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THE NEUROPROTECTIVE EFFECTS OF SGLT2 OR NOX1/NOX4 SELECTIVE INHIBITORS ON ALZHEIMER'S-LIKE SYMPTOMS DEVELOPMENT IN DIABETIC MICE

By
LEEN MOHAMAD ALI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
September, 2020

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ACKNOWLEDGEMENTS

To my mentor and advisor, Dr. Assaad Eid, I wish to extend my gratitude and appreciation. Thank you for giving me the opportunity to work on my thesis project under your supervision. I really appreciate everything you have done for us during this year. Despite all the unforeseeable circumstances, you have always been there for us and guided us in our times of need. Thank you for always listening to our concerns and making sure that our needs were met. I really admire your work ethic and appreciate the time I have spent working under your supervision because it has taught me a lot about dedication and perseverance in research. Lastly, thank you for giving me the opportunity to be a part of a lab that was also like a second family to me.

Thank you to all the members of the lab for always being there for me and ensuring a supportive and co-operative work environment. Despite all the hardships that we went through this year, working with you has been a highlight that has made this experience more bearable and memorable.

Lasty, I would like to extend my deepest gratitude and appreciation to Lynn Ala'eddine, Mohamad Noureddine and Dr. Fred. Thank you for teaching me everything I needed to make it this far into this project. Not only have you been there for me through all the technical difficulties, but you have also given me the motivation and emotional support that is much needed in our field of work.

Finally, I would like to thank my family for being there for me throughout this whole journey. None of this would have been possible without your never-ending support and motivation.

AN ABSTRACT OF THE THESIS OF

Leen Mohamad Ali for Master of Science
Major: Neuroscience

Title: The Neuroprotective Effects of SGLT2 or Nox1/Nox4 Selective Inhibitors on Alzheimer's-Like Symptoms Development in Diabetic Mice

Background: Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive loss of memory and cognitive function along with molecular alterations involving the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs). Epidemiological evidence has shown that type II diabetes mellitus (T2DM) is a major risk factor for the development of AD. Among the shared pathological features of both diseases are impaired insulin signaling and oxidative stress. However, the exact mechanism of how peripheral insulin resistance in diabetes may contribute to AD pathology remains largely unknown.

Aim: The aim of the present study is to determine the role of impaired insulin signaling along with reactive oxygen species (ROS) overproduction in the onset of AD-like pathology in an animal model of T2DM. We also investigate the potential ameliorative properties of dapagliflozin (SGLT2 inhibitor) and GKT137833 (dual Nox1/Nox4 inhibitor) on the Alzheimer's-like symptoms developed in T2DM.

Methods: Female C57BL/6J mice were used to conduct this study. T2DM was induced using a combination of high-fat diet and low doses of streptozotocin injections. Treatment groups were administered with either dapagliflozin (2mg/kg) or GKT (40mg/kg) over a period of 9 weeks. Cognitive function of the studied mice was assessed using the novel object recognition test and the spontaneous T-Maze alternation test. To test for sensory dysfunction associated with diabetes, mechanical and heat hyperalgesia were assessed using Hargreave's test and the electronic von Frey. The raised beam walking test was also conducted to detect any motor dysfunction. At the molecular level, western blots were used to assess the expression of NADPH oxidase-4 (Nox4), insulin receptor substrate-1 (IRS-1) and Tau phosphorylated at Ser404 in the hippocampus and prefrontal cortex. RT-PCR was also conducted to study the mRNA levels of Nox1, IRS-1 and IRS-2. Immunohistochemical staining was performed to detect the deposition of amyloid-beta plaques and tauS404 and S396 hyperphosphorylation in the brain.

Results: Compared to the control group, type 2 diabetic mice showed sensory, cognitive and motor dysfunction. Treatment with dapagliflozin and GKT reversed spatial and recognition memory loss, as well as sensory and motor dysfunction in mice. These observations were paralleled by the reestablishment of the insulin signaling activity and the inhibition of NADPH-dependent ROS overproduction.

Conclusion: This study reveals a potential neuroprotective role of SGLT2 inhibition as well as Nox1/Nox4 inhibition and highlights the involvement of insulin resistance and oxidative stress in the progression of Alzheimer's-like symptoms associated with T2DM. These findings put forward a potential novel therapeutic strategy for the treatment of AD.

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ABBREVIATIONS

AD	Alzheimer's Disease
Aβ	Amyloid-Beta
NFT	Neurofibrillary Tangles
T2DM	Type 2 Diabetes Mellitus
ROS	Reactive Oxygen Species
IRS	Insulin Receptor Substrate
NOX	NADPH-oxidase
APP	Amyloid-Precursor Protein
PSEN	Presenilin
APOE4	Apolipoprotein E4
CNS	Central Nervous System
GSK-3	Glycogen Synthase Kinase 3
DM	Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
AGE	Advanced Glycation End Products
RAGE	Receptor for Advanced Glycation End Products
HFD	High-Fat Diet
PI-3K	Phosphatidylinositol-3 Kinase
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PDK-1	Phosphoinositide-dependent Kinase-1
GS	Glycogen Synthase
MAPK	Mitogen-activated Protein Kinase
IGF	Insulin-like Growth Factor
BBB	Blood Brain Barrier
GLUT	Glucose transporter
RNS	Reactive Nitrogen Species
4-HNE	4-hydroxy-2-nonenal
GLP-1	Glucagon-like Peptide 1
SGLT2	Sodium-Glucose cotransporter 2
STZ	Streptozotocin
BGL	Blood Glucose Level
NOR	Novel Object Recognition
IHC	Immunohistochemical
RT-PCR	Real-Time Polymerase Chain Reaction

CHAPTER I

INTRODUCTION

A. Alzheimer's Disease: General Overview

Dementia is a group of symptoms affecting memory, cognition and behavior sufficient to interfere with daily activities. About 46.8 million people worldwide suffer from dementia, and its global health burden is on the rise due to an aging population, where numbers are predicted to increase to 131.5 million by 2050 [1-3]. The most common types of dementia are Alzheimer's disease (AD), vascular dementia, frontotemporal dementia and dementia with Lewy bodies. AD is the leading cause of dementia worldwide, accounting for 50-75% of cases [3]. It is described as a progressive neurodegenerative disorder characterized by cognitive dysfunction, memory loss and behavioral abnormalities gradually resulting in an inability to function independently [4, 5]. Moreover, it is an age-related disorder that is currently the third leading cause of death in the elderly [1].

AD can be categorized into two general types: familial AD or sporadic AD [2]. Familial AD is usually an early-onset disease that occurs between the ages of 30 and 50. It accounts for less than 1% of all AD cases, and it usually occurs due to rare autosomal dominant mutations in any one of three genes: amyloid-precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) [5]. On the other hand, the late-onset disease, also known as sporadic AD, accounts for more than 95% of AD cases [6]. It is a multifactorial disease that is driven by both genetic and environmental factors [2]. Genetic studies have identified multiple risk factors with apolipoprotein E4 (APOE4) allele as a primary genetic risk factor

for late-onset AD [7, 8]. For the most part, sporadic AD and familial AD share many clinical features [6].

1. The Clinical and Molecular Manifestations of AD

The core clinical criteria used to diagnose AD dementia include, but are not limited to: cognitive deficits such as an impairment in learning, inability to remember new information, impaired reasoning, poor judgement, inability to recognize faces or common objects, impaired language functions and changes in personality and behavior. For a patient to be diagnosed with AD, one must have a gradual progression of these symptoms over months to years [9]. AD is also characterized by morphological and physiological changes primarily expressed as the formation of intraneuronal neurofibrillary tangles (NFTs) and the extracellular deposition of beta-amyloid (Aβ) plaques [10]. In addition to that, AD is also strongly associated with neuroinflammation [11], oxidative stress [12] and impaired glucose metabolism [13], all of which ultimately leads to the progressive loss of synapses and the degeneration of neurons [1, 11, 14, 15].

Under physiological conditions, APP is cleaved and continuously cleared from the central nervous system (CNS) to prevent the accumulation of its products [16]. However, in pathological conditions, the expression and processing of APP becomes dysregulated where it is cleaved by β and y-secretases to form Aβ peptides (40-42 amino acids in length). These peptides can ultimately accumulate into oligomers and insoluble Aβ plaques due to an imbalance between their production and clearance, and the deposition of Aβ mainly originates in the frontal and temporal lobes, hippocampii and limbic system [6, 17]. This

dysregulation occurs as a consequence of a mutation in the APP, PSEN1 or PSEN2 genes in the familial form of AD; however, it remains unclear how Aβ accumulation originates in sporadic cases of AD [15]. This led to the development of the amyloid hypothesis where Aβ accumulation in the brain is considered as the primary driving force of neurodegeneration and other features of AD such as the formation of NTFs [17]. However, the deposition of Aβ plaques generally occurs way before the onset of cognitive decline in AD patients [18], and treatments specifically aimed at reducing Aβ formation such as those targeting β and y-secretases have proved ineffective or had undesirable side-effects [1].

NFTs consist of aggregated hyperphosphorylated tau proteins [10]. Tau protein is a microtubule-associated protein that is crucial for maintaining neuronal stability and interconnections; however, in pathological conditions, tau will be hyperphosphorylated through the over-activation of kinases such as glycogen synthase kinase-3 (GSK-3) among others. Hyperphosphorylated tau will then get ubiquitinated, misfold and self-aggregate to form NFTs and dystrophic neurites inside neurons. NFTs then contribute to the degeneration of neurons by disrupting the cytoskeletal framework and synaptic connections [18-20]. Hyperphosphorylation of tau at Ser262, Ser396, Ser404 and Thr231 among others have been shown to contribute to AD pathogenesis [21]. NFTs emerge in the medial temporal lobe and hippocampus and gradually spread into other areas [6], and hyperphosphorylated tau has been identified as a better predictor of disease progression and cognitive performance than Aβ accumulation [19]. This led to the development of the tau hypothesis where tau hyperphosphorylation and NFT formation act as the main trigger for other AD pathologies, but treatment strategies focused on blocking tau phosphorylation or aggregation remain challenging [1, 22].

2. A Novel Hypothesis for the Development of AD

Several hypotheses for the development of AD have been suggested, and the most popular of which are the previously mentioned amyloid hypothesis and tau hypothesis in addition to the vascular hypothesis and the cholinergic hypothesis [1]. However, despite advancements in our understanding of the disease over the years, no cure or definitive preventative strategies have yet been discovered, and the aetiology of the disease remains unclear. Due to its severity and rising prevalence, it is of utmost importance to explore other mechanisms for AD pathogenesis. In recent years, there has been an increasing body of evidence linking diabetes, impaired glucose metabolism and insulin signaling pathways to AD [23-27]. Type 2 diabetes mellitus (T2DM), mainly characterized by a state of insulin resistance [28], has been considered as a risk factor for AD [29]. In addition to that, studies have found that levels of insulin receptors and insulin-receptor mediated activity, such as the activation of kinases that are downstream of insulin signaling, are dysregulated in postmortem AD brains [30-34]. Other studies have shown that the levels of glucose uptake and metabolism are decreased in the brains of AD patients [35], and insulin deficiency and resistance were shown to increase with Braak stages in postmortem AD brains [33]. This led to the development of a new hypothesis that insulin resistance could be one of the driving forces contributing to the pathogenesis of AD. Owing to the fact that peripheral insulin resistance is the main trigger for T2DM [28], the term type 3 diabetes has been suggested for AD that originates due to insulin resistance in the brain [36]. According to this hypothesis, AD is described as a specific form of diabetes that explicitly targets the brain where insulin resistance is also accompanied by deficiencies in insulin levels [36].

B. Diabetes Mellitus - Background

Diabetes mellitus (DM) encompasses a group of heterogenous metabolic disorders characterized by chronic hyperglycemia due to disturbed insulin secretion or signaling or both [37]. The prevalence of diabetes has been increasing at an unsustainable rate where it is estimated that about 463 million people worldwide suffer from this disease, and the global health expenditure directed towards diabetes and its complications was estimated at 727 billion USD in 2017. The number of adults with diabetes is expected to reach 578 million by 2030 [38].

There are two major types of diabetes: type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [39]. T1DM is caused by an autoimmune response where the body's own immune system attacks the beta cells of the pancreas. This ultimately results in very little or no insulin production by the pancreas leading to a state of hyperglycemia. T1DM most commonly occurs at an early age, and it accounts for 5-10% of diabetes cases [40]. The most common type of diabetes is T2DM which accounts for about 90% of the cases [39]. T2DM, also known as non-insulin-dependent diabetes mellitus, is defined by fasting hyperglycemia that is largely due to disturbances in insulin signaling and action along with a compensatory hypersecretion of insulin. Thus, individuals with T2DM, at early stages of the disease, generally do not require treatment with insulin in order to survive, but defects in insulin secretion have also been shown to occur at later stages of the disease [40, 41]. In most cases, insulin receptor tyrosine-kinase activity is diminished mainly within skeletal muscles, liver and adipose tissue [42-44]. Age, obesity and lack of physical activity are the main risk factors for developing T2DM [39, 40].

1. Diabetic Complications

Diabetes is associated with numerous complications. Among the long-term vascular complications of diabetes are diabetic neuropathy, nephropathy, retinopathy and cardiovascular disease [45]. The microvascular complications are due to damage to small blood vessels and include neuropathy, nephropathy and retinopathy. The macrovascular complications are due to damages in the arteries, and these include cardiovascular disease leading to myocardial infarctions and cerebrovascular disease resulting in strokes [41].

Diabetic neuropathy is a neurodegenerative disorder of the peripheral and autonomic nervous system that primarily targets sensory axons and to a lesser extent, motor axons [46]. It is by far the most prevalent of the diabetic complications where it occurs in about half the patients with diabetes [47]. The most common form of diabetic neuropathy is distal symmetric polyneuropathy. It is generally characterized by a loss of sensory function in the hands and lower limbs associated with pain and morbidity [46]. Another major complication of diabetes is diabetic nephropathy or kidney disease. It is clinically characterized by persistent proteinuria with a decline in glomerular filtration rate. The features of nephropathy develop gradually over many years and can be fatal when left untreated. Hyperglycemia that occurs in diabetes is known to affect various types of kidney cells including endothelial cells, smooth muscle cells, podocytes and others, which is why the progression of nephropathy is a highly complex process [48]. Nephropathy is also considered a major risk factor for the progression of macrovascular complications [49].

Diabetic retinopathy is the leading cause of preventable blindness in adults [50], and it occurs in about a third of diabetic patients [51]. It is primarily characterized with alterations in vascular permeability and lesions within the retina. These develop gradually over the years

and can ultimately result in visual impairment at later stages [52]. In addition to that, diabetic patients have an increased risk of developing cardiovascular disease which accounts for more than half of the mortality cases seen in diabetes [53]. It can manifest as premature atherosclerosis leading to myocardial infarction, stroke and impaired cardiac function [41]. In order to reduce the risk of these diabetic complications, it is crucial to achieve optimal glycemic control as early as possible in the course of the disease [54, 55]. Unfortunately, clinical studies have shown that glycemic control on its own does not provide utmost protection from developing diabetic complications. Therefore, the search for novel therapeutic strategies targeting these long-term health complications is crucial to prevent their initiation and progression in diabetic patients.

