figure 4.3.II.

**Radial Arm Latency**

- Diab: I-I + Placebo  n= 6-9
- Diab: I-S + Placebo  n= 8-10

* p<0.0083

**Radial-Arm Latency**

- Diab: I-I + Placebo  n= 6-9
- Diab: I-S + Placebo  n= 8-10

* p<0.0083
Figure 4.3.II.

**Object Recognition Time (Novel-Known)**

- Diab: l-l + Placebo  n= 6-9
- Diab: l-S + Placebo  n= 8-10

* p< 0.0083

---

**Object Recognition Time (Novel-Known)**

- Diab: l-l + Placebo  n= 6-9
- Diab: l-S + Placebo  n= 8-10

* p< 0.0083

---

---

111
Figure 4.3.II.

Rotarod Latency

- Diab: I-L + Placebo  n= 6-9
- Diab: I-S + Placebo  n= 8-10

Time (s)

MR T2 Values

* P<0.0083

Whole Brain  S1  Caudate/Putamen  M1  Anterior Commissure  Corpus Callosum  Internal Capsule  CA1  CA2  CA3  V1  Posterior Commissure  Pons  Cerebellum

T2 Value (ms)
Figure 4.3.II.

G

MR MT Values

<table>
<thead>
<tr>
<th>Region</th>
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<th>Diab: I-I + Placebo</th>
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</tr>
<tr>
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<td>*</td>
<td></td>
</tr>
<tr>
<td>CA2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Corpus Callosum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior Commissure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internal Capsule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior Commissure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.0083

n=4

n=6

H

MR Volumes

<table>
<thead>
<tr>
<th>Region</th>
<th>Diab: I-S + Placebo</th>
<th>Diab: I-I + Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.0083

n=4

n=6
4.3.III Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Diabetic Mice Receiving I-I and API-2.

To study the outcome of Akt inhibition on the beneficiary effects of I-I, a group of diabetic mice (n=8-10) were enrolled to receive concurrent daily intranasal delivery of API-2, the Akt inhibitor, and I-I. Diabetic mice receiving I-I and Placebo performed better than mice receiving intranasal API-2 together with I-I, in water maze and holeboard tests over the final weeks of the study. The object recognition [4.3.III.D], and radial arm [4.3.III.D] tests demonstrated the better performance of diabetic mice receiving I-I compared to diabetic mice treated with I-I and API-2, clearly. AUC measurements showed better performances for the diabetic mice treated with I-I in comparison to diabetic mice treated with I-I and API-2, in object recognition and radial arm tests.

Volumetric measurements of the entire brain showed no significant difference between diabetic mice treated with I-I and placebo and diabetic mice treated with I-I and API-2 [4.3.III.H], T2 map values in different brain regions were significantly higher [4.3.III.F] and MTR values were significantly lower [4.3.III.G] for diabetic mice treated with both I-I and API-2 in comparison to diabetic mice treated with I-I and placebo. While I-I treated diabetic mice were partially protected from the elevation of T2 values, diabetic mice, treated with I-I and API-2 concurrently, demonstrated elevated T2 map values across grey and white matter regions of the brain. The significant elevation in T2 map values were significantly demonstrated across corpus callosum, internal capsule, CA1 and CA2 regions of the hippocampus and posterior commissure. There were no statistically significant differences between diabetic brains treated with I-I along with API-2 and diabetic brains treated with I-I and Placebo, in terms of the fractional anisotropy [4.3.III.I], and fiber tract numbers [4.3.III.J] of IC and CC.
This part of the results suggests that much of the I-I beneficial effects on diabetic brain could be blocked by Akt inhibition.

**Figure 4.3.III: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and Placebo and Diabetic Mice Receiving I-I and API-2.**

The graphs demonstrate performance of diabetic wildtype mice treated with intranasal Insulin (I-I) and Placebo (Diab: I-I + Placebo), and Diab wildtype mice treated with Intranasal Insulin(I-I) and API-2 (Diab: I-I + API-2), in watermaze [A], radial arm [B], holeboard [C], object recognition [D], and rotarod [E] tests. Categorization into five-week periods was shown in A1-D1. AUC measures were significant between the groups, in radial arm and object recognition tests [B and D, p<0.0083]. In different brain regions, T2 map values were significantly increased [F] and MTR values were significantly decreased [G] for Diab: I-I + API-2. The difference between the overall brain volumes of two groups was not statically significant [H]. FA values [I] and fiber numbers [J] in Diab and nonDiab mouse brains of first experimental cohort is shown. Error bars represent mean ± SEM.
Figure 4.3.III

**Watermaze Latency**

- Diab: I-I + Placebo  n= 6-9
- Diab: I-I + API-2  n= 8-10

* p< 0.0083

**Radial Arm Latency**

- Diab: I-I + Placebo  n= 6-9
- Diab: I-I + API-2  n= 8-10

* p< 0.0083

---

Weeks
Figure 4.3.III

Radial-Arm Latency

- Diab: I-I + Placebo  n = 6-9
- Diab: I-I + API-2  n = 8-10

HoleBoard Latency

- Diab: I-I + Placebo  n = 6-9
- Diab: I-I + API-2  n = 8-10

* p < 0.0083

Time (s)

Weeks

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.3.III

Holeboard Latency

- Diab: I-I + Placebo  n = 6-9
- Diab: I-I + API-2  n = 8-10

* p < 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Object Recognition Time (Novel-Known)

- Diab: I-I + Placebo  n = 6-9
- Diab: I-I + API-2  n = 8-10

* p < 0.0083

Time (s)

Weeks
Figure 4.3.III

Object Recognition Time (Novel-Known)

- ◊ Diab: I-I + Placebo  n= 6-9
- ▲ Diab: I-I + API-2  n= 8-10

Rotarod Latency

* p<0.0083

- ◊ Diab: I-I + Placebo  n= 6-9
- ▲ Diab: I-I + API-2  n= 8-10
Figure 4.3.III

**MR MT Values**

- Diab: I-I + Placebo  \( n = 6 \)
- Diab: I-I + API-2  \( n = 5 \)

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<tr>
<th>Tissue Type</th>
<th>MT Values</th>
<th>p-value</th>
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<tbody>
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<tr>
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<td>35</td>
<td>*</td>
</tr>
<tr>
<td>Posterior Commissure</td>
<td>30</td>
<td>*</td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td>35</td>
<td>*</td>
</tr>
<tr>
<td>Internal Capsule</td>
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<td>*</td>
</tr>
<tr>
<td>Anterior Commissure</td>
<td>35</td>
<td>*</td>
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</table>

**MR Volumes**

- Diab: I-I + Placebo  \( n = 6 \)
- Diab: I-I + API-2  \( n = 5 \)

<table>
<thead>
<tr>
<th>Tissue Type</th>
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<tbody>
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<td>310</td>
</tr>
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</table>

* indicates significance at p<0.0083.
4.3.IV Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-S and API-2 and Their Control Diabetic Littermates Receiving I-S and Placebo.

To test the effect of API-2 treatment in the diabetic mice and to show the effect of Akt further inhibition in diabetic animals, we compared the results of the diabetic mice receiving I-S and API-2 with their control diabetic littermates receiving I-S and placebo. The difference between control diabetic wildtype mice and API-2 treated diabetic mouse were not significant in four cognitive tests (MANOVA, p<0.0083) Additional assessments using the AUC for each intervention cohort showed no statistically different performances on cognitive testing [Fig 4.3.IV.A-D]. There were also no significant differences in MR T2 [4.3.IV.F], MTR [4.3.IV.G] FA values [4.3.III. I], or fiber numbers [4.3.III.J] measured between the two groups.

Detecting no difference between diabetic mice receiving API-2 and their control diabetic littermates suggests that API-2 delivery does not further impair cognitive function of DM mice.
Figure 4.3.IV: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-S and API-2 and Diabetic Mice Receiving I-S and Placebo.