2. Diabetes-Induced Alzheimer's Disease

Numerous signaling pathways and functions are halted in insulin resistant states [56]. Consequently, diabetes has been associated with other long-term complications including depression [57], sexual dysfunction [58] and dementia [59]. Peripheral insulin resistance is known to induce changes within the central nervous system where it is accompanied by reduced insulin levels and activity in the brain [60]. When T2DM is inadequately controlled, it can increase the risk of developing cognitive impairment [61], and it has been shown that diabetic patients are 1.4-2.0 fold more likely to develop AD than non-diabetic individuals [62].

Studies have shown that as T2DM progresses, it can be associated with the main features of AD pathology. To begin with, a study on diabetic mice shows significant accumulation of

Aβ plaques in the brain and decreased insulin-degrading enzyme activity which normally reduces Aβ build-up by degrading it [63]. It has also been shown that increased levels of advanced glycation end products (AGE) and their receptors (RAGE), which occurs in T2DM, mediate the transport of soluble Aβ across the blood brain barrier, thus contributing to its accumulation in the CNS [64-66]. In a study on cynomolgus monkeys, Aβ plaques were observed in the frontal and temporal lobes of T2DM-affected monkeys about 5 years earlier than in healthy controls [67]. In addition to that, increased phosphorylation of tau protein within the brain is evident in mouse models of T2DM [68].

Tau hyperphosphorylation is also evident in postmortem brain samples of T2DM patients [69], and several studies have shown that T2DM patients display signs of brain atrophy and neurodegeneration where hippocampal volume decreases with longer duration of diabetes [70, 71]. In addition to that, increased plasma insulin levels such as those present in an insulin resistant state were shown to increase Aβ and inflammatory agents in the brain [60]. Thus, systemic insulin resistance, such as that in T2DM, may be associated with an increased risk of developing the main features of AD pathology.

C. Diabetes and AD: Shared Mechanisms

1. Insulin Resistance and Type 2 Diabetes Mellitus

Insulin resistance generally occurs when the cells of the body have a reduced response to insulin which makes it unable to achieve its glucose-lowering function. This requires higher circulating levels of insulin to try to compensate for the hyperglycemia [44, 72, 73]. Under normal conditions, cells respond to insulin when it binds to its

receptor tyrosine kinase on the plasma membranes of target cells [74]. This initiates multiple phosphorylation events that ultimately activate enzymes controlling different aspects of cellular function (Fig. 1). These signaling pathways involve multiple points of regulation that ensure appropriate signal duration and intensity; however, disturbances in these pathways can lead to a state of insulin resistance [75]. Thus, several studies concluded that insulin resistance generally occurs due to both receptor defects such as decreased expression of insulin receptors leading to lower levels of binding, and post-receptor defects which are the disturbances in signal transduction following insulin binding [76, 77]. However, insulin resistance is not simply a switching off of all insulin signaling completely, which is why increasing the levels of insulin can be effective in preserving some insulin action in mild and moderate cases [44].

Insulin resistance usually develops due to triggering lifestyle factors such as the consumption of a high-fat diet (HFD) [72], and it has been linked to obesity, hypertension and T2DM [60]. As mentioned previously, T2DM is mainly characterized by a state of insulin resistance and hyperglycemia [40]. Although insulin resistance mostly affects skeletal muscles, liver and adipose tissue due to their notable requirements of glucose uptake and metabolism [41], it has also been shown to affect other cells at sites of diabetic complications such as the kidney [78]. One study showed that a deficiency in insulin receptor action in podocytes induced a disease state similar to diabetic nephropathy even during normal glycemic conditions [79]. Weight reduction and/or pharmacological intervention against hyperglycemia can improve insulin resistance in T2DM, but it is seldom restored to normal levels [40].

A state of insulin resistance can be very debilitating for cell function because insulin signaling has many physiological roles such as glucose uptake by cells, glycogen synthesis, cellular proliferation, metabolism and many others [75]. The phosphorylation of the insulin receptor induces phosphorylation of cellular substrates such as insulin receptor substrate 1-4 (IRS1-4) [80]. The resulting morphological change in IRS increases its binding to phosphatidylinositol-3 kinase (PI-3K) [81] which ultimately activates phosphatidylinositol 3,4,5-trisphosphate (PIP3). This then recruits phosphoinositide-dependent kinase-1 (PDK1) which activates AKT [82]. Through this pathway (Fig. 1) , IRS-1 and IRS-2 can control many cellular functions, and their roles tend to be tissue-specific [81]. The PI-3K signaling pathway, or the metabolic pathway, has many functions including regulation of glucose uptake, cell survival and metabolism. Another major pathway commonly affected by insulin resistance is the mitogen-activated protein kinase (MAPK) signaling pathway, or the mitogenic pathway, which plays a role in cellular proliferation and gene expression [75]. In addition to its overwhelming functions in the periphery, insulin signaling has also been shown to play a huge role in the CNS.

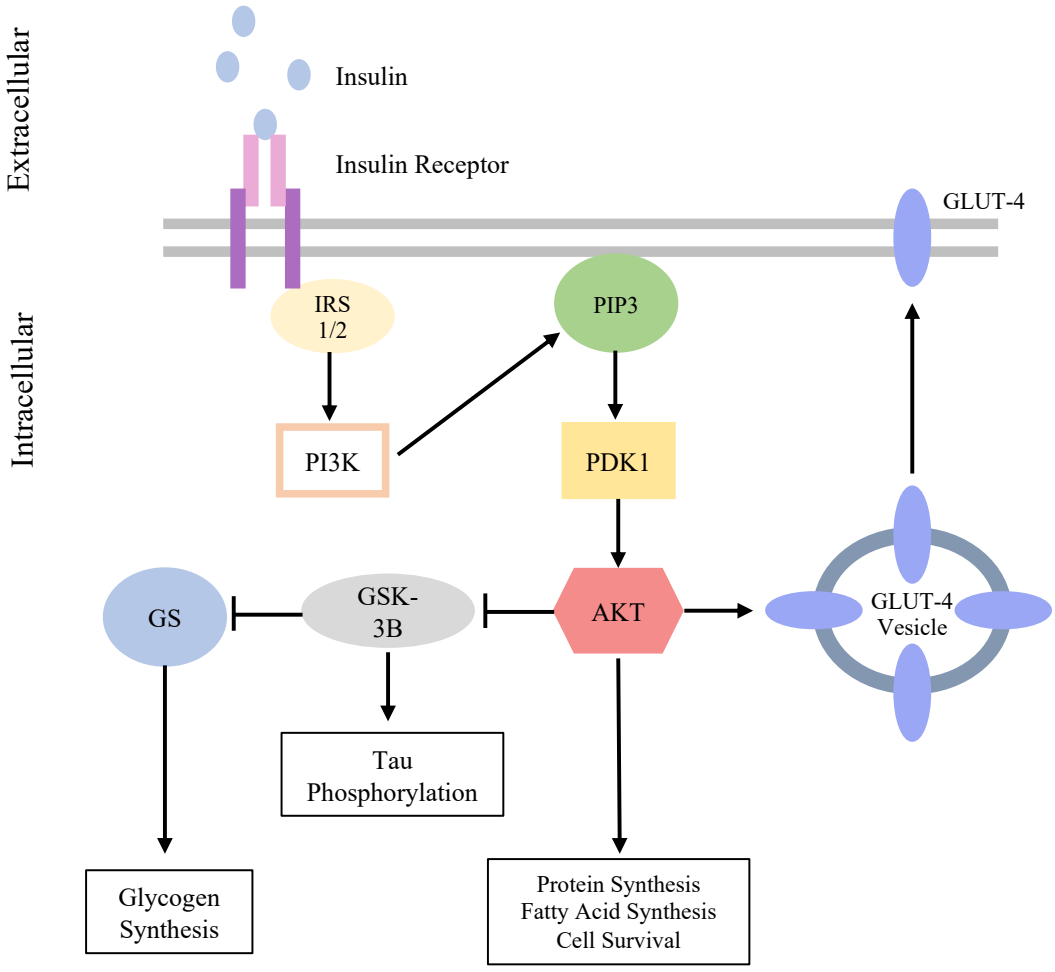


Figure 1. The metabolic pathway of insulin signaling under physiological conditions.
After insulin binds to its receptor, the activated receptor phosphorylates IRS-1/2 protein. IRS-1/2 can then activate PI-3K which in turn leads to the formation of PIP3. PDK1 is then recruited by PIP3 to phosphorylate AKT. AKT then catalyzes multiple reactions leading to GLUT-4 translocation to the cell membrane, glycogen synthesis through glycogen synthase (GS), protein synthesis, fatty acid synthesis and cell survival.

2. The Role of Insulin in the CNS

The brain had long been considered "insulin insensitive" because most of the glucose uptake by neurons is glucose transporter-3 (GLUT-3) dependent, which does not require insulin to perform its function [83]. However, neurons as well as the other cells of the brain (glial cells, pericytes and endothelial cells) have been shown to express insulin receptors

[84] which show an even distribution across all cell types of the human CNS [85]. Insulin receptors in the brain exist in two isoforms, A form and B form, which have different binding affinities to insulin and insulin-like growth factor (IGF), or they can form a heterodimer with the IGF-1 receptor [86, 87]. Multiple studies have demonstrated that insulin found in the brain can be actively transported across the blood brain barrier (BBB) [88, 89] or synthesized within the brain [90].

In the brain, and unlike its actions in the periphery, insulin acts more like a growth factor rather than a hormone that mainly regulates glucose uptake [89]. Insulin/IGF signaling elicits multiple effects such as promoting neuronal growth, learning and memory, inhibiting apoptosis, regulating metabolism, synthesizing proteins and maintaining synaptic plasticity [91, 92]. In a recent study, insulin/IGF signaling was shown to activate a pathway which results in the translocation of GLUT1 to the cell membrane in order to enhance glucose uptake on demand. GLUT1 is the main glucose transporter found in astrocytes and was previously thought to be an insulin-independent transporter only [93]. In addition to that, GLUT4 and GLUT8, which are insulin-dependent transporters, are mainly expressed in neurons of the hippocampus, amygdala, cortex and cerebellum. Thus, insulin-mediated translocation of these GLUTs is important in maintaining cognitive functions associated with the hippocampus [94]. Studies have demonstrated that upon treatment with insulin, the uptake of a glucose analog was significantly increased in the hippocampus [95], and normal adults treated with insulin showed improvements in memory formation [96]. Therefore, proper insulin action is essential for the normal functioning of the brain.

3. Insulin Resistance and Alzheimer's Disease

In line with its functions in cognition, memory and neuronal survival, studies have shown that disturbances in insulin signaling in the brain are associated with AD pathology [27], and insulin resistance is now considered a risk factor for AD [97]. In an experimental model of brain insulin resistance, silencing insulin or IGF receptor expression in the brain caused degeneration of the hippocampus and temporal lobes and impairments in learning and memory [98], and it also increased the phosphorylation of tau protein in another study [99]. This is because the phosphorylation of tau proteins can be regulated by the insulin signaling pathway through GSK-3B (Fig.1) [100]. When insulin receptors were downregulated within the hippocampus of rats, long-term memory was significantly impaired [101]. Taken together, these studies demonstrate the detrimental effects of insulin resistance on the brain.

In addition to that, studies have found that insulin receptors were desensitized in the brains of AD patients [60], and that the total and phosphorylated components of the insulin/PI-3K/AKT signaling pathway were downregulated in the cortices of AD patients [35]. In line with this study, AD brains with no history of T2DM showed reduced insulin response specifically at the level of the IRS-1/PI-3K signaling pathway. This was associated with an increase in Aβ plaques and a decrease in episodic and working memory [31]. In addition to that, postmortem AD brains showed diminished total levels of IRS-1 and IRS-2 [102], and insulin deficiency and resistance were shown to increase with Braak stage in AD brains [33]. Furthermore, multiple studies have found reductions in brain glucose metabolism in AD patients relative to control patients [103-105]. In addition to that, several studies have demonstrated that intranasal administration of insulin improves

working memory and cognitive function in patients with cognitive impairment such as AD [106, 107]. Thus, brain insulin resistance is one of the features of AD which may be disrupting major functions such as glucose utilization, metabolism, protein phosphorylation, cell survival and others.

4. Oxidative Stress in T2DM and AD

Another common pathological feature between T2DM and AD is oxidative stress. Oxidative stress occurs as a result of an imbalance between the biological system's ability to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) and to detoxify these reactive molecules (Fig. 2) [108]. Oxidative stress is a major hallmark of T1DM and T2DM [109, 110]. Hyperglycemia in T2DM is known to activate multiple pathways which ultimately produce AGEs. AGEs along with their receptors result in the formation of reactive oxygen species [111]. In addition to that, impaired glucose metabolism increases the production of ROS and RNS which can deplete the cell's antioxidant capacity leading to a state of oxidative stress [112]. ROS and RNS-induced protein and lipid peroxidation may lead to cellular damage and death, and it is increased in diabetic patients as compared to controls [113]. The overproduction of ROS through NADPH-oxidases has been shown to contribute to diabetic cardiomyopathy and nephropathy [114, 115], and oxidative stress in general has been a major contributing factor to diabetic neuropathy [109, 110].

Several studies have shown that oxidative stress not only impacts neurons in the periphery, but it is also a key mediator of neurodegeneration in AD since it can contribute to the damage of synapses, to the disruption of neuronal plasticity [116] and to the induction of

neuronal apoptosis [117]. Studies on cerebrospinal fluid and brains of AD patients have shown increased levels of 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde, which are markers of oxidative stress [118]. The level of oxidative stress has been shown to increase with Braak stage of AD in both CSF and postmortem brain tissue. 4-HNE is significantly higher in the CSF of patients at early stages of AD as compared to controls [32], and patients with mild cognitive impairment display increased levels of oxidized proteins in their parietal lobe, frontal lobe and hippocampus [119]. In addition to that, an increase in lipid peroxidation along with a decrease in antioxidant capacity were evident before the development of Aβ plaques, NFTs and cognitive decline in a transgenic mouse model of AD [120]. Thus, oxidative stress develops early on in the course of AD and may be used as a sign for early detection along with other factors. Although NADPH-oxidases are recognized as a major source of reactive oxygen species in neurological disorders [121, 122], their role in cognitive decline in T2DM has not yet been investigated.

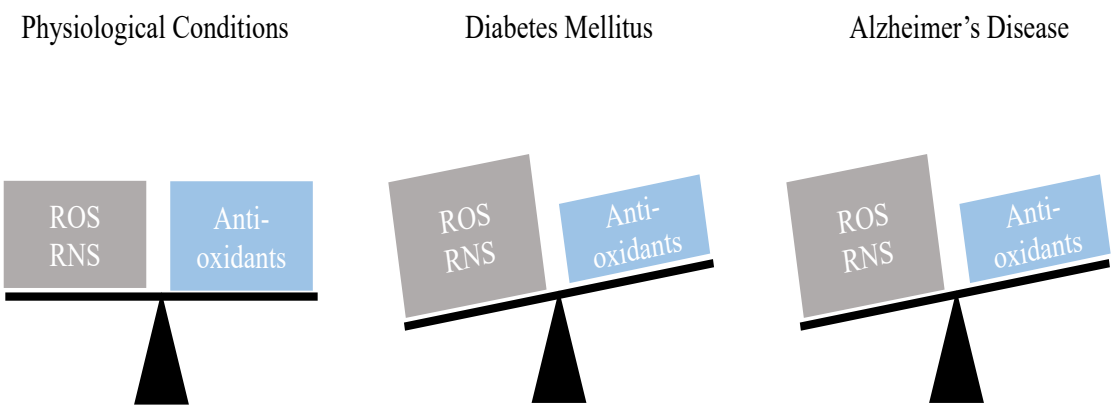


Figure 2. Schematic representation of oxidative Stress in diabetes mellitus and Alzheimer's disease.