Performances were similar in diabetic mice treated with I-S and API-2 (Diab: I-S + API-2) and control diabetic mice treated with I-S and Placebo (Diab: I-S + Placebo) (p<0.0083) for the watermaze [A], radial arm [B], holeboard [C], object recognition [D], and Rotarod [E] tests. AUC measurements also demonstrated no significant difference between the two groups. The difference between the overall brain volumes [F], T2 map values [G], and MTR values [H] of two groups were not statistically significant [G].
**Figure 4.3.IV**

Radial Arm Latency

- Diab: I-S + Placebo     n= 8-10
- Diab: I-S + API-2       n= 6-9

* p< 0.0083

Time (s)

Weeks

---

**Radial-Arm Latency**

- Diab: I-S + Placebo     n= 8-10
- Diab: I-S + API-2       n= 6-9

* p< 0.0083

Time (s)

Week 1 to 5   Week 6 to 10   Week 11 to 15   Week 16 to 20   Week 21 to 26
Figure 4.3.IV

HoleBoard Latency

- Diab: I-S + Placebo, n = 8-10
- Diab: I-S + API-2, n = 6-9

* p < 0.0083

C1

HoleBoard Latency

- Diab: I-S + Placebo, n = 8-10
- Diab: I-S + API-2, n = 6-9

* p < 0.0083
Figure 4.3.IV

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 8-10
- Diab: I-S + API-2  n= 6-9

* p< 0.0083

Time (s)

Weeks

Object Recognition Time (Novel-Known)

- Diab: I-S + Placebo  n= 8-10
- Diab: I-S + API-2  n= 6-9

Time (s)

Time

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.3.IV

Rotarod Latency

- **Diab: I-S + Placebo**  n= 8-10
- **Diab: I-S + API-2**  n= 6-9

Figure F

MR T2 Values

*P<0.0083

- **Diab: I-S + Placebo**  n=4
- **Diab: I-S + API-2**  n=4
**Figure 4.3.IV**

**MR MT Values**

* p<0.0083

- Diab: I-S + API-2  n=4
- Diab: I-S + Placebo  n=4

**MR Volumes**

- Diab: I-S + Placebo  n=4
- Diab: I-S + API-2  n=4

Whole Brain
4.3.V Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and API-2 and Diabetic Mice Receiving I-S and API-2.

To study the impact of Akt inhibition on I-I effect we compared the cognitive and MRI results of the diabetic mice receiving I-I and API-2 with the diabetic mice receiving I-S and API-2. API-2 Inhibition of the protective effects of insulin treatment in diabetic mice appeared partial. While the performance of diabetic mice receiving I-I along with Akt inhibitor didn’t significantly improve compared to mice treated with I-S and API-2 for the tests for object recognition and radial arm tasks [4.3.V.B, D], mice treated with I-I and API-2 still showed better performance in holeboard, and watermaze tests [4.3.V.C, A], mainly within the second half of the study (ANOVA, P < 0.0083). There was no significant difference between whole brain volume [4.3.V.F], MTR [4.3.V.H], Fractional anisotropy [4.3.III.I] and fiber tract numbers [4.3.III.J] of these two groups. T2 values of IC and CA1 were lower in mouse brain receiving both I-I and API-2 [4.3.V.G], suggesting a minor preservation in the protective effect for insulin despite API-2 application. The results suggest that Akt inhibition was able to partially block the beneficial effects of I-I in DM brain.

Figure 4.3.V: Cognitive Behavioural Data and MRI Results for Diabetic Mice Receiving I-I and API-2 and Diabetic Mice Receiving I-S and API-2.

The graphs demonstrate the performance of diabetic mice treated with I-S and API-2 (Diab: I-S + API-2) and diabetic mice treated with I-I and API-2 (Diab: I-I + API-2) in watermaze [A], holeboard [C], object recognition [D], and Rotarod [E] tests. Categorization into five-week periods [A1-D1] and AUC measures were also similar between the groups. The difference between the overall brain volumes [F], T2 map values [G], and MT values [H] of two groups
were not statically significant. (ANOVA, P<0.0083)
Figure 4.3.V

Radial Arm Latency
- Diab: I-S + API-2  n= 6-9
- Diab: I-I + API-2  n= 8-10

Radial-Arm Latency
- Diab: I-S + API-2  n= 6-9
- Diab: I-I + API-2  n= 8-10
Figure 4.3.V

HoleBoard Latency

- Diab: I-I + API-2  n= 8-10
- Diab: I-S + API-2  n= 6-9

* p<0.0083

Time (s)

Weeks

C1

HoleBoard Latency

- Diab: I-I + API-2  n= 8-10
- Diab: I-S + API-2  n= 6-9

* p<0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.3.V

Object Recognition Time (Novel-Known)

* p< 0.0083

- Diab: I-S + API-2 \( n=6-9 \)
- Diab: I-I + API-2 \( n=8-10 \)
Figure 4.3.V

Rotarod Latency

- Diab: I-S + API-2  n=6-9
- Diab: I-I + API-2  n=8-10

mm³  MR Volumes

- Diab: I-S + API-2  n=4
- Diab: I-I + API-2  n=5

Whole Brain
Figure 4.3.V

**MR T2 Values**

- Diab: I-S + API-2, n=4
- Diab: I-I + API-2, n=5

- *P<0.0083

---

**MR MT Values**

- Diab: I-S + API-2, n=4
- Diab: I-I + API-2, n=5

- *p< 0.0083
4.3.VI Cognitive Behavioural Data and MRI Results for Non-Diabetic Mice Receiving I-S and API-2 and Their Non-diabetic Control Littermates Receiving I-S and Placebo.

To investigate the effect of Akt inhibition in non-diabetic mice and to test whether Akt inhibition, as a proposed mechanism for DLE, will lead to similar cognitive and structural changes in non-diabetic mice, as seen in diabetic mice, we compared non-diabetic mice treated with I-S and API-2 with their control littermates treated with I-S and placebo. Cognitive decline for wildtype non-diabetic mice receiving intranasal API-2 was demonstrated at the final weeks of study, with longer escape latencies in water maze test [4.3.VI.A] compared to control non-diabetic mice. Similarly, radial arm [4.3.VI.B] and holeboard testing [4.3.VI.C] demonstrated significant loss of cognitive performances of the wildtype non-diabetic mice receiving the Akt inhibitor, as evident by the longer latencies to endpoint, compared to control non-diabetic Mice. No significant difference was detected between these groups for the object recognition task [4.3.VI.D] and AUC measurements in all four cognitive tests.

Higher MR T2 values [4.3.VI.F] and lower MTR [4.3.VI.G] was observed in non-diabetic mice treated with API-2 in comparison to control diabetic mice. However, FA values [4.3.III. I], fiber tract numbers [4.3.III. J], and whole brain volume was not significantly different between non-diabetic mice treated with API-2 and their control non-diabetic littermates receiving I-S and Placebo [4.3.VI.H]. These results suggested a partial role for Akt inhibition in the development of cognitive and structural changes in DLE.

Figure 4.3.VI: Cognitive Behavioural Data and MRI Results for non-Diabetic Mice Receiving I-S and API-2, and Their Non-diabetic Control Littermates Receiving I-S and Placebo.

The graphs demonstrate performance of nonDiab wildtype mice treated with I-S and placebo (nonDiab: I-S + Placebo), and nonDiab wildtype mice treated with I-S and API-2 (nonDiab: I-S
+ API-2), in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests. AUC measurement was not significant. Also shown for these groups are T2 map values [F], MTR values [G] and brain Volume measurements [H]. * P<0.0083.
Figure 4.3.VI

Radial Arm Latency

- nonDiab: I-S + Placebo  n= 8
- nonDiab: I-S + API-2     n= 8

* p< 0.0083

Time (s)

Weeks

Radial-Arm Latency

- nonDiab: I-S + Placebo  n= 8
- nonDiab: I-S + API-2     n= 8

* p< 0.0083

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.3.VI

HoleBoard Latency

- *p < 0.0083

- nonDiab: I-S + Placebo  n= 8
- nonDiab: I-S + API-2  n= 8

Weeks

Time (s)

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 25

Time
Figure 4.3 VI

Object Recognition Time (Novel-Known)

- nonDiab: I-S + Placebo  n= 8
- nonDiab: I-S + API-2    n= 8

* p<0.0083

Time (s)

Weeks

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.3.VI

**Rotarod Latency**

- nonDiab: I-S + Placebo  n= 8
- nonDiab: I-S + API-2  n= 8

**MR T2 Values**

- *P<0.0083

<table>
<thead>
<tr>
<th>Region</th>
<th>T2 Value (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td>40</td>
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<tr>
<td>SI</td>
<td>42</td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td>38</td>
</tr>
<tr>
<td>M1</td>
<td>36</td>
</tr>
<tr>
<td>Anterior Commissure</td>
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<tr>
<td>Corpus Callosum</td>
<td>37</td>
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<tr>
<td>Internal Capsule</td>
<td>39</td>
</tr>
<tr>
<td>CA1</td>
<td>41</td>
</tr>
<tr>
<td>CA2</td>
<td>40</td>
</tr>
<tr>
<td>CA3</td>
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<td>Pons</td>
<td>39</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>34</td>
</tr>
</tbody>
</table>
4.4 Results of Akt overexpression in mice

To examine whether the enhanced Akt expression in oligodendrocytes have any effect on diabetic brain changes or I-I treatment outcome, we enrolled 44 diabetic and non-diabetic Akt overexpressing (AKTO) transgenic mice in our study. The overexpression of Akt in cerebrum samples from Akt overexpressing transgenic mice was shown by Flores et al, using Western blot and Akt kinase assay, and was confirmed in our lab using a few hippocampal samples [A representative blot is presented in Figure 4.4.B]. Future molecular studies on collected tissues are needed to establish Akt overexpression in all of the mice enrolled in this study. Transgenic mice were randomly divided into two diabetic and two non-diabetic groups which contained approximately even ratios of males to females [Figure 4.1]. Relevant comparisons were performed between the groups according to the pre-planned scheme [Figure 4.4.A]. Similar to the previous mouse cohorts, cognitive testing began from first week of diabetes and was completed after 26 weeks of diabetes. There were no significant differences between the groups at baseline. (ANOVA with Bonferroni correction; p<0.016)
**Figure 4.4.A: Comparison Chart of Third Experimental Cohort**

Pre-planned comparisons between the groups were performed as follows. The groups, which are highlighted, compared for differences in the cognitive and MRI results. Three pairs of comparison were made. The cells in grey color are the mirror of the same pairs in the table. Numbers show the order of results being shown.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic Akt Over-expressing Transgenic Mice</th>
<th>Non-Diabetic Akt Over-expressing Transgenic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-I</td>
<td>I-S</td>
</tr>
<tr>
<td>Diabetic Akt Over-expressing Transgenic Mice</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>I-S</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Non-Diabetic Akt Over-expressing Transgenic Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-I</td>
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<tr>
<td>I-S</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Figure 4.4.B: Representative Western blot analyses of pAkt in AKTO mice.**

Western blot shows high pAkt level in Akt overexpressing (AKTO) transgenic hippocampal samples. Courtesy of Dr. Alma Rosales, from the laboratory of Dr. C. Toth.
To investigate whether the enhanced Akt expression in oligodendrocytes have any beneficiary effect on diabetic brain changes, we compared diabetic AKTO mice receiving I-S with their non-diabetic control littermates. Akt overexpressing transgenic diabetic mice spent less time with the novel object in object recognition task at the end of study compared to their non-diabetic transgenic littermates [4.4.I.D]. However, the performances of diabetic and non-diabetic transgenic mice were comparable in watermaze [4.4.I.A], radial arm [4.4.I.B] and holeboard [4.4.I.C] tests. Results for cognitive behavioural tasks are also presented as the average of each of 5 consecutive weeks [4.2.1 A1-D1]. For A-D, AUC measurements were performed and demonstrated no significant difference between the two groups. These findings suggest that Akt overexpression in the diabetic brain can ameliorate cognitive impairment in mice.

Whole brain volume was not significantly different between diabetic and non-diabetic Akt overexpressing mice at the endpoint [4.4.I.F]. Higher T2 values in diabetic brain was observed in CC and CA1 region of hippocampus [4.2.I.G] and lower MT ratios were seen in different areas of the diabetic brain [4.2.I.H]. While the FA values [4.4.I.I] were comparable between diabetic and non-diabetic Akt overexpressing brains, the calculated numbers of tract fibers [4.4.I.J] in CC was lower in diabetic mouse brain, suggesting axonal loss in these areas of the brain due to diabetes. Further histological analysis of brain morphology and pathology is required to interpret these findings. Taken together, these results suggest a protective role for Akt activation in the development of DLE.
Figure 4.4.I: Cognitive Behavioural Data and MRI Results for Diabetic AKTO Transgenic Mice Receiving I-S and Their Non-Diabetic Transgenic Littermates receiving I-S.

The graphs demonstrate performance of non-diabetic AKTO mice treated with I-S (nonDiab: AKTO, I-S), and diabetic AKTO mice treated with I-S (Diab: AKTO, I-S), in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests. Categorization into five-week periods [A1-D1] and AUC measures were also similar between the groups [A-D, p<0.0083]. The charts show T2 map values [F] and MTR [G] volumetric measurements of the entire brain [H] for nonDiab: AKTO, I-S and Diab: AKTO, I-S. FA values [I] and fiber numbers [J] in Diab and nonDiab AKTO mouse brains are shown. Error bars represent mean ± SEM.
Figure 4.4.1

Radial Arm Latency

* p<0.016

- Diab: AKTO, I-S n= 9-12
- nonDiab:AKTO, I-S n= 9-10
Figure 4.4.1

HoleBoard Latency

- Diab: AKTO, I-S  n= 9-12
- nonDiab: AKTO, I-S  n= 9-10

* p < 0.016

Weeks

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.4.1

Object Recognition Time (Novel-Known)

- Diab: AKTO, I-S  n= 9-12
- nonDiab:AKTO, I-S  n= 9-10

* p<0.016

Time (s)

Weeks

Object Recognition Time (Novel-Known)

- Diab: AKTO, I-S  n= 9-12
- nonDiab:AKTO, I-S  n= 9-10

* p<0.016

Time (s)

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26

Time
Figure 4.4.1

Rotarod Latency

- **Diab: AKTO, I-S**  \( n=9-12 \)
- **nonDiab: AKTO, I-S**  \( n=9-10 \)

![Rotarod Latency Graph]

Figure F

MR T2 Values

- **nonDiab: AKTO I-S**  \( n=4 \)
- **Diab: AKTO I-S**  \( n=4 \)

![MR T2 Values Graph]
Figure 4.4.1

**MR MT Values**

- Diab: AKTO, I-S $n=4$
- nonDiab: AKTO, I-S $n=4$

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diab: AKTO, I-S</th>
<th>nonDiab: AKTO, I-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td>* p&lt;0.016</td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corpus Callosum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior Commissure</td>
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<tr>
<td>Caudate/Putamen</td>
<td></td>
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<tr>
<td>Internal Capsule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior Commissure</td>
<td></td>
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</tbody>
</table>

**MR Volumes**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Diab: AKTO, I-S</th>
<th>nonDiab: AKTO, I-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Brain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM.
Figure 4.4.1

Fractional Anisotropy

FA Value

* P<0.016

Diab: AKTO, I-S
Diab: AKTO, I-H
nonDiab: AKTO, I-S
nonDiab: AKTO, I-H

Internal Capsule

Corpus Callosum

Total Tract Fibers
4.4.II Cognitive Behavioural Data and MRI Results for Diabetic AKTO Transgenic Mice Receiving I-S and Diabetic AKTO Transgenic Mice Receiving I-I.

Over seven months, a group of diabetic AKTO mice (n=11-12) were provided with daily intranasal delivery of I-I, to study the I-I treatment effects on these mice. Diabetic AKTO mice receiving I-I performed better than mice receiving I-S, over the final weeks of the study in the water maze test. However, the performances of the diabetic AKTO mice receiving I-I in the other cognitive behavioural tasks were not significantly different from the diabetic AKTO mice treated with I-S [4.4.II. B-D].

Volumetric measurements of the entire brain showed no significant difference between diabetic AKTO mice treated with I-I and diabetic AKTO mice treated with I-S [4.4.II. H].

Neither T2 values nor MTR of different brain regions significantly changed with I-I treatments [4.4.II. G&H]. I-I treatment did not affect the FA values, and fiber tract numbers [4.4.I. I&J] of IC and CC in diabetic AKTO brains. The results show that I-I treatment did not have further significant beneficial effects on Akt overexpressing diabetic brain.