D. Anti-diabetic Drugs in AD

Although numerous clinical trials have been conducted on AD patients, the FDA has only approved five drugs; however, these only manage to relieve symptoms temporarily and are unable to slow the progression of AD [1]. Therefore, there is an urgent need to develop novel and more effective therapies for AD. Due to the common underlying mechanisms between AD and diabetes, researchers have started looking into anti-diabetic drugs as a possible treatment for AD. Multiple studies have looked into metformin and glucagon-like peptide 1 (GLP-1) receptor agonists, in addition to other anti-diabetic drugs [123].

Metformin is the most commonly prescribed medication to combat T2DM since it increases insulin sensitivity and lowers glucose levels [124]. In many studies, metformin has shown potential in treatment of AD pathology. In an ICV-STZ model of sporadic AD, treatment with metformin has shown improvement of spatial learning and memory. In cultured hippocampal neurons, metformin inhibited Aβ-induced toxicity and apoptosis by inhibiting the hyperphosphorylation of c-Jun N-terminal kinase (JNK) [125]. In the SAMP8 mouse model of AD, metformin was shown to improve both learning and memory in the T-maze foot shock avoidance, object recognition and the Barnes maze by decreasing the accumulation of Aβ and pTau [126]. In another study on transgenic AD mice, metformin treatment ameliorated Aβ accumulation and neuroinflammation, as well as impairment in spatial memory and neuronal loss in the hippocampus [127]. In several studies, non-diabetic subjects with MCI or mild AD were treated with metformin, and they showed positive effects on executive function, learning, memory and attention [128, 129]. In another clinical trial, metformin was given to diabetic patients at high doses over a long period of time which was associated with a lower risk of developing AD [130].

GLP-1 is a hormone that helps in lowering glucose levels, so GLP-1 receptor agonists are currently used in the treatment of T2DM [131]. They are also being investigated as a potential treatment for AD [132, 133]. Liraglutide, which is a GLP-1 receptor agonist, shows protective effects on impaired spatial learning and memory by ameliorating the hyperphosphorylation of tau and dysfunctions in insulin signaling in the ICV-STZ mouse model of AD. It was also able to reduce neuronal degeneration [134, 135]. In another study on a transgenic mouse model of AD, treatment with GLP-1 receptor agonist was shown to reduce Aβ plaques, microglial activation and synapse loss [136]. Dulaglutide, another GLP-1 receptor agonist, was administered to a sporadic AD mouse model resulting in protection against cognitive impairment and decrease in tau hyperphosphorylation by stimulating the PI-3K/AKT/GSK-3B pathway [123]. In a non-human primate model of AD, treatment with GLP-1 receptor agonist reduced tau phosphorylation and synapse loss [137]. In addition to that, GLP-1 receptor agonists given to AD patients have shown a decrease in glucose hypometabolism in clinical trials [138].

Sodium-Glucose cotransporter 2 (SGLT2) inhibitors are another class of drugs prescribed for diabetics to lower blood glucose by inhibiting glucose reabsorption in the kidneys [139]. Preliminary studies have started looking into their effects on cognitive function. In a randomized clinical trial on T2DM patients, treatment with SGLT2 inhibitors for 12 months prevented any reduction in cognitive performance during the study period [140]. In addition to that, SGLT2 inhibitor prevented impairment in cognitive function along with a reduction of cerebral oxidative stress in a mouse model of T2DM [141]. It also improved brain insulin signaling and mitochondrial function, averted cognitive impairment and increased synaptic plasticity in the hippocampus of obese-insulin resistant rats [142]. Thus, preliminary

evidence has shown some protective effects of SGLT2 inhibitors on cognitive function insinuating that SGLT2 inhibitors may be used as a novel therapeutic strategy in AD.

E. Hypothesis and Aim of the Study

Although diabetes has been considered as a major risk factor for the development of AD, the mechanisms underlying this association remain uncertain. Increasing evidence supports shared pathological features between diabetes and AD such as insulin resistance and oxidative stress. Understanding the mechanisms by which diabetes may be inducing AD is crucial for the development of preventative methods and treatment strategies targeting diabetes-induced AD. Thus, this study aims to determine the role of impaired insulin signaling and ROS overproduction in the development of AD-like symptoms in diabetes (Fig. 3). We hypothesize that in a mouse model of T2DM, the expression of IRS-1 and IRS-2 in the brain may be altered paralleled by increased ROS production through the NADPH oxidase family of enzymes, specifically Nox1 and Nox4. These changes will ultimately contribute to AD-like symptoms of cognitive decline and Aβ and tau pathology. In addition to that, we hypothesize that treatment with dapagliflozin, an SGLT2-inhibitor, and GKT137833, a Nox1/Nox4 inhibitor, will work to alleviate the AD-like symptoms associated with T2DM.

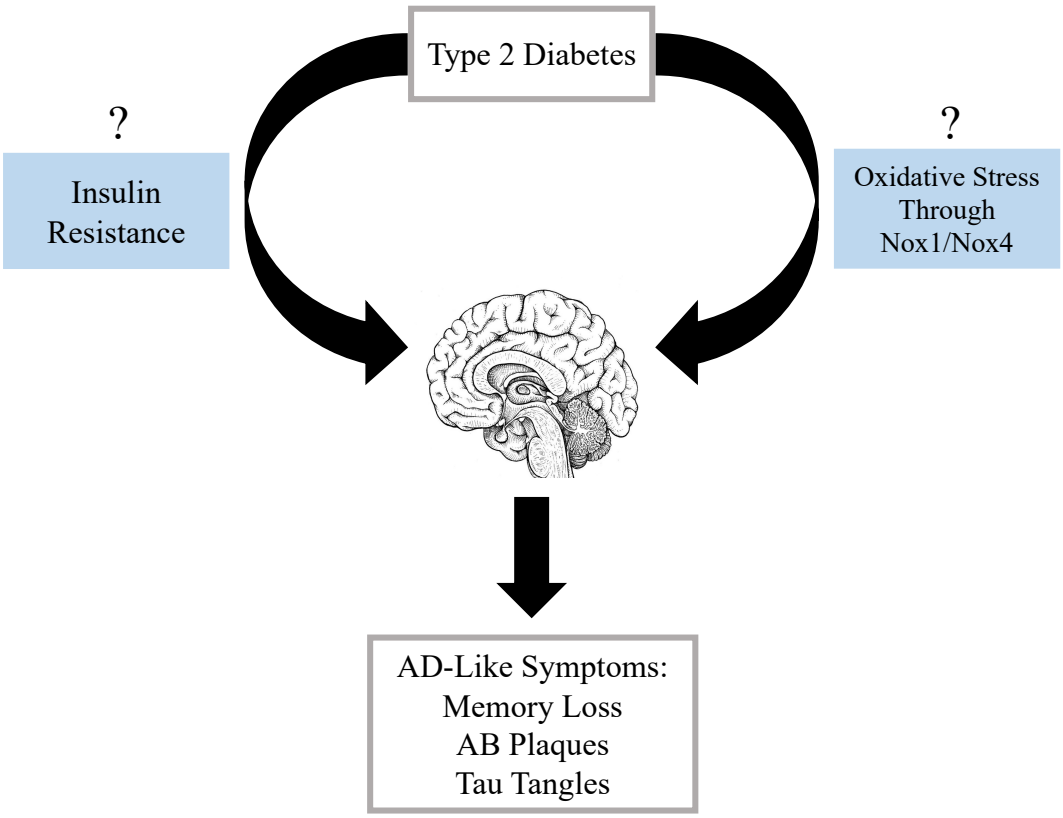


Figure 3. Proposed model for the development of AD-like symptoms in T2DM.
T2DM induces a state of brain insulin resistance as well as ROS overproduction, through an upregulation of Nox1 and Nox4 in the brain, ultimately contributing to AD-like pathology of memory loss, Aβ plaque accumulation and tau tangles.

CHAPTER II

MATERIALS AND METHODS

A. Animal Models

C57BL/6J mice were used to conduct this study. This strain is susceptible to the induction of T2DM rendering it an excellent model for studying the onset of T2DM and its associated complications.

Sixteen 9-week-old female mice were separated into 4 groups: control, diabetic, diabetic treated with dapagliflozin (SGLT2-inhibitor) and diabetic treated with GKT #137833 as shown in Table 1. To induce T2DM, mice were fed a 60% HFD for a period of 5 weeks followed by intraperitoneal streptozotocin (STZ) injections. STZ was administered following the *Low-Dose Streptozotocin Induction* protocol described by the Diabetic Complications Consortium (Diacomp) at a dose of 75mg/kg but for 5 consecutive days. STZ was freshly prepared before injection by dissolving it in a citrate buffer (pH 4.5). Diabetic groups were kept on a HFD until the end of the experiment. Dapagliflozin was dissolved in water and administered through oral gavage at a dose of 2 mg/kg 6 times per week for a period of 9 weeks. GKT was dissolved in methyl cellulose and administered through oral gavage at a dose of 40 mg/kg 3 times per week for a period of 9 weeks.

All animals were housed in a temperature-controlled room (20° to 22°C) with a 12:12 h light: dark cycle (light period from 7:00 am to 7:00 pm). All mice had *ad libitum* access to drinking water and food. The control group was provided with standard rodent chow, whereas the diabetic groups were provided with HFD chow. All behavioral assessments were performed

in the morning between the hours of 8:00 am and 12:00 pm. All procedures and protocols were conducted in accordance with the ethical guidelines and under the approval of the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB).

Condition	Number of animals
Control	5
Diabetic (T2DM)	3
Diabetic treated with Dapagliflozin (T2DM+SGLT2i)	4
Diabetic treated with GKT (T2DM+GKT)	4

Table 1. Experimental Groups.

B. Experimental Design

Upon arrival, mice were separated into their respective groups then diabetic groups started their HFD regimen for five weeks. Body weights and random blood glucose levels (BGL) were recorded once per week until the end of the study. BGL were measured using an Accu-Check Performa® glucometer following a tail-prick. Following the implementation of the T2DM protocol, blood glucose levels were recorded during the next week to ensure that T2DM was properly induced. Mice with plasma glucose of 250 mg/dL or above were considered diabetic. The glucose tolerance test was conducted for the control and T2DM group where 2g/kg of glucose were injected intraperitoneally after 6 hours of fasting. BGL were measured before

injection and at 15, 30, 45, 60 and 90 mins after injection to determine how quickly glucose is cleared from the blood.

At 8 weeks of diabetes, treatment groups were administered with either dapagliflozin or GKT for a period of 9 weeks. After 16 weeks from diabetes induction, behavioural tests were conducted for all groups over a period of one week. All mice were then euthanized and required organs/tissues were harvested. The brains were removed carefully, after which the prefrontal cortex and hippocampus were extracted, and collected blood was analyzed for HbA1c levels.

C. Behavioral Tests

1. Spontaneous T-maze Alternation

To assess the cognitive ability of the mice, the T-maze test was conducted. The setup consists of an elevated and enclosed apparatus in the shape of a T with one starting arm and two goal arms separated by a long central partition. The natural tendency of rodents is to alternate their choice of goal arm when two trials are performed in quick succession. This is known as spontaneous alternation, and it is used as an indication of spatial working memory. This spontaneous alternation is very sensitive to dysfunctions of the hippocampus, but other brain structures such as the thalamus, cerebellum and substantia innominate are also involved [143, 144]. Before this test can be conducted, the mice need to be accustomed to the investigator's touch by placing hands inside their cage and gently picking them up.

Animals were left in the behavioural room for 10-15 minutes prior to the testing session to allow for an optimal state of arousal. Each mouse was placed at the base of the T facing away from the goal arms and allowed to explore freely until it chose one of the two available

arms. Once the mouse reached its goal arm, the investigator closed the entrance to that arm by a plastic door and allowed the mouse to explore the arm freely for 30 seconds. The criterion for the arm choice was met when the mouse had fully entered the arm, including the tip of its tail.

After 30 seconds, the mouse was slowly picked up, and the door was reopened. The mouse was then placed back at the base of the T facing away from the goal arms, and it was allowed to choose an arm again. If the mouse chose the arm that was not visited on its first trial, then it has passed as this is an indication of its memory of the first arm chosen. Mice with normal spatial working memory would have a natural tendency to choose the new arm in an effort to locate potential food, water, shelter or mates [143]. However, if it chose the arm that was previously visited, then it failed.

Each mouse was given 3 trials, and each trial had to be completed in under 2 minutes with an interval of 5 minutes between each trial. If the animal spent more than 90 seconds in the maze without choosing an arm, then it was returned to its cage and the trial was performed at a later time. The percentage of successful trials was calculated based on the 3 trials performed.

2. Novel Object Recognition

The novel object recognition (NOR) test is conducted to assess recognition memory and learning in mice [145]. Recognition memory, which is the ability to recognize a previously encountered item, is a subcategory of declarative memory which relies on the integrity of the medial temporal lobe [146]. The mice are initially presented with two identical copies of an object to explore, one of which is later replaced with a novel object. The amount of time spent

exploring the novel object is used as an indicator for recognition memory. This is based on the innate tendency of the animal to explore a novel object which makes it spend more time exploring the novel object only if it recognizes the familiar one. This test consists of three phases: habituation, familiarization and testing [145].

Before the habituation phase, mice were initially placed in the testing room for 15 minutes and had been previously accustomed to the investigator's handling to avoid any novelty-induced stress. During the habituation phase, each mouse was placed in an opaque open field box (acrylic square box, 30 x 30 x 30 cm) and allowed to explore freely for 5 minutes. It was then returned to its cage, and the box was cleaned with 70% ethanol. The habituation phase was set to occur 24 hours before familiarization and testing.

During the familiarization phase, each mouse was placed inside the same open field box where two copies of the same object were placed in two corners of the box 5 cm away from the walls. The mouse was placed in an empty corner facing the walls of the box, and it was allowed to explore freely for 5 minutes. The mouse was then returned to its cage, and the objects as well as the apparatus were thoroughly cleaned with 70% ethanol to remove any odours. An intersession interval (ISI) of 5 minutes was implemented between the familiarization phase and the testing phase.

One of the familiar objects was then removed and replaced with a novel object. During the testing phase, the mouse was placed back in the open field box and allowed to explore freely for 5 minutes. A camera was placed on top of the box to record each testing phase for later analysis. The investigator was at least one meter away from the box.

In order to collect the data, the same experimenter thoroughly examined the video recordings for any exploratory behaviour. Exploration of an object was defined by directing

the nose towards the object when it was 2 cm or less away from it. Climbing over the object or leaning on to it was not considered an explorative behaviour, unless it was accompanied with directing the nose towards the object [145]. The time spent by each mouse on the familiar object and the novel object was recorded using a stopwatch. The choice of familiar versus novel object was randomized for each mouse, and their placement within the box was randomized as well [145].

3. Hind Paw Withdrawal Response to Thermal Stimulation (Hargreave's Test)

Hind paw withdrawal latency was measured using Hargreave's Method which tests for the animal's sensitivity to thermal pain. The IITC Plantar Analgesia Meter was set up according to the manufacturer's protocol (IITC Life Science) to measure the withdrawal latency (in seconds) to an infrared heat stimulus when it is applied to the plantar surface of the hind paw.