Figure 4.4.II: Cognitive Behavioural Data and MRI Results for Diabetic AKTO Transgenic Mice Receiving I-S and Diabetic AKTO Transgenic mice receiving I-I.

The graphs demonstrate performance of diabetic AKTO mice treated with I-S (Diab: AKTO, I-S), and diabetic AKTO mice treated with I-I (Diab: AKTO, I-I), in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests. For A1-D1 twenty six weeks were categorized into five-week periods of time. The fifth period is considered weeks 21 through 26 (21-26). AUC measurements demonstrated no significant differences between the groups for all four
cognitive behavioural tests [A-D, p<0.016]. The charts show volumetric measurements of the entire brain, T2 map values, and MTR [H] for Diab:AKTO, I-I and Diab:AKTO,I-S.
Figure 4.4.II

Hole Board Latency

- Diab: AKTO, I-S n= 9-12
- Diab: AKTO, I-I n= 11-12

* p < 0.016

Time (s)

Weeks

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Hole Board Latency

- Diab: AKTO, I-S n= 9-12
- Diab: AKTO, I-I n= 11-12

Time (s)

Week 1 to 5 Week 6 to 10 Week 11 to 15 Week 16 to 20 Week 21 to 26

Time
Figure 4.4.II

Object Recognition Time (Novel-Known)

- Diab: AKTO, I-S  n= 9-12
- Diab: AKTO, I-I  n= 11-12

* p< 0.016

Time (s)

Weeks

Object Recognition Time (Novel-Known)

- Diab: AKTO, I-S  n= 9-12
- Diab: AKTO, I-I  n= 11-12

* p< 0.016

Time (s)

Time

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.4.II

Rotarod Latency

- Diab: AKTO, I-S n= 9-12
- Diab: AKTO, I-I n= 11-12

MR T2 Values

- Diab: AKTO I-I n=4
- Diab: AKTO I-S n=4

T2 Value (ms)

- Whole Brain
- Caudate/Putamen
- M1
- Anterior Commissure
- Corpus Callosum
- Internal Capsule
- CA1
- CA2
- CA3
- V1
- Posterior Commissure
- Pons
- Cerebellum
4.4.III Cognitive Behavioural Data and MRI Results for Non-Diabetic AKTO Transgenic Mice Receiving I-S and Non-Diabetic AKTO Transgenic mice receiving I-I.

To check the effect of I-I treatment in non-diabetic AKTO mice, we compared the results of non-diabetic AKTO mice receiving I-I with their control non-diabetic littermates. The results of cognitive behavioural tests for non-diabetic AKTO mice receiving I-I or I-S were similar in all four cognitive tests over the 7 months of the study. No significant difference was detected between AUC measurements in all four cognitive tests. (ANOVA, P < 0.0083).

Whole brain volume, MR T2 values [4.4.III.F] and MTR [4.4.III.G] in non-diabetic mice treated with I-I or I-S were comparable in different areas of the brain.

No statically significant difference was detected between the two groups in terms of their FA values [4.4.I. I] and fiber tract numbers [4.4.I. J].

Figure 4.4.III: Cognitive Behavioural Data and MRI Results for Non-Diabetic AKTO Transgenic Mice Receiving I-S and Non-Diabetic AKTO Transgenic mice receiving I-I.

The graphs demonstrate performance of non-diabetic AKTO mice treated with I-S (non-Diab: AKTO, I-S), and non-diabetic AKTO mice treated with I-I (nonDiab: AKTO, I-I), in watermaze [A], radial arm [B], holeboard [C], object recognition [D] and Rotarod [E] tests. Categorization into five-week periods [A1-D1] and AUC measures were also similar between the groups [A-D, p<0.0083]. The charts show volumetric measurements of the entire brain, T2 map values, and MTR [H] for nonDiab: AKTO, I-I and nonDiab: AKTO, I-S.
Figure 4.4.III

WaterMaze Latency

* p < 0.016

- nonDiab:AKTO, I-S n= 9-10
- nonDiab:AKTO, I-I n= 9-10

Weeks

Time (s)

WaterMaze Latency

* p < 0.016

- nonDiab:AKTO, I-S n= 9-10
- nonDiab:AKTO, I-I n= 9-10

Week 1 to 5  Week 6 to 10  Week 11 to 15  Week 16 to 20  Week 21 to 26
Figure 4.4.III

Radial Arm Latency

* p<0.016

- nonDiab:AKTO, I-l  n= 9-10
- nonDiab:AKTO, I-S  n= 9-10
Figure 4.4.III

Object Recognition Time (Novel-Known)

* $p < 0.016$

- nonDiab: AKTO, I-S
  - $n = 9-10$

- nonDiab: AKTO, I-I
  - $n = 9-10$

Time (s)

Weeks

Object Recognition Time (Novel-Known)

* $p < 0.016$

- nonDiab: AKTO, I-S
  - $n = 9-10$

- nonDiab: AKTO, I-I
  - $n = 9-10$

Time (s)

Week 1 to 5, Week 6 to 10, Week 11 to 15, Week 16 to 20, Week 21 to 26
Figure 4.4.III

MR MT Values
- nonDiab:AKTO, I-I  n=3
- nonDiab:AKTO, I-S  n=4

MR Volumes
- nonDiab:AKTO, I-I  n=3
- nonDiab:AKTO, I-S  n=4
Chapter Five: DISCUSSION

5.1 General Remarks and Major Findings

The present study examined the cognitive component of DLE and the associated brain structural abnormalities in a murine model of type 1 DM along with the role of PI3K-Akt signalling pathway as a possible mechanism to target the prevention of DM-mediated impairment. Previous studies have described various cerebral abnormalities such as cognitive deficits, brain atrophy and white matter abnormalities in both murine models of diabetes and in human DM1 and DM2 [43, 101, 105, 251-253]. It is known that these abnormalities are correlated with deficiency of insulin, its receptor and associated signalling machinery in both rat and mouse DM brains [9, 15]. The present findings are consistent with previous results from our lab which showed the beneficial effects of intranasal insulin (I-I) on prevention of DM-mediated cerebral changes, without causing prominent systemic effects or modification of glycemia levels [9].

Our laboratory's previous study demonstrated that I-I partially preserved the PI3K-Akt pathway, which is responsible for most of the metabolic actions of the insulin signalling machinery [9, 22]. Yet, to date, no precise study on the role of PI3K-Akt pathway in the pathogenesis of DLE has been performed. To examine if diabetic brain changes can be attributed to dysfunctional PI3K-Akt signalling, we studied the pharmacological inhibition of PI3K-Akt in both diabetic and non-diabetic mice. The sequential monitoring of cognitive changes along with endpoint MRI studies showed that DM mice receiving PI3K and Akt inhibitors lost the beneficial effects of receiving I-I, and that non-DM mice subjected to PI3K and Akt inhibition demonstrated cognitive and structural changes similar to the DM mice. This novel finding elucidates the important role of PI3K-Akt Pathway in the pathogenesis of the diabetic brain and
suggests insulin signalling loss through PI3K-Akt pathway as a major role player in the pathogenesis of DLE. In addition, we examined effects in diabetic and non-diabetic Akt overexpressing transgenic mice to determine if promotion of downstream insulin signalling prevents cognitive and structural changes in the diabetic brain. Interestingly, Akt overexpression in diabetic mice prevented significant adverse changes in their performance in the radial arm, holeboard and water maze tests in comparison to non-diabetic mice, as well as limiting the dysfunction of mice in their object recognition until a later stage of DM. *Taken together, these results suggest a crucial role for the PI3K-Akt signalling in the development of diabetic brain impairment due to type 1 DM.*

5.2 Methods and Limitations to Be Considered

Prior to summarizing our results and conclusions, reviewing the strengths and limitations of the applied methods, as well as the approaches taken to control for confounders, will help us to interpret our final results more accurately and provide information on how future studies can be improved.

5.2.1 Cognitive Test Battery

The battery of behavioral studies used in present study includes the Morris water maze, radial arm test, holeboard test, and object recognition task. Such a battery of tests is often required for added validity. The purpose of using a battery of four cognitive tests over a long-term period of time was to assess general cognition through the course of DM. As well, it is important to also use tasks that do not require optimal motor function, as mice with long term DM do develop diabetic peripheral neuropathy contributing to motor and coordination deficits which can
confound test results [19]. Utilizing multiple cognitive paradigms from the time of disease onset through disease progression is an inclusive approach to study changes in cognitive abilities associated with the disease progression over a long period of time. Our current results are analogous to previous findings which showed poor cognitive performance in diabetic mice in comparison to non-diabetic ones.

As the duration of DM lengthens, the rate and severity of various complications of DM increases [3]; this can also impact upon the sensory-motor function of the diabetic mice and their interaction with the environmental context. It is clear that both active and inactive interaction with the environment affects cognitive processing [254]. Therefore, diabetic complications can potentially affect cognitive testing. Considering these limitations, we have assessed visuospatial and procedural memory in mice using a battery of cognitive tests.

These cognitive tests were selected for several reasons. The Morris water maze was chosen since it is a well-recognized test for learning and memory and it is the test principally used to examine cognition in DM rodent models [36, 38, 72, 244, 245]. The holeboard and the radial arm tests were included since they are spatial tasks traditionally used in rodents and similarly assess learning and memory [242, 243, 247, 255]. The holeboard test, the radial arm test, and the Morris water maze primarily evaluate spatial information processing and long-term memory [247, 255]. To learn where the food reward or hidden platform is located, both spatial strategy and procedural understanding are necessary. The spatial information processing is used in order to form multiple associations between cues in order to make a cognitive map of the environment and is mostly dependent upon the hippocampus [243]. The amygdala is involved in learning about stimuli that correlate with biologically significant stimuli (e.g. escape platform or food reward) [256]. The dorsal striatal system, on the other hand, is involved in making associations,
linking a learned response to a perceived stimulus. The repetition of a learned sequence, such as a series of turns from an initial position, which is known as the stimulus–response strategy, is dependent on the dorsal striatum function [257]. Although these three systems are functionally and anatomically dissociable there is evidence that they can process information simultaneously and in parallel. Once the correct location is encoded, the information has to be transferred into long-term memory for prolonged storage and subsequent retrieval. This mainly involves the frontal cortex [247]. In general, control diabetic mice initially learned water maze, holeboard and radial arm tasks as equally well as their non-diabetic peers; but showed waning performances on each of the behavioural tasks after 11–15 weeks of DM, with further impairment in performances continuing throughout the rest of the 26 weeks of testing.

The object recognition task was incorporated to examine novelty preference and short-term memory. This task is less dependent upon need for mobility, making the presence of diabetic peripheral neuropathy less of a confounder than with the other three cognitive tests [247]. It does not need exposure to aversive stimuli and food or water restriction and has been replicated in many laboratories using a variety of apparatus designs for mice and rats. To discriminate between the similar object from the novel object, mice need to learn the object features; this requires the intact function of visual cortex, the hippocampus, and the parahippocampus [246]. For novelty preference, short-term memory is necessary and primarily involves the perirhinal cortex, used for visual perception and visual memory [258]. The object recognition results suggest that short term memory formation is also disrupted after 11-15 weeks of DM.

Most of the previous studies assessing diabetic mice either examined the cognitive function using a limited number of tests over a short period of time, or tested memory acquired through fear conditioning, which due to its huge emotional component is known as an implicit memory
involving mostly the amygdala. In contrast, our test battery was administered over a very long duration of DM, simulating the human population who develops cognitive deficits at a more elderly age. The tests used in this study engaged the function of the main brain structures involved in memory, including the cortical function, sensory and motor regions and their association areas; but collectively the battery chiefly assessed hippocampal function [247]. The hippocampus, which is an important structure for learning and memory, has high levels of IR expression [72, 73, 172, 259] and has been shown to exhibit detrimental changes in DM [38, 75, 79, 107, 108].

In general, it is important to understand that the exact types of learning and memory being measured with these tests, the precise cognitive processes which are used, and the associated anatomical structures and neural pathways cannot be definitively determined. Thus localizing the cerebral deficits in diabetic mice is difficult based on the results of cognitive tests alone. Therefore, the cognitive test battery was only used as an assessment of general alterations in cognition.

5.2.1.1 Confounding Factors for Behavioural Testing

As it was mentioned before, DM is a systemic disease and the multiple diabetic complications and sickness phenotype can have potential effects on each of these cognitive tests. Although previous research has indicated that sickness behaviours and diabetic complications (such as neuropathy or retinopathy) do not impact cognitive performance in cognitive behavioural tests [34, 43], this is not clear in diabetic models where multiple complications due to DM may occur.

By designing and comparing diabetic groups receiving different treatments, we tried to control the confounding effects of factors not related to the intervention. The use of different control diabetic groups in our study design helps to minimize the confounding factors related to
DM, yet, we are aware of our study's potential limitations due to various diabetic complications (reintopathy, neuropathy, and overall illness) which were seen in the diabetic groups receiving dissimilar treatments.

To limit the impact of motor complications of DM on the cognitive test results, mice were monitored for their motor functions monthly using the Rotarod test. The results showed that there is no significant difference between diabetic cohorts for their motor function, until near the end of study and weeks after cognitive dysfunction detected by behavioural tests. Likewise, there were no significant differences between non-diabetic cohorts for Rotarod testing. Previous studies also suggested that diabetic mice usually develop motor comorbidities at the later time points of DM [9, 19, 46].

In terms of sensory dysfunction, however, previous studies from our laboratory have shown that CD1 diabetic mice developed sensory behavioural changes earlier than has been reported in other rodent models of DM, and that I-I delivery significantly reduced neuropathic pain and later sensory loss in diabetic mice [19]. Thus, tactile allodynia could limit diabetic mice movement during the cognitive testing and affect the exploratory behaviour of mice. Another sense that may be affected by DM is olfaction. Researches in diabetic patients showed that DM can cause decreased olfactory sensory abilities [260, 261]. Moreover, links have been found between olfactory dysfunction and neurodegenerative disorders, such as AD [262]. Therefore, olfactory deficits are possibly another diabetic complication affecting performance on the cognitive tests. Although it has not been determined if olfaction is affected in DM mouse models, olfaction is a central sense in rodents and constitutes a significant portion of the rodent brain [263]. To prevent olfactory cues from guiding cognitive behaviour, a neutral flavour, relatively odorless food reward (Cheerio) was used and the testing environment was cleaned between mice. The
holeboard test, the radial arm test, and swimming in the Morris water maze especially, require sensorimotor skills. The object recognition task was thus added as it is less based on sensorimotor abilities [247], yet certainly requires visual skills to discriminate objects and still can be affected by DM neuropathic pain. The holeboard test, the radial arm test, and the Morris water maze also rely on vision for spatial learning.

Other factors that may play a role contributing to discrepancies between cognitive behavioural results may be the handling of mice by more than one experimenter. Two undergraduate students aided in portions of behavioural testing. It was tried to set a specific test for each observer, to control the impact of different handlers upon the results. Additionally, testing conditions for the most part were kept consistent throughout testing.

We utilized the cognitive test battery to increase inclusiveness and provide an overarching assessment of cognition, but use of multiple tests such as this may contribute to testing fatigue. The intensive testing throughout the mouse’s lifetime, may have led to stress impacting on the cognitive test results. To reduce these limitations, the uniformity of testing conditions was maintained throughout the testing regimen and between the groups as far as possible; however, some unclear changes in the testing environment and psychological state of the mice might occur during the long experimental timeline.

Baseline data was recorded prior to experimental group induction and during training periods. The experimental groups were randomly assigned at the beginning and there were no baseline differences in cognitive testing between any of the mouse cohorts identified. Our study was a blinded experiment -observers were all unaware of the experimental groups of the mice and their treatments. We used this study design to control other confounding factors and to avoid different types of bias.
5.2.2 MRI studies

Although the usefulness of the applied MRI techniques cannot be denied, they also have some limitations. For example, we used a region of interest (ROI) approach to analyze the MRI data. The ROI are usually manually traced and therefore rely on tester’s anatomical knowledge, which makes it heavily operator dependent [264, 265]. In this regard, the validity of DTI results is even harder to address, as the previous studies on mice are limited and there is no systematic accepted gold standard. Previous DTI studies on human brain, however, showed that it is possible to reconstruct the major white matter tracts, comparable to the classical post-mortem descriptions of the standard anatomical reference works. Further, the ROI approach, which was used in this study, may show a degree of intra- and inter-subject variation of the FA values, even within a highly homogenous tract[264], like IC in our study. Although limitations exist for performance of ROI measures, we reduced variability and bias by having one well trained investigator, with self-taught criteria followed for regional selection with blinding of treatment groups, performed the analysis.