Each mouse was placed in a separate compartment on a preheated elevated glass plate (32 °C) and allowed to habituate for 30 minutes. The infrared idle intensity was set to 2% while the active intensity was set to 25% for a maximum duration of 20 seconds to avoid any damage to the skin. After the habituation period, the movable infrared emitter was placed below the glass plate and positioned at the mid-plantar surface of the hind paw for each mouse. The emitter was activated and then manually stopped as soon as the animal elicits a withdrawal response, and the withdrawal latency was then recorded. If the animal moved its paw arbitrarily, this movement was not counted as a withdrawal response, and the trial was disregarded. Typically, the animal's withdrawal response to thermal stimulation was accompanied by licking or observing the paw. Six trials were conducted for each animal with

an intertrial interval of 5 minutes while alternating the targeted paw, and the average for all trials was then calculated.

4. Hind Paw Withdrawal Response to Mechanical Stimulation (Electronic von Frey)

To test for tactile sensitivity and nociception, mechanical stimulation of the hind paws with the von Frey Filament (15g) was conducted using the Dynamic Plantar Aesthesiometer (DPA) by Ugo Basile. The apparatus consists of an elevated metallic mesh with animal enclosures, a portable force transducer unit with a von Frey filament to stimulate the mechanical force, and an electronic unit for setting the required force.

Each mouse was placed in its own enclosure on top of the metallic mesh and allowed to accommodate for 30 minutes. The mice were separated by opaque walls to avoid seeing each other. The force delivered by the filament was set at 15 g to activate both mechanoreceptors and nociceptors. The movable force generator was positioned such that the von Frey filament was applied perpendicularly to the center of the plantar surface of the hind paw (the force should not be applied on fat pads). As soon as the mouse elicited a withdrawal response, the DPA automatically recorded the time and the maximum force (in grams) that elicited that response.

Six trials were conducted for each mouse with an intertrial interval of 5 minutes. The force required and the time taken to elicit a withdrawal response were recorded.

5. Raised Beam Walking

To assess for motor dysfunction and peripheral nerve injury in animals, the raised beam walking test was conducted. The apparatus consists of an elevated narrow beam (1.2 cm in diameter, 70 cm in length and 50 cm elevation) with a starting open platform at one end and a closed platform to house the mouse once it has crossed at the other end. The goal of the test is for the mouse to walk across the length of the beam from the starting platform until it reaches the closed housing platform.

The first day consists of a training session where each mouse was placed at the starting platform and allowed to adapt to the set-up by crossing the length multiple times. Once the mouse succeeded in reaching the housing platform several times, the testing session was conducted the following day. During the test, each mouse was given 3 trials with an intertrial interval of 5 minutes. Each trial was video recorded and subsequently analyzed for the time required to cross the beam, the speed of the animal, the number of slips and the number of stops.

D. Molecular Experiments

In order to assess changes at the molecular level, brain tissue (prefrontal cortex and hippocampus) were carefully collected from each mouse.

1. Immunohistochemical (IHC) Staining

In order to detect Aβ plaques and tau hyperphosphorylation in the brain, the Novolink Polymer Detection kit (RE7150-K) was utilized. Using a freezing microtome, extracted brains

were cut into sections of 7um in thickness. Brain sections were then fixed with pre-cooled methanol, washed with 0.025% Triton-X TBS, incubated with blocks provided with the kit and then with a rabbit polyclonal Aβ antibody (1:500, Cusabio), TauS404 antibody (1:250, abcam) or TauS396 antibody (1:4000, abcam) overnight. The following day, sections were incubated with post-primary solutions and counterstained with haematoxylin. The slides were later mounted, and beta-amyloid plaques and hyperphosphorylated tau were visualized using Olympus light microscope where they appeared brown in color.

2. Western Blot

In order to detect changes at the protein level between our groups, the western blot technique was utilized. Initially, prefrontal cortex and hippocampus samples were each lysed using RIPA buffer (0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1 mM phenylmethylsulfonyl fluoride). The lysates were left to rotate overnight at 4°C, and then they were centrifuged at 13,600 rpm for 40 minutes at 4°C. The supernatants containing the extracted proteins were collected, and the pellets were discarded.

The concentration of proteins within each sample was quantified using the Lowry protein assay. For immunoblotting, equal amounts of protein (40 μg) were loaded into 8-12% polyacrylamide gels. The proteins were subjected to an SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred onto nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were then blocked with 5%

Bovine Serum Albumin (BSA) dissolved in Tris-buffered saline and later incubated with the primary antibody overnight at 4°C. The primary antibodies used were anti-IRS1 (1:500, 06-248, Millipore), anti-Nox4 (1:500, ab133303, Abcam), anti-TauS404 (1:500, ab92676, Abcam), anti-HSC70 (1:500, sc7298, Santa Cruz) and anti-alpha tubulin (1:500, sc5286, Santa Cruz). The primary antibodies were then detected by horseradish peroxidase-conjugated anti-rabbit IgG (1:10000, Bio-Rad) or anti-mouse IgG. Protein bands were visualized by enhanced chemiluminescence using the ChemiDocTM imaging system (Bio-Rad Laboratory, CA, USA). Densitometric analysis was performed using Image J software.

3. Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from hippocampus and prefrontal cortex samples using TRIzol reagent (Sigma Aldrich, Steinheim, Germany) according to the manufacturer's protocol. Extracted RNA samples were then quantified using the NanoDrop and converted to cDNA using the iSCRIPT cDNA Synthesis Kit (Bio-Rad) starting with 500 ng of total RNA.

cDNA was analyzed by real-time PCR using SYBR Green dye (iTOP Universal SYBR Green Supermix, Bio-Rad). Forward and reverse primers shown in Table 2 were used to amplify their complementary DNA sequences using the Bio-Rad CFX 384 machine according to the thermal cycling protocol. cDNA was quantified using the comparative Ct method where YWHAZ was used as the housekeeping gene.

Primers	Sequence	Annealing T (°C)
Nox 1	F : 5'-TCGACACACAGGAATCAGGA-3' R: 3'-TTACACGAGAGAAATTCTTGGG-5'	58.4
Nox 4	F: 5'-GAAGGGGTTAAACACCTCTGC-3' R: 5'-ATGCTCTGCTTAAACACAATCCT-3'	61.3 59.3
IRS-1	F: 5' - TCC CAA ACA GAA GGA TG - 3' R: 5' - CAT TCC GAG GAG AGC TTT TG - 3'	58.4
IRS-2	F: 5' - GTA GTT CAG GTC GCC TCT GC - 3' R: 5' - TTG GGA CCA CCA CTC CTA AG - 3'	62.5 60.5
YWHAZ	F: 5' - GGT GAT GAC AAG AAA GGA ATT GTG - 3' R: 5' – GCA TCT CCT TTT TGC TGA TTT CA- 3'	61.8 59.3

Table 2. Forward and reverse primer sequences and conditions employed for real-time PCR.

E. Statistical Analysis

Analysis was performed using Graphpad Prism 8 (GraphPad Software, La Jolla, CA, USA). For behavioural and molecular data, statistical significance was determined using the t-test when comparing two groups. Following a normality test to detect a normal (Gaussian) distribution, parametric or non-parametric testing was conducted. One-way analysis of variance (ANOVA) or two-way ANOVA with Fisher's LSD test were conducted when comparing all groups. The results of the diabetic and treated groups were compared to those of the controls, and the results of the treated groups were also compared to those of the diabetic group. Data are presented as mean ± the standard deviation (SD) or standard error of the mean (SEM). Differences with a p value <0.05 were considered to be statistically significant.

CHAPTER III

RESULTS

A. Metabolic Parameters indicate a state of insulin resistance

The glucose tolerance test was conducted for the control and T2DM groups to measure blood glucose levels; it shows that the diabetic mice had significantly higher blood glucose levels compared to the control mice at 45, 60 and 90 minutes after injection with glucose (Fig. 4). HbA1c levels (%) were also significantly higher in all groups as compared to the control group; however, the levels of the SGLT2 inhibitor-treated group were significantly lower than those of the T2DM group while remaining significantly higher than the control (Table 3).

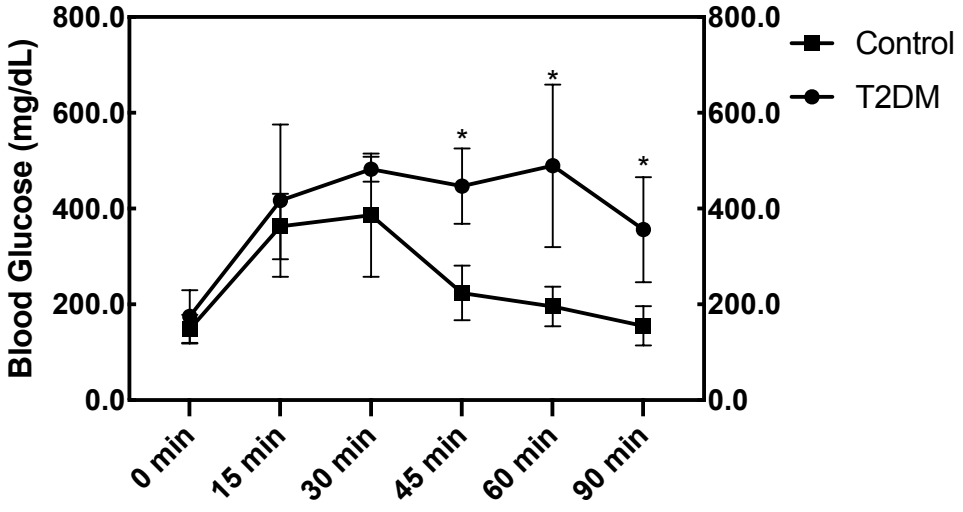


Figure 4. Glucose tolerance test results.
Histograms representing blood glucose levels (mg/dL) following the induction of the glucose tolerance test for the control (n=5) and diabetic (n=3) groups at different time intervals. Values represent the mean ± SD. *p<0.05 compared to control.

Group	HbA1c (%)
Control	5.82 ± 0.47644517
T2DM	8.933333333 ± 0.25166115 *
T2DM + SGLT2i	7.875 ± 0.33040379 * #
T2DM + GKT	9.175 ± 0.34034296 *

Table 3. HbA1c levels in blood collected from all groups.
HbA1c (%) analysis done by HPLC using the variant II Hemoglobin Testing System (Biorad). Analysis was done on blood collected from the control (n=5), diabetic (n=3), dapagliflozin-treated (n=4) and GKT-treated (n=4) groups. Values represent the mean ± SD. *p<0.05 compared to control. #p<0.05 compared to T2DM.

B. Type 2 Diabetes induces Alzheimer's-like symptoms

T2DM has been considered a risk factor for AD [61]. Two major hallmarks of AD are the accumulation of Aβ plaques and hyperphosphorylated tau which forms neurofibrillary tangles [17, 147]. Phosphorylation of tau protein at sites Ser396-404 are found in both mild and severe AD cases [148]. The expression of tau phosphorylated at Ser404 was quantified through western blot analysis at 17 weeks of diabetes onset. Compared to the control group, the levels of ptau Ser404 in the T2DM group tended to increase in the prefrontal cortex (Fig. 5B) and the hippocampus (Fig. 5D), although not significant. In addition to that, immunohistochemical staining of brain tissue was used to detect amyloid-beta plaques and phosphorylation of tau at sites S404 and S396 around the hippocampal area. In our representative images, control mice do not show any accumulation of Aβ plaques (Fig. 5E), whereas T2DM mice do show plaque accumulation (Fig. 5F). In addition to that, controls do not display any hyperphosphorylation of tau at S404 (Fig. 5G) or S396 (Fig. 5I). However, T2DM mice show tau hyperphosphorylation within neurons at S404 (Fig. 5H) and at S396 (Fig. 5J).

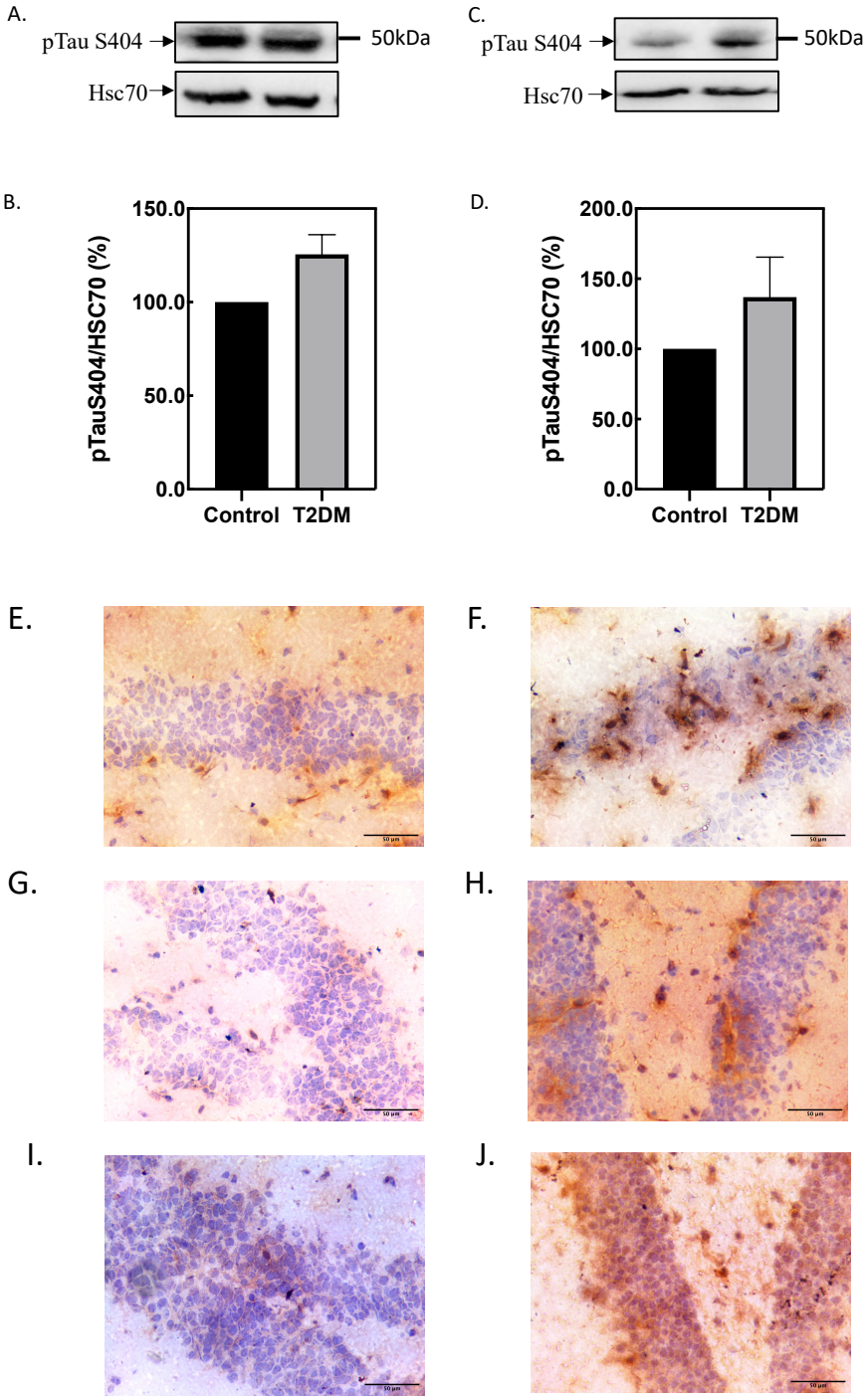


Figure 5. Molecular alterations indicative of AD develop in a mouse model of T2DM.
Representative image of phosphorylated tau (ptauS404) expression along with its western blot quantification in the prefrontal cortex (A,B) and the hippocampus (C,D) where Hsc70 was used as a loading control. Values represent the mean ± SEM for the control and diabetic groups (n=3). Representative images of immunohistochemical staining to determine the deposition of Aβ (E,F), phosphorylation of tau at S404 (G,H) and S396 (I,J) in controls (E,G,I) and T2DM (F,H,J) with n=1.