There are also some limitations to the MTR measures. ROI determinations of the MTR were performed in several clinical and animal studies. From these studies, we learned that white matter lesions can have a range of lower abnormal MTR values that could represent the spectrum of pathological changes. However, it is shown that different factors (e.g. coil hardware) can influence the MTR values which can hinder its application in multicenter studies [266-268]. Moreover, calculation of MTR depends on registration of unsaturated and saturated images. A small shift due to mouse motion may be enough to affect the calculated MTR value, especially with small lesions or at tissue boundaries. The images which showed significant motion of mice
as can occur with irregular respiration, despite isoflurane anesthesia, were excluded from analysis.

Future improvements in MRI techniques, using tract-specific measurements in DTI studies [264] and the combination of DTI tractography with other MRI techniques will help in resolving these problems.

Recent studies showed that there are changes in white matter microstructure through the life span which are related to development or deterioration of specific cognitive skills [269]. DTI studies on human subjects, for example, showed that FA increases sharply during early brain development, continues to increase more gradually through childhood and adolescence, stabilizes during middle adulthood, and then declines in older adulthood [269]. In order to consider the impact of aging, we assessed all mice at the same age, including non-diabetic control mice, at 7–8 months of age.

5.2.3 Animal models

Although STZ induced diabetic mouse models are often used to explore cognition, DM, and the diabetic brain, it is important to note that the details of behaviours, cognitive tests, neural pathways, brain anatomy, disease pathophysiology, and drug effects differ from rodent to human. Therefore, these models cannot precisely imitate the disease condition as it occurs in humans and caution is needed when interpreting results from mice for human use.

Akt over-expressed mice were bred in animal research facilities at the University of Calgary; since the exact number of male mice needed for each group was not provided according to our timeline, we enrolled both male and female transgenic mice in this part of study. Although mouse groups contained approximately even ratios of males to females, the known variations
between male and female phenotypes and functions in cognitive behavioural tests [270] may cause higher variation within the experimental groups.

Moreover, it must be noted that the mouse strain utilized as an Akt overexpressed model is of SJL/SWR background while the mice that were used in other cohorts were of CD1 background. Since the genetic background influences mice phenotypes [271], our final analysis and comparison of results between the mouse cohorts were limited. Thus, we only compared Akt overexpressed mice results within their groups.

Mouse mortality was greater than expected over the study duration for the first cohort of mice, although mortality only seemed to occur in conjunction with presence with DM and not interventions. Thus, supplementary mice were added as needed.

5.2.4 Drug Treatment

We started our study with the lowest dosage shown to be effective in blocking the PI3K-Akt pathway, according to our preliminary data. The inhibitory effects of these drugs in different areas of the brain and the level of inhibition could vary between the mice. Further molecular studies are needed to confirm the inhibitory effects of these drugs for all tested mice.

As discussed earlier, insulin can also exert its effects through the MAPK-ERK pathway, which also can be blocked by Wortmannin [272]. However, according to our preliminary data, the downstream regulators of this pathway do not develop chronic changes in the DM brain. Still, the possibility exists that insulin’s effects may be facilitated through other unknown downstream molecules.
5.2.5 Statistical Analysis

The MANOVA was decided upon since it is robust to detect significant differences is differences between the means of three or more independent (unrelated) groups. For ANOVA to be valid the normality and homogeneity assumptions have to be met. However, the assumption of normality and the homogeneity of variances were not always met at each time point in our cognitive behavioural tests. The random noise which could be seen in weekly behavioural tests between and within the groups was previously reported in other behavioural studies and was predictable. Additional AUC measurements and analyzing the results using the average of each 5 consecutive weeks helped address these violations. The variation of MRI results within the groups was minimal which lead to more consistent results and powerful analysis based on ANOVA.

All cognitive data was based upon a minimum of six mice in each cohort group at all time-points. However, the minimum number of mice tested for MRI studies were mostly lower than calculated N number, leading to high probability of type 2 error.

A missing value analysis was performed for missing data to account for mice that died before the study endpoint. These imputed values could bias the data as it is not true data, but based on existing data patterns, and a survival effect may have occurred in cases of high mortality.

Despite all these considered limitations, our study produced several robust findings which added significant information to the growing body of knowledge about cognitive dysfunction in DLE. In the next sections, I will review these findings and discuss their significance.
5.3 Diabetes and White Matter Abnormalities

“Nothing defines the function of a neuron better than its connections” (Mesulem) [273] White matter fills nearly half of the human brain and is of importance for mastering and executing mental and physical activities, affecting learning tasks, as well as mental and social skills. Despite white matter’s extent, it often receives less attention than its associated grey matter region. The perfect conduction of physiological signals across a brain network will depend on the condition of the white matter pathways that link network nodes [269]. Recent studies using new magnetic resonance imaging techniques provide exciting ideas that even normal individual differences in white matter structure have consequences for behavioural variation [269, 274].

The relationship between WMA, mostly identified using MRI, upon the brains of adult and elderly subjects, and cognitive function has been the focus of several studies since the first reports of these changes [34, 93, 121, 275, 276]. When WMA develop, intraneural communications become disrupted leading to cognitive deficits, and problems with mental and executive function. In the human brain, WMA are commonly identified using MRI. Recently, with newly developed MRI techniques in combination with the conventional ones, researchers are able to detect abnormalities in white matter that are not visible with conventional magnetic resonance imaging alone. The latest development in MRI brain-scanning technology, as a non-invasive method for mapping tissue macrostructure and microstructure, has led to a revival in study brain connections in vivo. Our laboratory’s previous studies showed that development of brain atrophy and WMA, identified with both MRI T2 and magnetization transfer (MT) imaging as well as with structural examination of myelination, occurs in diabetic mice [9].

In this study, in addition to acquisition of conventional T2 images, two other sets of MR scans were obtained using DTI and MT based imaging for each mouse. DTI and MTR images allow
quantitative measurements of microstructural integrity of the regions of interest (ROI) with a better anatomical localization [266, 277]. In the present study, experimental DM was shown to be associated with regional abnormalities, demonstrated by higher T2 signal as well as lower MTR in different areas of the brain in comparison to non-diabetic mice. The increase in T2 values evident in the diabetic brain suggesting an increase in water content in the brain and disruption of white matter structures, that has previously been found to be associated with a loss of myelin content [11]. The delivery of I-I in diabetic mice, however, led to reductions in T2 values. Furthermore, as we will discuss later, concurrent intranasal administration of both I-I and PI3K-Akt inhibitors led to a significant increase in T2 values in comparison to administration of I-I alone. These results are consistent with our cognitive behavioural findings, showing the crucial role of PI3k and Akt in the pathogenesis of the disease.

Magnetization transfer imaging is one of the imaging techniques, usually used in the study of white matter changes, such as in multiple sclerosis (MS) [278]. Since, MT images provide both morphological and pathological information with a higher specificity than conventional MRI, it is considered as a very useful method to monitor the myelin destruction [267]. Also, it is argued that MT imaging enables us to measure the ‘invisible’ lesion burden in the so called normal appearing white matter (NAMW), i.e. the brain tissue which does not show abnormalities on conventional MRI [279]. In the present study, the decreased MTR values observed under the control diabetic conditions confirm the presence of disruption of tissue integrity; and, those diabetic mice exposed to I-I intervention were protected against loss of MT values. Thus as for the T2 results, the MT data support the occurrence of disruption in overall tissue integrity in the chronic diabetic condition related to the insulin signalling in the brain. The MT values were decreased in diabetic and non-diabetic mice receiving Wortmannin (PI3K inhibitor) and API-2
(Akt inhibitor), confirming the importance of the insulin-PI3K-Akt pathway in the physiopathology of changes seen in the diabetic brain. The MRI results were generally consistent and supportive of our behavioural findings.