In order to determine whether diabetes induces AD-like symptoms of memory loss as it progresses, we performed two behavioral tests targeting cognitive function on C57BL/6J mice after 16 weeks of diabetes onset. The spontaneous T-maze alternation was used to assess spatial memory. When compared to the control group, diabetic mice showed a decreased percentage of spontaneous alternation of goal arm in the T-maze (Fig. 6A). The novel object recognition test was used to assess recognition memory. In the control group, exploration time of the novel object versus the familiar object was statistically significant with more time being spent on the novel object (Fig. 6B). However, in the T2DM group, the exploration times of the novel and familiar objects were almost the same with no statistical significance (Fig. 6B).

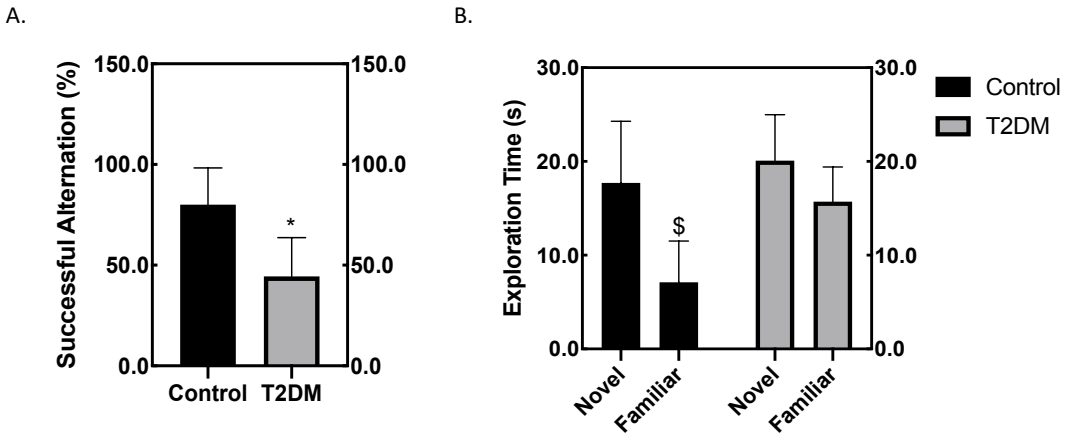


Figure 6. AD-like symptoms of memory loss develop in a mouse model of T2DM.
Histograms representing percentage of successful alternation in T-maze (A) and the time spent exploring the novel and familiar objects in the NOR test (B) for the control (n=5) and diabetic (n=3) groups. Values represent the mean ± SD. *p<0.05 compared to control. $p<0.05 compared to novel object.

C. Dapagliflozin and GKT alleviate Alzheimer's-like symptoms induced in a mouse model of T2DM

Diabetic mice that were treated either with dapagliflozin (SGLT2-inhibitor) or GKT had a significantly higher percentage of spontaneous alternation as compared to the untreated diabetic mice (Fig. 7A). In addition to that, the time spent exploring the novel versus the familiar object were significantly different in both treatment groups where more time was spent exploring the novel object (Fig. 7B).

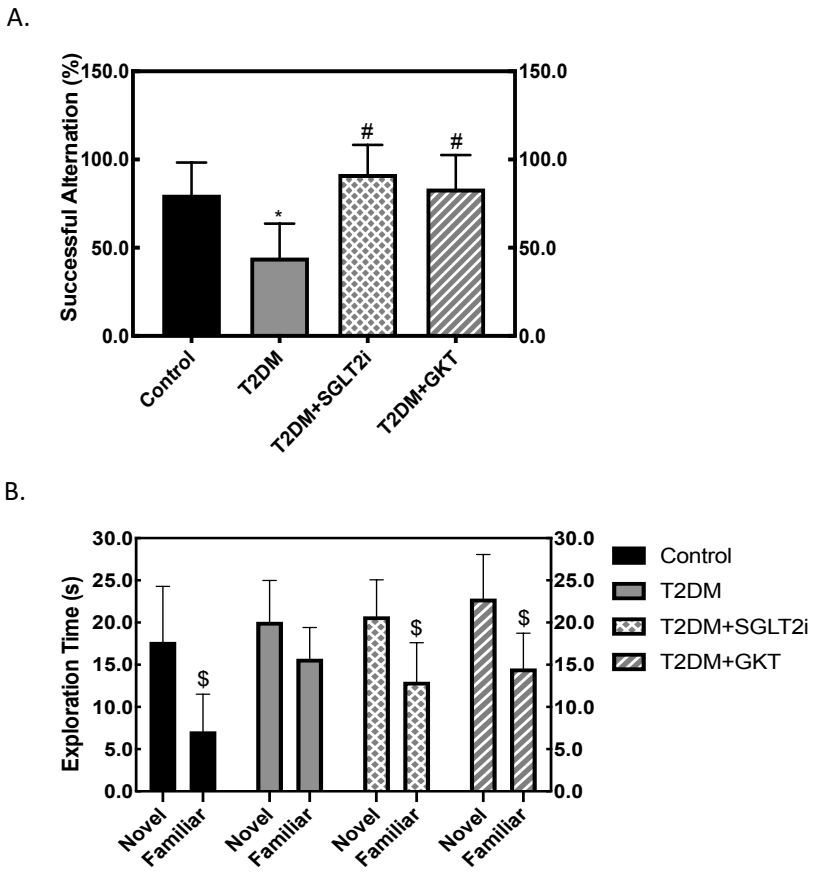


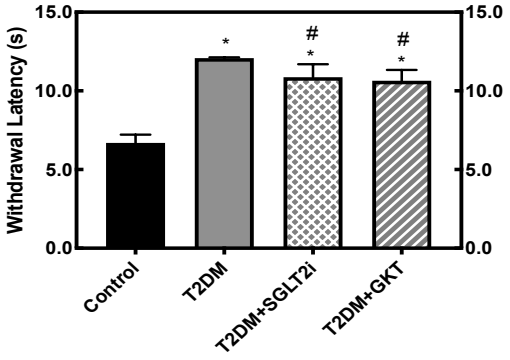
Figure 7. Dapagliflozin and GKT alleviate cognitive dysfunction associated with T2DM.
Histograms representing percentage of successful alternation in T-maze (A) and time spent exploring the novel and familiar objects in the NOR test (B) for the control (n=5), diabetic (n=3), dapagliflozin-treated (n=4) and GKT-treated (n=4) groups. Values represent the mean ± SD. *p<0.05 compared to control. #p<0.05 compared to T2DM. $p<0.05 compared to novel object.

D. Dapagliflozin and GKT alleviate thermal and mechanical hypoalgesia observed in Type 2 diabetic mice

In order to test for dysfunctions within sensory neurons, hind paw withdrawal response to a thermal or mechanical stimulus was recorded as a measure of pain sensitivity. To assess thermally-induced pain behaviour, Hargreave's test was conducted. T2DM mice had a significantly higher hind paw withdrawal latency to the thermal stimulus when compared to the control indicating an increased threshold to thermal stimuli (Fig. 8A). However, the dapagliflozin-treated and the GKT-treated groups showed a significantly decreased withdrawal latency when compared to the untreated diabetic group, although it was not returned to control levels (Fig. 8A).

To assess mechanically-evoked pain behaviour, the electronic von Frey was utilized. When a mechanical stimulus of a 15g force was applied to the hind paws, a similar trend was observed where diabetic mice had a significantly higher withdrawal latency when compared to the controls (Fig. 8B). This was reversed in the treatment groups which displayed significantly lower withdrawal latencies as compared to the T2DM group (Fig. 8B). These results were paralleled when measuring the force (g) at which withdrawal took place (Fig. 8C) where diabetic mice displayed withdrawal behavior at a significantly higher force compared to the control group, and treatment with dapagliflozin and GKT significantly reduced this withdrawal force.

A.



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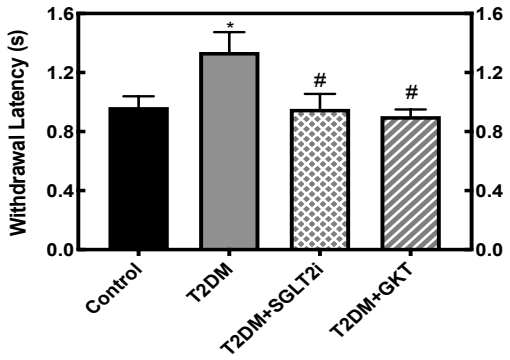

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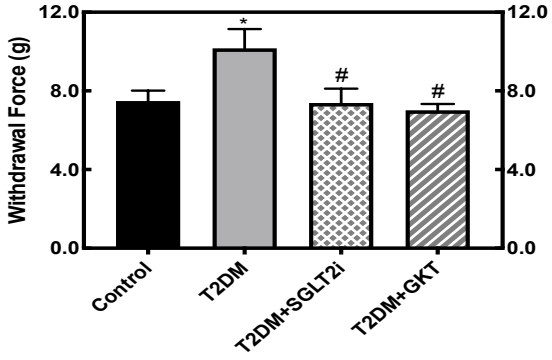

Figure 8. The effect of dapagliflozin and GKT on sensory dysfunction associated with T2DM.
Histograms representing hind paw withdrawal latency in Hargreave's test (A), hind paw withdrawal latency in the electronic von Frey (B) and withdrawal force in the electronic von Frey (C) for the control (n=5), diabetic (n=3), dapagliflozin-treated (n=4) and GKT-treated (n=4) groups. Values represent the mean ± SD. *$p<0.05$ compared to control. #$p<0.05$ compared to T2DM.

E. Dapagliflozin and GKT prevent motor dysfunction in T2DM

Diabetic neuropathy can also be associated with poor balance and co-ordination and frequent falls [149]. In order to assess fine motor co-ordination, the raised beam walking test was conducted on a mouse model of T2DM. Peripheral neuropathy assessment for the T2DM group showed a significantly higher average number of slips (Fig. 9A) and stops (Fig. 9B) when compared to the controls. However, the increase in the number of slips was reversed and returned back to control levels with the administration of dapagliflozin and GKT (Fig. 9A), and the number of stops was significantly decreased with the administration of GKT (Fig. 9B).

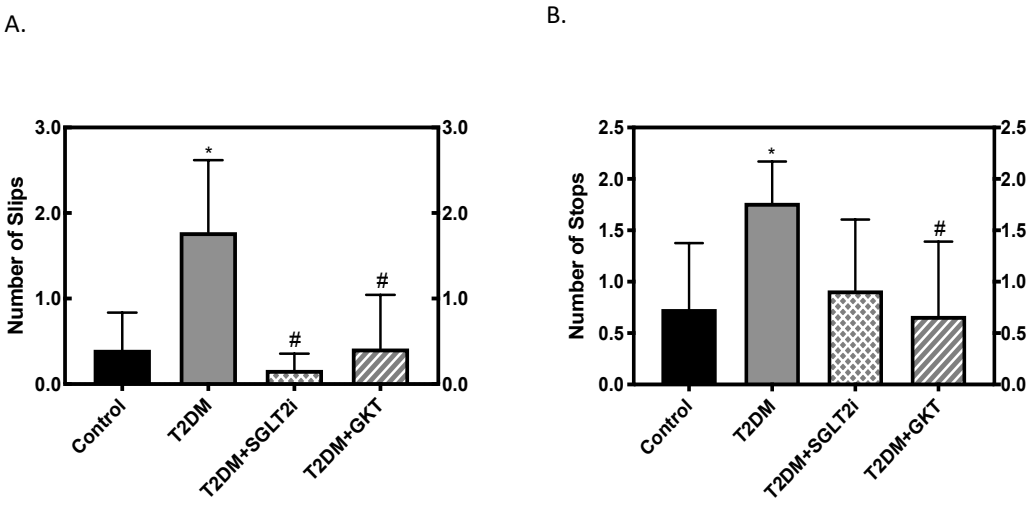

Figure 9. The effect of dapagliflozin and GKT on fine motor co-ordination in a model of T2DM.
Histograms representing the average number of slips (A) and the average number of stops (B) in the raised beam walking test for the control (n=5), diabetic (n=3), dapagliflozin-treated (n=4) and GKT-treated (n=4) groups. Values represent the mean ± SD. *$p<0.05$ compared to control. #$p<0.05$ compared to T2DM.

F. T2DM is associated with dysfunctions in the levels of IRS-1 and IRS-2 in the brain

Dysregulations in the IRS proteins have been implicated in the development of diabetic complications such as cardiomyopathy, nephropathy, retinopathy and neuropathy [150]. To detect any changes in the IRS proteins, western blot analysis and RT-PCR were performed on the prefrontal cortex and hippocampus of all groups. Although not significantly changed, the levels of IRS-1 protein decreased to about 50% of the control levels in the prefrontal cortex of T2DM mice (Fig. 10B). However, IRS-1 levels were significantly increased compared to the T2DM group when treated with dapagliflozin and GKT, and levels for treatment with dapagliflozin were also significantly higher than those seen in the control group. In line with these results, IRS-1 and IRS-2 mRNA levels in the T2DM group were decreased to about half the values of the control group in the prefrontal cortex, although they were not statistically significant (Fig. 10 C,D). Treatment with GKT showed an obvious trend of increase for IRS-1 and IRS-2 mRNA levels compared to the T2DM group; however, levels after treatment with dapagliflozin were significantly higher than those of the T2DM group for both IRS-1 and IRS-2 mRNA levels.

In the hippocampus, the levels of IRS-1 protein were not altered in the T2DM mice as compared to the controls, but treatment with dapagliflozin displayed a trend of increase in IRS-1 protein levels (Fig. 10F). At the mRNA level, IRS-1 and IRS-2 expression in the hippocampus was not changed in the T2DM group as compared to the controls, and treatments displayed no significant changes either (Fig. 10 G,H).