DTI images provide unique insights into the fine architecture of neuronal tissues and to changes associated with various pathological states. For example fractional anisotropy (FA), an index that measures the directionality of diffusion on a scale from zero (when diffusion is totally random) to one (when water molecules are able to diffuse along one direction only), has been shown to be extremely sensitive to the degeneration of corticospinal tracts in stroke patients [117, 280]. Also, DTI tractography reconstructs axonal trajectories virtually by measuring the diffusivity of water along different directions on a voxel-by-voxel basis. Our results showed that diabetic mice had lower FA and tract quantification numbers in CC and IC; I-I treatment improved FA and tract numbers in diabetic mice, while PI3K-Akt inhibition removed I-I effect, and led to lower FA values and tract numbers, indicating the importance of PI3K-Akt signalling for the I-I effect.

Not only is DM associated with WMAs, but it is also associated with both cortical and subcortical atrophy. Diabetic brain atrophy can be identified in humans by neuroimaging techniques including CT or MRI. By these means, cerebral atrophy, which is demonstrated by widened sulci and enlarged ventricles, is shown to be more pronounced in individuals with DM than in age-matched controls [106, 281]. In animal models of DM such as the STZ-induced diabetic mouse, cerebral atrophy has also been shown to be evident across several brain regions [9, 13]. Similarly, our present results showed brain atrophy in diabetic mice, along with WMAs and loss of FA and total tract fibers of CC. The brain atrophy could be prevented by I-I treatment and PI3K-Akt inhibition was able to reduce the I-I effect. Moreover, diabetic transgenic mice
overexpressing cerebral Akt did not show significant loss of whole brain volume due to DM state. The etiology of brain atrophy is unclear and not well understood. While some researchers propose that it may relate to neuronal loss, our laboratory’s previous findings showed brain atrophy in mouse and rat models of DM despite absence of neuronal loss in these murine models [11, 31, 282]. Later studies in rat have identified loss of neurons within the hippocampus [248], occurring months after the presence of synaptic loss. Other previously described mechanisms in the development of WMAs and atrophy in the diabetic brain include vascular border zone hypoperfusion, subclinical ischemia [283], axonal degeneration and abnormalities in the blood-brain barrier and cerebrospinal fluid dynamics [121]. Lastly, detected cerebral atrophy may also be related to loss of brain synapses, which has also been detected in both Type 1 and Type 2 diabetic models [9, 15] and patients with AD [284, 285].

To conclude, our current results confirmed the existence of WMA in STZ-induced diabetic mouse model, demonstrated the beneficial effects of I-I in prevention of WMA, and revealed the important role of PI3K-Akt signalling pathway in the pathogenesis of WMA in diabetic animals.

5.4 Diabetes Brain and Intranasal Insulin

The present study showed that diabetic mice had measurable changes in cognitive behavioural testing, as well as both cerebral atrophy and white matter deficiencies identified with magnetic resonance imaging. Intranasal insulin delivery was associated with partial protection against such pathological changes and significantly improved cognitive outcomes in mice. Our study of intranasal insulin in diabetic mice confirmed that noted cognitive decline, brain atrophy and white matter changes can be at least partially related to a relative insulin signalling deficiency.
As it was noted earlier in the background section, the insulin signalling pathways are activated by binding of insulin to insulin receptor, which belongs to a family of receptor tyrosine kinases (RTK)s that contains the Insulin-like Growth Factor (IGF) receptor. The IGF receptors are both structurally and functionally related to insulin receptors [142, 172, 286]. Although the IGF receptors are predominantly activated by IGF-I and IGF-II, they can also be activated by insulin at a lower affinity; the IGF/insulin receptor cross-reactivity is widely accepted [72, 142, 287]. The activation of the IGF receptor is followed by recruitment of insulin receptor substrate (IRS) adapter proteins and activation of the common downstream intracellular signalling pathways shared by the IGF and insulin. Therefore, to discuss I-I effects in our study, the uncertain activation of the IGF receptors due to I-I delivery should be also considered.

Intranasal insulin delivery in humans has also been of benefit for cognition in AD human patients [150, 221], but investigations in patients with DM without AD remains to be performed; these patients are difficult to define as presence of AD neuropathology may also occur in patients with DM suffering from cognitive impairment [56, 78, 81, 91, 288, 289]. Although delivery of intranasal insulin to human subjects with DM with the goal of preventing neurodegenerative changes has not yet been attempted, evidence to date and our current results suggests that the method would be safe, without adverse nasal effects and with minimal chance of hypoglycaemia [153].

In summary, our current results confirmed the effectiveness and safety of intranasal insulin treatment and demonstrated its beneficial effects on both cognition and white matter changes in a mouse model of diabetes.
5.5 Diabetes Brain and PI3K Signalling

Previous studies have shown the down-regulation of PI3K-Akt signalling pathway in diabetic mouse brain that could be reversed by Insulin replacement [9]. The results of the present study showed that inhibition of PI3K activity within the diabetic brain can nullify beneficiary effects of Insulin. Likewise, Non-diabetic mice receiving PI3K inhibitor developed similar cognitive and brain structural changes as seen in diabetic mice without intervention. This finding, therefore, highlights the central role of PI3K signalling deficiency as the causative factor in the development of the diabetic brain. How exactly insulin mediated PI3k signalling impairment lead to cognitive dysfunction is not known. Insulin signalling involves activation of PI3K, which, in turn, activates Akt permitting Akt phosphorylation of isoforms of GSK-3 [9, 290], thereby inhibiting GSK-3 activity. In this way, insulin regulates tau protein phosphorylation [291]. Insulin-mediated GSK-3 inhibition may therefore have beneficial effects in preventing tau protein hyperphosphorylation. Phosphorylated tau protein level correlates with cognitive decline in subjects with mild cognitive impairment [292], predicts the rate of cognitive decline in different stages of AD [293, 294], and correlates significantly at baseline with the rate of hippocampal atrophy in mild to moderate AD, acting as an indicator of disease progression [295]. GSK-3 inhibition also leads to reduction of Aβ protein accumulation [296].

PI3K's role in LTP, which is a cellular model of memory formation and consolidation, should also be considered. Accumulating evidence suggest that PI3K activation is critical for trafficking of AMPAR (a-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptor), which is an ion channel involved in the formation of synaptic plasticity and LTP [297]. Synaptic targeting of AMPAR could also be regulated by PI3K activation during synaptic plasticity and cause long-lasting alternations in the efficacy of synaptic transmission. It is worth noting that a similar role
in synaptic plasticity has been reflected for insulin showing that insulin modulates components of synaptic plasticity including recruitment of AMPA receptors [298, 299], and N-methyl-D-aspartate receptor (NMDAR) trafficking and function [300]. It was also shown that IRS2 deficiency impairs tetanus-induced LTP in the CA1 region [202]. These findings together suggest a strong link between insulin mediated PI3K signalling and synaptic plasticity in brain. The question of how PI3K is involved in diverse forms of synaptic plasticity and related cognitive and emotional functions, such as spatial memory, inhibitory avoidance conditioning, and contextual fear memory, however, remains unanswered.

**Lastly, our results point out the important role of PI3K signalling in I-I effect within the brain.** However, it remains possible that insulin’s effects may also be mediated in part through other pathways that we have not considered or which have not yet been discovered.

### 5.6 Diabetes Brain and Akt Signalling

Although a simple scheme of divergent pathways looks sufficient to explain insulin signalling, a sheer number of gene and protein isoforms is involved in the activation of Akt and the generation of metabolic effects. Among the hundreds of molecules that have been shown to be involved in the insulin signalling pathway, the IR/IRS, the PI3K and Akt were determined as the three best-defined critical nodes [173]. That is because each of these network components is essential for insulin action, consisting of several isoforms with unique functions featuring extensive regulation mediating crosstalk with other signalling cascades. Our study tested the effects of both inhibition and overexpression of Akt signal on development of cognitive impairment and WMAs in diabetic brain. As we have mentioned before, there are three mammalian isoforms of this enzyme, Akt1, Akt2, and Akt3, which share high homology and
similar structure. API-2, the Akt inhibitor which was used in our study, is capable of inhibiting all three isoforms of Akt [209, 210]. However, in the transgenic mice used in the third cohort of study, the PLP promoter drives expression of constitutively active Akt1 [236]. While, Akt2 is mostly known to have role in the regulation of glucose metabolism [179], the major impact of Akt1 overexpression in oligodendrocytes was enhanced myelination [236]. Future studies are required to clarify distinct and overlapping functions of Akt isoforms in the brain.