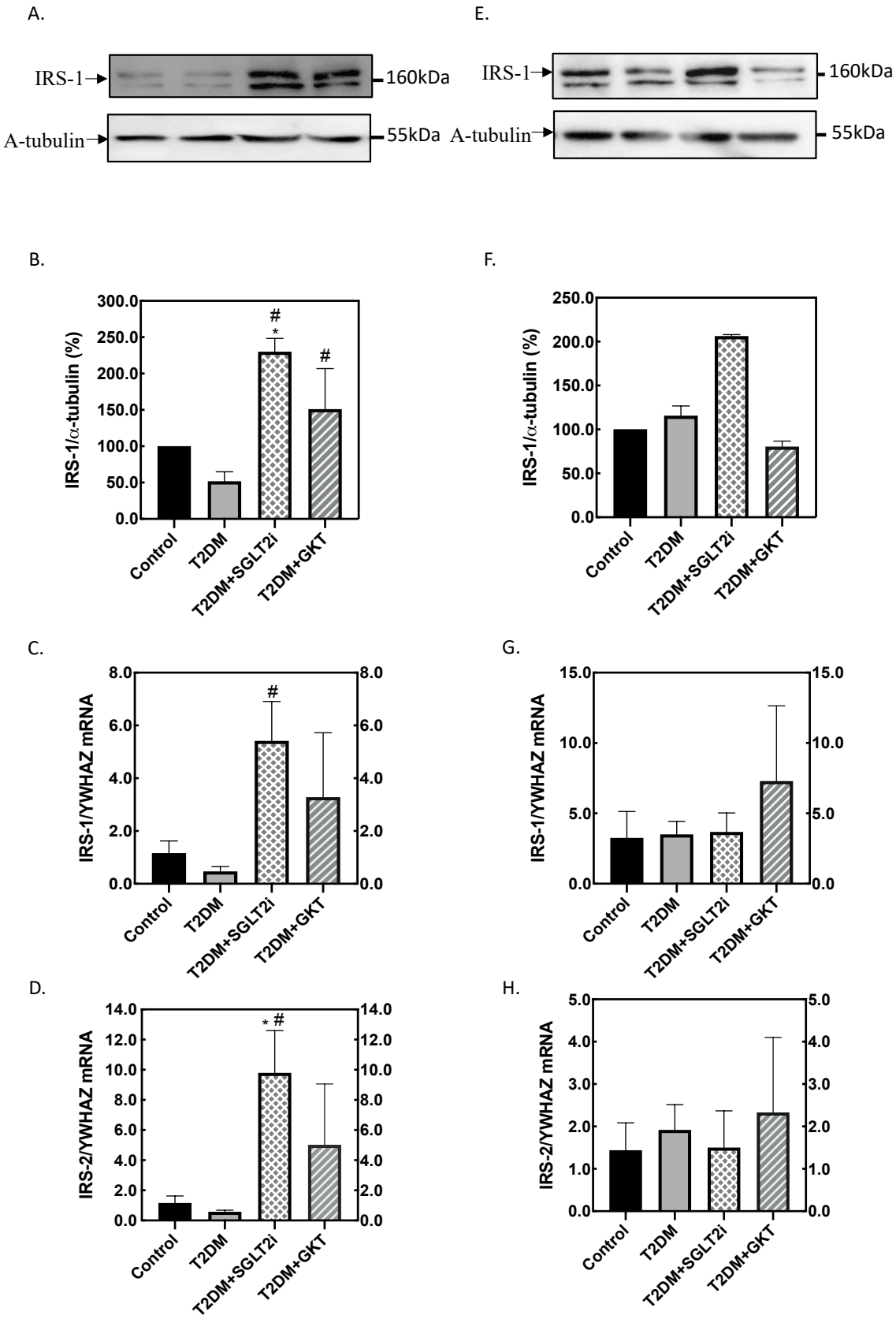
A.
IRS-1
160kDa
A-tubulin
55kDa
E.
IRS-1
160kDa
A-tubulin
55kDa
B.
IRS-1/α-tubulin (%)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
#
*
#
Control
T2DM
T2DM+SGLT2i
T2DM+GKT
F.
IRS-1/α-tubulin (%)
250.0
200.0
150.0
100.0
50.0
0.0
Control
T2DM
T2DM+SGLT2i
T2DM+GKT
C.
IRS-1/YWHAZ mRNA
8.0
6.0
4.0
2.0
0.0
#
Control
T2DM
T2DM+SGLT2i
T2DM+GKT
G.
IRS-1/YWHAZ mRNA
15.0
10.0
5.0
0.0
Control
T2DM
T2DM+SGLT2i
T2DM+GKT
D.
IRS-2/YWHAZ mRNA
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
#
Control
T2DM
T2DM+SGLT2i
T2DM+GKT
H.
IRS-2/YWHAZ mRNA
5.0
4.0
3.0
2.0
1.0
0.0
Control
T2DM
T2DM+SGLT2i
T2DM+GKT

Figure 10. The downregulation of IRS-1 and IRS-2 in the prefrontal cortex of a model of T2DM.
IRS-1 protein and mRNA expression were quantified using western blot analysis (B) and RT-PCR (C) in the prefrontal cortex. Representative image of the western blot gel is presented in A. mRNA levels of IRS-2 were also assessed in the prefrontal cortex (D). Expression was also studied in the hippocampus for IRS-1 protein using western blot analysis (F) with its mRNA expression measured using RT-PCR (G). A representative image for the western blot is presented in E. IRS-2 mRNA levels in the hippocampus were also assessed by RT-PCR (H). Analysis was performed with alpha tubulin as a loading control for western blots and YWHAZ as a housekeeping gene for RT-PCR. Values represent the mean ± SEM for control, diabetic, dapagliflozin-treated and GKT-treated groups (n=3), except for IRS-1 protein levels in the hippocampus where n=2 (F). *$p<0.05$ compared to control. #$p<0.05$ compared to T2DM.

G. The effect of Dapagliflozin and GKT on T2DM-induced alterations in the levels of Nox1 and Nox4 in the brain

To assess the involvement of NADPH oxidases in AD-like symptoms induced by T2DM, we examined the expression of Nox1 and Nox4 in the brain. We assessed the protein levels of Nox4 in the prefrontal cortex, and our results indicate a significantly increased level of Nox4 in the diabetic mice compared to the controls (Fig. 11B). Diabetic mice treated with both dapagliflozin and GKT had significantly reduced Nox4 protein expression when compared to the untreated diabetic group. In the hippocampus, there is an apparent trend of increase of Nox4 in the T2DM mice as compared to the control mice, although not significant (Fig. 11E). This trend was abolished when treated with GKT, where Nox4 was significantly decreased compared to the diabetic group.

We also assessed the mRNA levels of Nox1 in the prefrontal cortex which showed a 2-fold increase in the diabetic group compared to the control group, although not significant (Fig.11 C). Interestingly, treatment with dapagliflozin significantly downregulated the mRNA expression of Nox1 compared to the diabetic group whereas GKT had no apparent effect. In the hippocampus, mRNA levels of Nox1 displayed a trend of increase in the diabetic mice compared to the controls (Fig.11 F). These levels showed a trend of downregulation with GKT treatment.

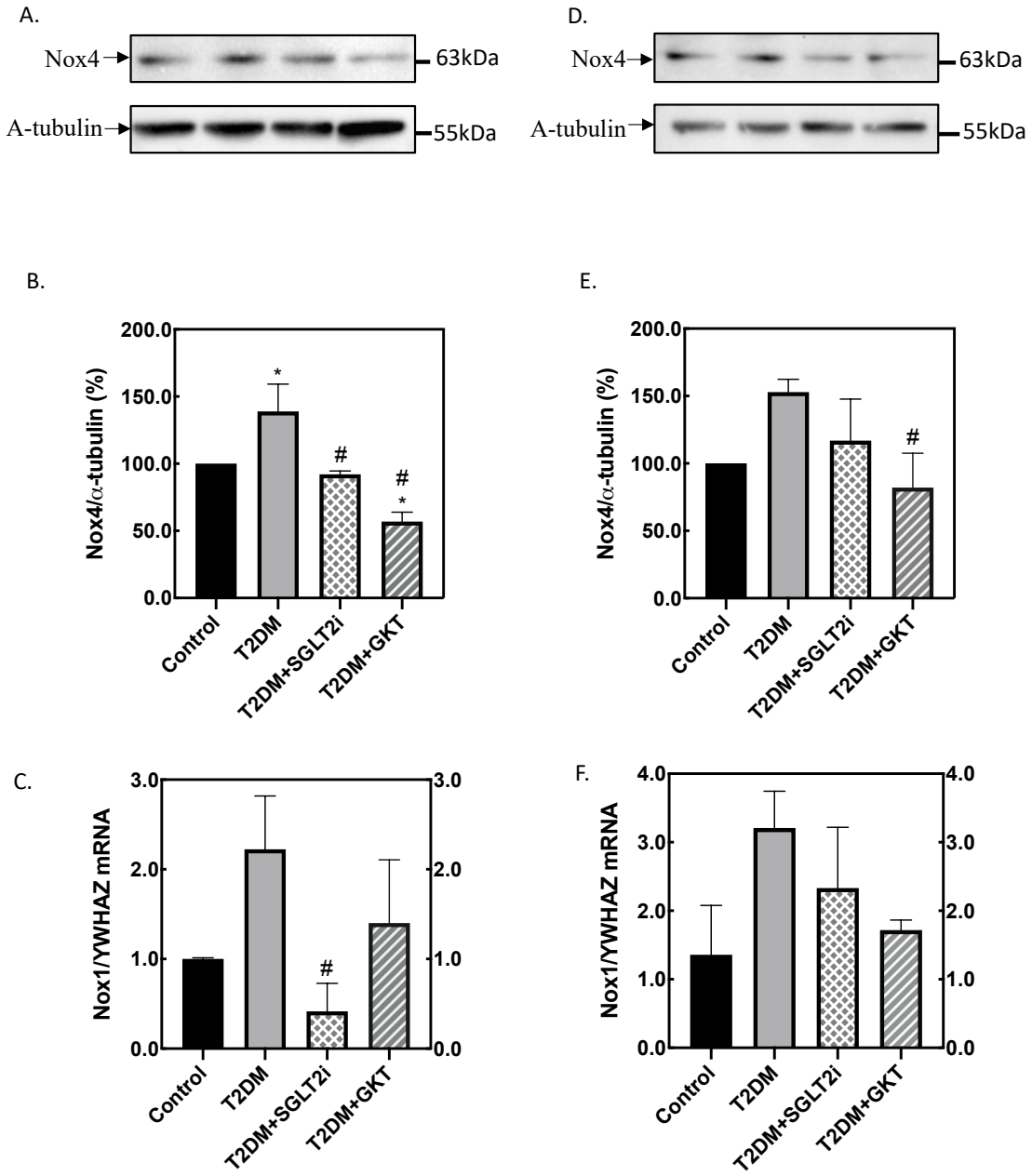

Figure 11. Upregulation of Nox4 and Nox1 in the prefrontal cortex and hippocampus of diabetic mice.
Representative images of the expression of Nox4 and histograms representing western blot quantification in the prefrontal cortex (A,B) and the hippocampus (D,E) with alpha-tubulin used as a loading control. Histograms representing Nox1 mRNA levels measured by RT-PCR in the prefrontal cortex (C) and the hippocampus (F) with YWHAZ as a housekeeping gene. Values represent the mean ± SEM for control, diabetic, dapagliflozin-treated and GKT-treated groups (n=3). *p<0.05 compared to control. #p<0.05 compared to T2DM.

CHAPTER IV

DISCUSSION

Both diabetes mellitus and Alzheimer's disease are age-related disorders which are increasing in prevalence, and a growing body of evidence has demonstrated that the risk of developing AD increases in diabetic patients as compared to healthy individuals [112, 151, 152]. However, the biological mechanisms underlying this association are yet to be understood. In an effort to investigate the possible pathogenic mechanisms underlying the onset of AD-like symptoms in T2DM, we employed a mouse model of T2DM through a combination of HFD and STZ injections.

In order to confirm blood glucose levels, we used the glucose tolerance test and HbA1c test which showed a significant increase in the diabetic group as compared to the controls indicating a state of insulin resistance. In addition to that, we assessed the incidence of diabetic neuropathy which is the most prevalent form of the diabetic complications [47] because identifying a common signaling pathway underlying different diabetic complications could allow us to identify novel therapeutic strategies that may help in preventing the progression of these complications altogether. Diabetic neuropathy is mainly characterized by the loss of peripheral nerve function leading primarily to sensory symptoms as well as poor co-ordination and balance. This can be associated with pain and numbness [149]. In the early stages of diabetic neuropathy, damage to small nerve fibers occurs and results in pain and paresthesias. However, damage to the large nerve fibers occurs at later stages and can result in numbness and loss of protective sensation which may eventually lead to several complications such as

foot ulcerations and falls [153, 154]. The increased threshold to thermal and mechanical stimuli that was seen in the diabetic group as compared to the controls when using Hargreave's method and the electronic von Frey is an indication of hypoalgesia, which is a measure of sensory loss [155]. Thus, the untreated diabetic mice were characterized with a loss of protective sensation. In addition to that, treatment with SGLT2-inhibitor and GKT ameliorated this state of hypoalgesia indicating a relief of this sensory dysfunction. However, this relief was less pronounced in response to the thermal stimulation where levels were not returned to control levels. This may be an indication of an irreversible damage that occurred to the fibers which respond to thermal stimulation before the treatments were given. The T2DM mice also displayed decreased motor nerve function relative to the control mice which was evident through a significantly higher average number of slips and stops. Thus, the T2DM group displayed clear signs of diabetic neuropathy which were ameliorated when treated with either SGLT2-inhibitor or GKT.

We then assessed the presence of AD-like features in our mouse model of T2DM. AD is primarily characterized with the extracellular deposition of Aβ plaques and intracellular neurofibrillary tangles made from hyperphosphorylated tau proteins [10]. Immunohistochemical staining of Aβ in the T2DM mouse brain showed the deposition of Aβ plaques around hippocampal neurons which concurs with previous reports of the development of Aβ pathology in diabetic brains [60, 63].

An appropriate balance in the phosphorylation and dephosphorylation of tau residues is needed to maintain its physiological roles [156]. However, in an insulin resistant state, the IRS-1/PI3K pathway is diminished leading to an activation of GSK-3B and lower phosphatase function which can lead to an increased phosphorylation of tau proteins [15].

Studying the protein expression of ptauS404 in the prefrontal cortex and hippocampus of T2DM mice showed a trend of increase compared to the controls which may indicate that the hyperphosphorylation is still at its earlier stages. Tau pathology is known to spread in a well-defined manner starting at the transentorhinal region then spreading to other areas of the brain, and its spreading is strongly correlated with the extent of cognitive decline in AD [22]. Since multiple phosphorylation sites are implicated in AD pathogenesis, we should also examine the hyperphosphorylation of tau at other sites. The IHC staining for ptauS404 and ptauS396 in T2DM demonstrates that the hyperphosphorylation of tau has begun around the hippocampal area. Tau is localized within axons under physiological conditions; however, when it gets hyperphosphorylated in AD, it tends to accumulate in the soma and proximal dendrites [157] which can be detected in our staining results. In line with our results, several studies have previously reported tau hyperphosphorylation in diabetic brains [68, 69].

To test for AD-like cognitive impairment in our T2DM model, two cognitive behavioral tests were employed. Due to the damage of the medial temporal lobes in Alzheimer's disease, the first symptoms often experienced by AD patients are impairments to spatial and episodic memory [158]. The most common clinical features of AD are impairments in learning and recall of recently-learned information; this also tends to be accompanied by other cognitive deficits such as object agnosia [9]. This is due to the involvement of the medial temporal lobe in the acquisition of new memories [159]. However, the prefrontal cortex is also involved in the processing of working memory [144].

After analyzing the results of the spontaneous alternation in the T-maze, the T2DM mice displayed a significantly decreased alternation of goal arm as compared to the controls suggesting an inability of the mice to remember the first arm that was previously visited. This

is an indication of impaired spatial working memory. The novel object recognition test relies on a visual-recall task which is a component of recognition memory. In general, mice are known to have an innate preference for novelty, so when they recognize a familiar object, they are likely to spend more time on the novel object instead [160]. Thus, when the T2DM mice spent almost the same amount of time on both objects, they were unable to discriminate between the two objects indicating an impairment in their recognition memory. However, when treated with either dapagliflozin or GKT, these impairments were ameliorated in both tests. This is an indication of the treatments' ability to rescue both spatial working memory and recognition memory in a model of T2DM. These results concur with previously published data on the ability of SGLT2-inhibitor to prevent cognitive impairment in insulin resistance models [141, 142], and to our knowledge, this is the first report of GKT's ameliorative properties towards cognitive decline. Taken together, our results demonstrate that AD-like symptoms can be induced by T2DM which is in accordance with previously published data where T2DM was associated with cognitive decline, Aβ accumulation and tau hyperphosphorylation. Along with the previously mentioned damages induced to the peripheral nervous system, these changes clearly demonstrate that T2DM is inducing changes within the whole nervous system ultimately leading to a generalized state of dysfunction.

Diabetes and AD share multiple underlying pathogenic features including insulin resistance, oxidative stress, inflammation and impaired glucose metabolism [39, 71]. Thus, the association between them may be a multifactorial one. In this study, we investigated the involvement of both insulin resistance and oxidative stress. The downregulation of insulin signaling and IRS proteins is crucial for the progression of diabetic complications [150], and insulin resistance is known to increase the risk of memory loss and Alzheimer's disease [60].

IRS-1 and IRS-2 are among the most important adaptor proteins in insulin signaling because they take part in many essential cellular functions [81]. In the brain, IRS-1 and IRS-2 are distributed throughout various structures including olfactory bulb, cortex, hippocampus, hypothalamus and cerebellum [161], and they are highly expressed in the hippocampus and cerebral cortex [162]. Studying postmortem AD brains has shown diminished levels of total IRS-1 and IRS-2 with increased phosphorylation of IRS-1 at Ser312 and Ser616 [102]. These reductions in the expression of IRS-1 and IRS-2 will ultimately result in decreased activation of downstream components in the insulin signalling pathway which is indicative of insulin resistance. Our results parallel these findings, where IRS-1 and IRS-2 protein and mRNA levels were decreased with T2DM in the prefrontal cortex. In addition to that, both treatments seem to be working to upregulate IRS1 and IRS2 indicating their possible effects on the insulin signalling pathway. Previous reports of treatment with SGLT-2 inhibitors have demonstrated an improvement of insulin resistance in obesity and T2DM [163, 164].

In the hippocampus we see no changes in IRS-1 and IRS-2 levels which requires further investigation in future studies since the hippocampus is typically one of the first structures to undergo pathological changes in AD [165]. One study has demonstrated a trend of increase in IRS-1 levels in the hippocampus in human AD brains [31]; however, this does not refute the hypothesis of the development of impaired insulin signalling within the hippocampus. This calls for further analysis of different proteins involved in insulin signalling such as the insulin receptor itself, while also taking into consideration any changes in IRS-1 and IRS-2 at the phosphorylation level.

To investigate the involvement of oxidative stress in the development of AD-like symptoms in T2DM, we focussed on the NADPH oxidase system which is among the major

contributors to ROS production in neurological disorders [166]. A previous study has shown that Nox activity is upregulated in mild cognitive impairment [167]; however, the roles of Nox1 and Nox4 in cognitive impairment have not been fully investigated. Although they are not the only Nox isoforms in the central nervous system, several studies have previously reported their presence in the brain [122, 166, 168]. Our results demonstrate that Nox4 protein and Nox1 mRNA levels show an increase in the prefrontal cortex and hippocampus of T2DM mice suggesting elevated ROS production within these structures. To our knowledge, this is the first report showing increased Nox1 and Nox4 levels associated with AD-like symptoms in T2DM. Treatment with GKT, which is a dual Nox1/Nox4 inhibitor, was able to reduce Nox4 protein levels in the prefrontal cortex and hippocampus while showing a trend of decrease in the mRNA levels of Nox1in the hippocampus. Treatment with SGLT-2 inhibitor was able to downregulate Nox1 and Nox4 in the prefrontal cortex indicating a possible mechanism by which it reduces ROS production. Several studies have previously reported the effects of SGLT-2 inhibitors on reducing oxidative stress associated with diabetic complications such as cardiovascular disease and retinopathy [139, 169].

Although these underlying mechanisms may seem independent at first, they also tend to show significant interactions with each other. One of the potential sources of oxidative stress is impaired insulin/IGF signaling. Insulin-resistant human populations have shown evidence of decreased mitochondrial activity [170, 171], and a mouse model which lacks 95% of the insulin receptors within the brain exhibited impairments in mitochondrial function [172]. These impairments are known to reduce ATP production while increasing the generation of ROS [15]. However, other studies have also focused on oxidative stress as a main trigger for the development of insulin resistance [173, 174]. Thus, there could be a complex interplay

between the 2 mechanisms which may ultimately contribute to the pathogenesis of AD-like symptoms in diabetes. Understanding the mechanisms and pathogenesis of AD-like symptoms associated with diabetes is important for preventing its progression and finding alternative treatments. Overall, our results show that AD-like symptoms develop in a model of T2DM and are associated with a trend of decrease in IRS-1 and IRS-2 levels and increase in Nox1 and Nox4 levels. Our findings suggest that T2DM induces a state of insulin resistance in the brain paralleled by ROS overproduction through Nox1 and Nox4 isoforms to induce AD-like pathology of memory loss, Aβ plaque deposition and tau hyperphosphorylation. Treatment with SGLT2-inhibitor or GKT can ameliorate cognitive dysfunction associated with T2DM by targeting these pathways which suggests their possible usage as novel treatment strategies for the prevention of T2DM-induced AD.

Limitations and Future Perspectives

This study had some limitations primarily beginning with a small number of animals. Increasing the number of mice used in future studies will help make the results more reliable, especially for the behavioral tests. In addition to that, working with prefrontal cortex and hippocampal tissue limits the number of molecular experiments that can be performed on such small tissue. In the future, an interesting experiment to add would be the NADPH Oxidase Assay. Furthermore, the immunohistochemical staining was done on only one animal from each group to provide representative images; however, increasing the number of sections from each group to allow quantification of Aβ and ptau is also necessary in the future. We also need to quantify the levels of total tau, and study the effects of both

treatments on the Aβ and tau pathology. It will be interesting as well to focus on other indicators of insulin resistance especially in the hippocampus which showed no change in IRS levels. Additionally, we would also like to see the involvement of neuroinflammation in diabetes-induced AD by assessing the astrocytes and microglia in T2DM, and it would be of great value to this study to add an experimental group only implicated with AD and vehicle-treated groups. In addition to that, we would like to see the effect of combining the two treatments together.

REFERENCES

1. Du, X., X. Wang, and M. Geng, *Alzheimer's disease hypothesis and related therapies.* Transl Neurodegener, 2018. **7**: p. 2.
2. Lane, C.A., J. Hardy, and J.M. Schott, *Alzheimer's disease.* Eur J Neurol, 2018. **25**(1): p. 59-70.
3. Prince, M., et al., *World Alzheimer Report 2014*. Dementia and risk reduction: An analysis of protective and modifiable risk factors. 2014, London: Alzheimer's Disease International.
4. Zhang, L., et al., *Advance of sporadic Alzheimer's disease animal models.* Med Res Rev, 2020. **40**(1): p. 431-458.
5. Bateman, R.J., et al., *Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease.* Alzheimers Research & Therapy, 2011. **3**(1).
6. Masters, C.L., et al., *Alzheimer's disease.* Nature Reviews Disease Primers, 2015. **1**(1): p. 15056.
7. Saunders, A.M., et al., *Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease.* Neurology, 1993. **43**(8): p. 1467-72.
8. Verghese, P.B., J.M. Castellano, and D.M. Holtzman, *Apolipoprotein E in Alzheimer's disease and other neurological disorders.* Lancet Neurol, 2011. **10**(3): p. 241-52.
9. McKhann, G.M., et al., *The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.* Alzheimer's & dementia : the journal of the Alzheimer's Association, 2011. **7**(3): p. 263-269.
10. Nakabeppu, Y., *Molecular Pathophysiology of Insulin Depletion, Mitochondrial Dysfunction, and Oxidative Stress in Alzheimer's Disease Brain.* Adv Exp Med Biol, 2019. **1128**: p. 27-44.
11. Zhang, B., et al., *Integrated systems approach identifies genetic nodes and networks in late-onset Alzheimer's disease.* Cell, 2013. **153**(3): p. 707-20.
12. Pratico, D., *Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows.* Ann N Y Acad Sci, 2008. **1147**: p. 70-8.
13. Ryu, J.C., et al., *Consequences of Metabolic Disruption in Alzheimer's Disease Pathology.* Neurotherapeutics, 2019. **16**(3): p. 600-610.
14. Karran, E., M. Mercken, and B. De Strooper, *The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics.* Nat Rev Drug Discov, 2011. **10**(9): p. 698-712.
15. de la Monte, S.M., *Contributions of brain insulin resistance and deficiency in amyloid-related neurodegeneration in Alzheimer's disease.* Drugs, 2012. **72**(1): p. 49-66.
16. Ueno, M., et al., *Clearance of beta-amyloid in the brain.* Curr Med Chem, 2014. **21**(35): p. 4085-90.
17. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics.* Science, 2002. **297**(5580): p. 353-6.
18. Serrano-Pozo, A., et al., *Neuropathological alterations in Alzheimer disease.* Cold Spring Harb Perspect Med, 2011. **1**(1): p. a006189.
19. Brier, M.R., et al., *Tau and Abeta imaging, CSF measures, and cognition in Alzheimer's disease.* Sci Transl Med, 2016. **8**(338): p. 338ra66.

20. Montine, T.J., et al., *National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach.* Acta Neuropathol, 2012. **123**(1): p. 1-11.
21. Lee, G. and C.J. Leugers, *Tau and tauopathies.* Prog Mol Biol Transl Sci, 2012. **107**: p. 263-93.
22. Kametani, F. and M. Hasegawa, *Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease.* Front Neurosci, 2018. **12**: p. 25.
23. Leibson, C.L., et al., *Risk of dementia among persons with diabetes mellitus: a population-based cohort study.* Am J Epidemiol, 1997. **145**(4): p. 301-8.
24. Ott, A., et al., *Association of diabetes mellitus and dementia: the Rotterdam Study.* Diabetologia, 1996. **39**(11): p. 1392-7.
25. Ott, A., et al., *Diabetes mellitus and the risk of dementia: The Rotterdam Study.* Neurology, 1999. **53**(9): p. 1937-42.
26. Luchsinger, J.A., et al., *Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort.* Am J Epidemiol, 2001. **154**(7): p. 635-41.
27. Kuusisto, J., et al., *Association between features of the insulin resistance syndrome and Alzheimer's disease independently of apolipoprotein E4 phenotype: cross sectional population based study.* Bmj, 1997. **315**(7115): p. 1045-9.
28. Lillioja, S., et al., *Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians.* N Engl J Med, 1993. **329**(27): p. 1988-92.
29. Zhang, J., et al., *An updated meta-analysis of cohort studies: Diabetes and risk of Alzheimer's disease.* Diabetes Res Clin Pract, 2017. **124**: p. 41-47.
30. Frolich, L., et al., *A disturbance in the neuronal insulin receptor signal transduction in sporadic Alzheimer's disease.* Ann N Y Acad Sci, 1999. **893**: p. 290-3.
31. Talbot, K., et al., *Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline.* J Clin Invest, 2012. **122**(4): p. 1316-38.
32. Lee, S., et al., *CSF and Brain Indices of Insulin Resistance, Oxidative Stress and Neuro-Inflammation in Early versus Late Alzheimer's Disease.* J Alzheimers Dis Parkinsonism, 2013. **3**: p. 128.
33. Rivera, E.J., et al., *Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine.* J Alzheimers Dis, 2005. **8**(3): p. 247-68.
34. Steen, E., et al., *Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes?* J Alzheimers Dis, 2005. **7**(1): p. 63-80.
35. Liu, Y., et al., *Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes.* J Pathol, 2011. **225**(1): p. 54-62.
36. de la Monte, S.M. and J.R. Wands, *Alzheimer's disease is type 3 diabetes-evidence reviewed.* J Diabetes Sci Technol, 2008. **2**(6): p. 1101-13.
37. Petersmann, A., et al., *Definition, Classification and Diagnosis of Diabetes Mellitus.* Exp Clin Endocrinol Diabetes, 2019. **127**(S 01): p. S1-S7.
38. Saeedi, P., et al., *Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition.* Diabetes Res Clin Pract, 2019. **157**: p. 107843.

39. Chen, Y., Q. Yu, and C.-X. Gong, *Molecular Connection Between Diabetes and Dementia*, in *Diabetes Mellitus: A risk factor for Alzheimer's Disease*, Y. Nakabeppu and T. Ninomiya, Editors. 2019, Springer Singapore: Singapore. p. 103-131.
40. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes care, 2010. **33 Suppl 1**(Suppl 1): p. S62-S69.
41. Forbes, J.M. and M.E. Cooper, *Mechanisms of diabetic complications.* Physiol Rev, 2013. **93**(1): p. 137-88.
42. DeFronzo, R.A. and D. Tripathy, *Skeletal muscle insulin resistance is the primary defect in type 2 diabetes.* Diabetes Care, 2009. **32 Suppl 2**: p. S157-63.
43. Freidenberg, G.R., et al., *Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetic subjects.* The Journal of Clinical Investigation, 1987. **79**(1): p. 240-250.
44. Petersen, M.C. and G.I. Shulman, *Mechanisms of Insulin Action and Insulin Resistance.* Physiol Rev, 2018. **98**(4): p. 2133-2223.
45. Li, L. and C. Holscher, *Common pathological processes in Alzheimer disease and type 2 diabetes: a review.* Brain Res Rev, 2007. **56**(2): p. 384-402.
46. Feldman, E.L., et al., *Diabetic neuropathy.* Nat Rev Dis Primers, 2019. **5**(1): p. 41.
47. Callaghan, B.C., et al., *The Importance of Rare Subtypes in Diagnosis and Treatment of Peripheral Neuropathy: A Review.* JAMA Neurol, 2015. **72**(12): p. 1510-8.
48. Mogensen, C.E., C.K. Christensen, and E. Vittinghus, *The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy.* Diabetes, 1983. **32 Suppl 2**: p. 64-78.
49. Chronic Kidney Disease Prognosis, C., et al., *Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis.* Lancet, 2010. **375**(9731): p. 2073-81.
50. Mohamed, Q., M.C. Gillies, and T.Y. Wong, *Management of diabetic retinopathy: a systematic review.* JAMA, 2007. **298**(8): p. 902-16.
51. Cheung, N., P. Mitchell, and T.Y. Wong, *Diabetic retinopathy.* Lancet, 2010. **376**(9735): p. 124-36.
52. Frank, R.N., *Diabetic retinopathy.* N Engl J Med, 2004. **350**(1): p. 48-58.
53. Haffner, S.M., et al., *Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction.* N Engl J Med, 1998. **339**(4): p. 229-34.
54. *Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group.* Lancet, 1998. **352**(9131): p. 837-53.
55. Nathan, D.M., et al., *The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus.* N Engl J Med, 1993. **329**(14): p. 977-86.
56. Gasparini, L., et al., *Does insulin dysfunction play a role in Alzheimer's disease?* Trends Pharmacol Sci, 2002. **23**(6): p. 288-93.
57. Nouwen, A., et al., *Prevalence of depression in individuals with impaired glucose metabolism or undiagnosed diabetes: a systematic review and meta-analysis of the European Depression in Diabetes (EDID) Research Consortium.* Diabetes Care, 2011. **34**(3): p. 752-62.