5.6.1 Akt Pharmacological Inhibition

Akt inhibition in diabetic mice could partially block I-I effects on diabetic mouse cognition in our study, which suggests Akt as an important role player in development of diabetic brain. Moreover, the blockage of Akt in non-diabetic mice caused significant abnormalities in cognitive function and brain structure, as demonstrated in MRI studies. The observation that Akt inhibition in diabetic brain could not completely suppress I-I beneficiary effects on cognition and brain structural changes, may suggest an imperfect inhibition of Akt by intranasal API-2, despite our lab preliminary western blot analysis confirming substantial inhibition. This should be investigated further, using the brain tissue samples collected from our treated mice at euthanasia. It is also possible that another signal in the insulin-PI3K pathway sharing a key role with Akt in mediating the studied effects of insulin.

5.6.2 Akt Overexpression

Mild cognitive dysfunction, that only occurred at the final stages of the object recognition tests, was detected in diabetic Akt overexpressing mice. These mice were also protected from significant brain atrophy due to DM, and detected white matter changes were restricted to smaller numbers of brain regions as compared to wild type diabetic mice tested in the first and second cohorts of the study.
These observations not only demonstrate the role of Akt signalling impairment in development of diabetic brain but, as Akt overexpression is localized to oligodendrocytes in these transgenic mice, also suggest a critical role for oligodendrocytes in the pathophysiology of the disease. The overexpression of Akt in diabetic mouse brain regions and oligodendrocytes was shown before [236] and should be confirmed with our mouse brain samples in future.

5.7 Intranasal Drug Delivery

The intranasal technique can permit selective targeting of the brain for inhibition of PI3K, while avoiding systemic effects. It is a safe, non-invasive, and rapid way to administer drugs to the CNS. Drugs enter olfactory and trigeminal pathways that bypass the blood-brain barrier to have direct effects in the brain. Previous studies have successfully used the intranasal technique to treat diseases [9, 19, 225, 229]. However, since it is a novel technique, there are still many unanswered questions about its use and effects. It is important to note again that how much of the drug is absorbed, the uptake mechanism, and the localization within the brain for its penetration is not specifically known and may differ between species. Even if the dosage was optimal, different drug levels may have actually penetrated to different brain regions. Therefore, the inhibition of PI3k and Akt pathways in different areas of the brain should be confirmed later, using molecular techniques. Although intranasal therapy targets the brain, the drug may still enter and affect the rest of the body. The PI3K pathway is crucial for normal function throughout the body and therefore its inhibition can be harmful, though this seems unlikely as neither the mortality rates nor the body weight of the mice receiving PI3K and Akt inhibitors were significantly affected by these drugs in our study.
Wortmannin, a fungal metabolite that irreversibly inhibits PI3k by reacting covalently with its catalytic site, was used in the present study. As discussed earlier in the background section, Wortmannin has some off-target activity [272]. As a result, the effects of intranasal Wortmannin might not solely be a consequence of PI3K inhibition. However, the concentration of the drug required to inhibit the other protein kinases is considered to be higher than what was reported for the PI3K inhibition. For example, Davies et al. reported that while PI3K activity was completely inhibited in vitro by 1 µM Wortmannin, a large panel of protein kinases, including MAPK and PKA, were unaffected at this concentration[208]. Since, we used the lowest dosage shown to be effective in blocking the PI3K pathway the other kinases are unlikely to be greatly affected to a large extent. However, further molecular studies on collected tissues may be needed to exclude any other off-target effects of Wortmannin in our study. Nevertheless, our current findings confirm previous reports showing that therapeutics given by the intranasal route are delivered to the CNS and have the potential to target brain directly.

5.8 Cognitive Impairment in Mouse Model of Diabetes

Cognition is a complex process which implicates a collection of mental skills, such as attention, perception, comprehension, learning, remembering, problem solving, reasoning and so forth [254]. It is evident that the cognitive skills and processes, neural pathways, anatomical structures, and various other factors differ between rodents and humans. Despite these limitations, animal models permit research to be performed that are not possible in humans and expand our understanding of learning and memory, and therefore have led to many discoveries and insights related to human cognition [10, 128, 129]. As a result, non-human DM models have made significant contributions to the understanding of the disease and the discovery of
treatments, and rodent DM models are currently the best available non-human model of human type 1 DM, particularly to explore the physiopathology of the disease and the new treatments before human clinical trials [10]. Since understanding of the effects of DM upon cognition are in their infancy, cognitive research in diabetic mouse models provides better understanding of the disease pathophysiology and the possibility to select the most promising treatments thereby ensuring a better translation to human research. Our laboratory's previous findings in the rodent DM model began to explain diabetic brain abnormalities, including the newly emerging role of insulin [9]. Our present results not only demonstrated the pattern of cognitive impairment in diabetic mouse, but also described the role of Insulin-PI3K-Akt pathway in DM associated changes in the brain. Learning processes for the cognitive behavioural tasks appeared to be similar between diabetic and non-diabetic mice over the first several weeks. After 11-15 weeks of DM, diabetic mice demonstrated waning performances on each of the behavioural tasks, with impaired performances continuing throughout the remainder of the 26 weeks of testing. Behavioural experiments demonstrated better maintenance of visuospatial, procedural and object recognition memory functioning in the DM exposed brain receiving I-I as compared to the diabetic mouse brain not receiving. PI3K and Akt inhibition nullify I-I effect in diabetic mice and cause deterioration of cognitive performance in non-diabetic animals.

The present study supports the previous findings of our lab which demonstrated cognitive impairment in mouse model of DM. These findings extend our knowledge of the nature of cognitive dysfunction in DM and provide strong evidence for the role of insulin-PI3K-Akt pathway in the pathogenesis of DM mediated brain disease.


5.9 Summary and Conclusions

Our results suggest that deficiencies in insulin signalling are primarily responsible for DM-mediated abnormalities in the brain, yet are potentially reversible. Intranasal insulin delivery is a potential therapy to ameliorate behavioural and structural changes occurring in brain exposed to DM over time. Our results provide strong evidence for benefits of insulin without impact upon blood glucose levels, indicating that insulin is an important neurotrophic factor in the management of DM mediated brain disease. According to our findings, the beneficial effects of insulin are mediated through PI3K-Akt pathway within the brain. While inhibition of PI3K-Akt pathway impaired insulin function, upregulation of this pathway improved the outcome in the diabetic mouse model. Moreover, cognitive impairment in diabetic mice was a significant correlate of WMA detected by MRI; this suggests the use of MRI findings as a potent histopathological marker during the course of the disease.

5.10 Future Directions

As DM continues to grow in prevalence, DM-mediated brain changes are expected to contribute to accelerating rates of human dementia. We are optimistic that our current study, combined with our planned investigation of the structural and molecular changes that occur as a result of long term PI3K-Akt blockage, will be instrumental in determining mechanisms by which the brain can be protected from the effects of DM. The present study focused on cognition, but, as discussed earlier, DLE encompasses other components such as cerebral signal conduction, neurotransmission and synaptic plasticity, and underlying structural pathology. Future studies are needed to focus on changes that occur at the synaptic level, to clarify the impact of DM on the brain.
There are also many links between neurodegeneration and cognitive impairment in DM. For example, there are known associations between AD and DM. PI3K-Akt signalling pathway is one of the major links between these two diseases; but still there are major mechanisms common in both diseases, such as insulin resistance and insulin degrading enzyme (IDE), which require further study. Future studies could examine therapies to treat both diseases.

The role of alternative pathways with a potential role in DLE, such as AGE-RAGE and Polyol pathways in the DM brain is also being investigated in our laboratory. The role of IDE in the DM brain, as mentioned earlier, as well as the role of insulin-like growth factor-1 (IGF-1), a hormone which is structurally and functionally related to insulin, is still unknown; pending future studies in our laboratory.

In summary, future directions in the field of DLE should aim to further examine cognition in DM, pathways and mechanisms that are changed in the diabetic brain, and the various aspects of DLE and neurodegeneration, with the ultimate goal of developing a therapy to treat cognitive impairment within DM.
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