58. Adeniyi, A.F., J.O. Adeleye, and C.Y. Adeniyi, *Diabetes, sexual dysfunction and therapeutic exercise: a 20 year review.* Curr Diabetes Rev, 2010. **6**(4): p. 201-6.
59. Cukierman, T., H.C. Gerstein, and J.D. Williamson, *Cognitive decline and dementia in diabetes--systematic overview of prospective observational studies.* Diabetologia, 2005. **48**(12): p. 2460-9.
60. Craft, S., *Insulin resistance and Alzheimer's disease pathogenesis: potential mechanisms and implications for treatment.* Curr Alzheimer Res, 2007. **4**(2): p. 147-52.
61. Sridhar, G.R., G. Lakshmi, and G. Nagamani, *Emerging links between type 2 diabetes and Alzheimer's disease.* World journal of diabetes, 2015. **6**(5): p. 744-751.
62. Xu, W., et al., *Meta-analysis of modifiable risk factors for Alzheimer's disease.* J Neurol Neurosurg Psychiatry, 2015. **86**(12): p. 1299-306.
63. Zhao, L., et al., *Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention.* J Neurosci, 2004. **24**(49): p. 11120-6.
64. Deane, R., et al., *RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain.* Nat Med, 2003. **9**(7): p. 907-13.
65. Donahue, J.E., et al., *RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease.* Acta Neuropathol, 2006. **112**(4): p. 405-15.
66. Lovestone, S. and U. Smith, *Advanced glycation end products, dementia, and diabetes.* Proc Natl Acad Sci U S A, 2014. **111**(13): p. 4743-4.
67. Kimura, N., *Type II Diabetes Mellitus Accelerates Age-Dependent Aβ Pathology in Cynomolgus Monkey Brain.* Adv Exp Med Biol, 2019. **1128**: p. 133-145.
68. Kim, B., et al., *Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes.* Endocrinology, 2009. **150**(12): p. 5294-301.
69. Liu, Y., et al., *Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease.* J Neurochem, 2009. **111**(1): p. 242-9.
70. Last, D., et al., *Global and regional effects of type 2 diabetes on brain tissue volumes and cerebral vasoreactivity.* Diabetes Care, 2007. **30**(5): p. 1193-9.
71. Ninomiya, T., *Epidemiological Evidence of the Relationship Between Diabetes and Dementia.* Diabetes Mellitus: A Risk Factor for Alzheimer's Disease, 2019. **1128**: p. 13-25.
72. Czech, M.P., *Insulin action and resistance in obesity and type 2 diabetes.* Nat Med, 2017. **23**(7): p. 804-814.
73. Kahn, S.E., *The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes.* Diabetologia, 2003. **46**(1): p. 3-19.
74. Hubbard, S.R., *The insulin receptor: both a prototypical and atypical receptor tyrosine kinase.* Cold Spring Harb Perspect Biol, 2013. **5**(3): p. a008946.
75. Boucher, J., A. Kleinridders, and C.R. Kahn, *Insulin receptor signaling in normal and insulin-resistant states.* Cold Spring Harb Perspect Biol, 2014. **6**(1).
76. Kolterman, O.G., et al., *Receptor and postreceptor defects contribute to the insulin resistance in noninsulin-dependent diabetes mellitus.* J Clin Invest, 1981. **68**(4): p. 957-69.
77. Olefsky, J.M., O.G. Kolterman, and J.A. Scarlett, *Insulin action and resistance in obesity and noninsulin-dependent type II diabetes mellitus.* Am J Physiol, 1982. **243**(1): p. E15-30.

78. Tiwari, S., et al., *Reduced expression of insulin receptors in the kidneys of insulin-resistant rats.* J Am Soc Nephrol, 2007. **18**(10): p. 2661-71.
79. Welsh, G.I., et al., *Insulin signaling to the glomerular podocyte is critical for normal kidney function.* Cell Metab, 2010. **12**(4): p. 329-340.
80. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.
81. Hanke, S. and M. Mann, *The phosphotyrosine interactome of the insulin receptor family and its substrates IRS-1 and IRS-2.* Molecular & cellular proteomics : MCP, 2009. **8**(3): p. 519-534.
82. Franke, T.F., et al., *Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate.* Science, 1997. **275**(5300): p. 665-8.
83. Gray, S.M., R.I. Meijer, and E.J. Barrett, *Insulin regulates brain function, but how does it get there?* Diabetes, 2014. **63**(12): p. 3992-3997.
84. Plum, L., M. Schubert, and J.C. Bruning, *The role of insulin receptor signaling in the brain.* Trends Endocrinol Metab, 2005. **16**(2): p. 59-65.
85. Zhang, Y., et al., *An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex.* J Neurosci, 2014. **34**(36): p. 11929-47.
86. Siddle, K., *Signalling by insulin and IGF receptors: supporting acts and new players.* J Mol Endocrinol, 2011. **47**(1): p. R1-10.
87. Bedinger, D.H. and S.H. Adams, *Metabolic, anabolic, and mitogenic insulin responses: A tissue-specific perspective for insulin receptor activators.* Mol Cell Endocrinol, 2015. **415**: p. 143-56.
88. Baura, G.D., et al., *Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain.* J Clin Invest, 1993. **92**(4): p. 1824-30.
89. Banks, W.A., J.B. Owen, and M.A. Erickson, *Insulin in the brain: there and back again.* Pharmacol Ther, 2012. **136**(1): p. 82-93.
90. Csajbok, E.A. and G. Tamas, *Cerebral cortex: a target and source of insulin?* Diabetologia, 2016. **59**(8): p. 1609-15.
91. de la Monte, S.M. and J.R. Wands, *Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease.* J Alzheimers Dis, 2005. **7**(1): p. 45-61.
92. Rhea, E.M. and W.A. Banks, *Role of the Blood-Brain Barrier in Central Nervous System Insulin Resistance.* Frontiers in neuroscience, 2019. **13**: p. 521-521.
93. Fernandez, A.M., et al., *Insulin Regulates Astrocytic Glucose Handling Through Cooperation With IGF-I.* Diabetes, 2017. **66**(1): p. 64-74.
94. Jurcovicova, J., *Glucose transport in brain - effect of inflammation.* Endocr Regul, 2014. **48**(1): p. 35-48.
95. Debons, A.F., I. Krimsky, and A. From, *A direct action of insulin on the hypothalamic satiety center.* Am J Physiol, 1970. **219**(4): p. 938-43.
96. Craft, S., et al., *Insulin dose-response effects on memory and plasma amyloid precursor protein in Alzheimer's disease: interactions with apolipoprotein E genotype.* Psychoneuroendocrinology, 2003. **28**(6): p. 809-22.
97. Haan, M.N., *Therapy Insight: type 2 diabetes mellitus and the risk of late-onset Alzheimer's disease.* Nat Clin Pract Neurol, 2006. **2**(3): p. 159-66.

98. de la Monte, S.M., et al., *si-RNA inhibition of brain insulin or insulin-like growth factor receptors causes developmental cerebellar abnormalities: relevance to fetal alcohol spectrum disorder.* Mol Brain, 2011. **4**: p. 13.
99. Kimura, T., et al., *Sequential changes of tau-site-specific phosphorylation during development of paired helical filaments.* Dementia, 1996. **7**(4): p. 177-81.
100. Schubert, M., et al., *Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation.* J Neurosci, 2003. **23**(18): p. 7084-92.
101. Grillo, C.A., et al., *Hippocampal Insulin Resistance Impairs Spatial Learning and Synaptic Plasticity.* Diabetes, 2015. **64**(11): p. 3927-36.
102. Moloney, A.M., et al., *Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling.* Neurobiol Aging, 2010. **31**(2): p. 224-43.
103. Faulstich, M.E., *Brain imaging in dementia of the Alzheimer type.* Int J Neurosci, 1991. **57**(1-2): p. 39-49.
104. Waldron, A.M., et al., *In vivo molecular neuroimaging of glucose utilization and its association with fibrillar amyloid-beta load in aged APPPS1-21 mice.* Alzheimers Res Ther, 2015. **7**(1): p. 76.
105. Wurtman, R., *Biomarkers in the diagnosis and management of Alzheimer's disease.* Metabolism, 2015. **64**(3 Suppl 1): p. S47-50.
106. Benedict, C., et al., *Intranasal insulin as a therapeutic option in the treatment of cognitive impairments.* Exp Gerontol, 2011. **46**(2-3): p. 112-5.
107. Reger, M.A., et al., *Intranasal insulin improves cognition and modulates beta-amyloid in early AD.* Neurology, 2008. **70**(6): p. 440-8.
108. Sies, H., C. Berndt, and D.P. Jones, *Oxidative Stress.* Annual Review of Biochemistry, 2017. **86**(1): p. 715-748.
109. Russell, J.W., et al., *Oxidative injury and neuropathy in diabetes and impaired glucose tolerance.* Neurobiol Dis, 2008. **30**(3): p. 420-9.
110. Vincent, A.M., et al., *Oxidative stress in the pathogenesis of diabetic neuropathy.* Endocr Rev, 2004. **25**(4): p. 612-28.
111. Brownlee, M., *Negative consequences of glycation.* Metabolism, 2000. **49**(2 Suppl 1): p. 9-13.
112. Sims-Robinson, C., et al., *How does diabetes accelerate Alzheimer disease pathology?* Nat Rev Neurol, 2010. **6**(10): p. 551-9.
113. Giugliano, D., A. Ceriello, and G. Paolisso, *Oxidative stress and diabetic vascular complications.* Diabetes Care, 1996. **19**(3): p. 257-67.
114. Eid, A.A., et al., *Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases.* Diabetes, 2009. **58**(5): p. 1201-11.
115. Zhao, Q.D., et al., *NADPH oxidase 4 induces cardiac fibrosis and hypertrophy through activating Akt/mTOR and NFκB signaling pathways.* Circulation, 2015. **131**(7): p. 643-55.
116. Agostinho, P., R.A. Cunha, and C. Oliveira, *Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease.* Curr Pharm Des, 2010. **16**(25): p. 2766-78.
117. Wan, T., et al., *FA-97, a New Synthetic Caffeic Acid Phenethyl Ester Derivative, Protects against Oxidative Stress-Mediated Neuronal Cell Apoptosis and Scopolamine-Induced Cognitive Impairment by Activating Nrf2/HO-1 Signaling.* Oxid Med Cell Longev, 2019. **2019**: p. 8239642.

118. Reddy, V.P., et al., *Oxidative stress in diabetes and Alzheimer's disease.* J Alzheimers Dis, 2009. **16**(4): p. 763-74.
119. Butterfield, D.A., et al., *Elevated levels of 3-nitrotyrosine in brain from subjects with amnestic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease.* Brain Res, 2007. **1148**: p. 243-8.
120. Resende, R., et al., *Brain oxidative stress in a triple-transgenic mouse model of Alzheimer disease.* Free Radic Biol Med, 2008. **44**(12): p. 2051-7.
121. Angeloni, C., et al., *Traumatic brain injury and NADPH oxidase: a deep relationship.* Oxid Med Cell Longev, 2015. **2015**: p. 370312.
122. Vallet, P., et al., *Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia.* Neuroscience, 2005. **132**(2): p. 233-8.
123. Zhou, M., et al., *Dulaglutide ameliorates STZ induced AD-like impairment of learning and memory ability by modulating hyperphosphorylation of tau and NFs through GSK3beta.* Biochem Biophys Res Commun, 2019. **511**(1): p. 154-160.
124. Bendlin, B.B., *Antidiabetic therapies and Alzheimer disease.* Dialogues Clin Neurosci, 2019. **21**(1): p. 83-91.
125. Chen, B., et al., *Metformin Alleviated Abeta-Induced Apoptosis via the Suppression of JNK MAPK Signaling Pathway in Cultured Hippocampal Neurons.* Biomed Res Int, 2016. **2016**: p. 1421430.
126. Farr, S.A., et al., *Metformin Improves Learning and Memory in the SAMP8 Mouse Model of Alzheimer's Disease.* J Alzheimers Dis, 2019. **68**(4): p. 1699-1710.
127. Ou, Z., et al., *Metformin treatment prevents amyloid plaque deposition and memory impairment in APP/PS1 mice.* Brain Behav Immun, 2018. **69**: p. 351-363.
128. Koenig, A.M., et al., *Effects of the Insulin Sensitizer Metformin in Alzheimer Disease: Pilot Data From a Randomized Placebo-controlled Crossover Study.* Alzheimer Dis Assoc Disord, 2017. **31**(2): p. 107-113.
129. Luchsinger, J.A., et al., *Metformin in Amnestic Mild Cognitive Impairment: Results of a Pilot Randomized Placebo Controlled Clinical Trial.* J Alzheimers Dis, 2016. **51**(2): p. 501-14.
130. Sluggett, J.K., et al., *Metformin and risk of Alzheimer's disease among community-dwelling people with diabetes: a national case-control study.* J Clin Endocrinol Metab, 2019.
131. Nadkarni, P., O.G. Chepurny, and G.G. Holz, *Regulation of glucose homeostasis by GLP-1.* Prog Mol Biol Transl Sci, 2014. **121**: p. 23-65.
132. Calsolaro, V. and P. Edison, *Novel GLP-1 (Glucagon-Like Peptide-1) Analogues and Insulin in the Treatment for Alzheimer's Disease and Other Neurodegenerative Diseases.* CNS Drugs, 2015. **29**(12): p. 1023-39.
133. Reitz, C., C. Brayne, and R. Mayeux, *Epidemiology of Alzheimer disease.* Nature Reviews Neurology, 2011. **7**(3): p. 137-152.
134. Xiong, H., et al., *The neuroprotection of liraglutide on Alzheimer-like learning and memory impairment by modulating the hyperphosphorylation of tau and neurofilament proteins and insulin signaling pathways in mice.* J Alzheimers Dis, 2013. **37**(3): p. 623-35.
135. Chen, S., et al., *Liraglutide Improves Water Maze Learning and Memory Performance While Reduces Hyperphosphorylation of Tau and Neurofilaments in APP/PS1/Tau Triple Transgenic Mice.* Neurochem Res, 2017. **42**(8): p. 2326-2335.

136. McClean, P.L., et al., *The diabetes drug liraglutide prevents degenerative processes in a mouse model of Alzheimer's disease.* J Neurosci, 2011. **31**(17): p. 6587-94.
137. Batista, A.F., et al., *The diabetes drug liraglutide reverses cognitive impairment in mice and attenuates insulin receptor and synaptic pathology in a non-human primate model of Alzheimer's disease.* Journal of Pathology, 2018. **245**(1): p. 85-100.
138. Gejl, M., et al., *In Alzheimer's Disease, 6-Month Treatment with GLP-1 Analog Prevents Decline of Brain Glucose Metabolism: Randomized, Placebo-Controlled, Double-Blind Clinical Trial.* Front Aging Neurosci, 2016. **8**: p. 108.
139. Yaribeygi, H., et al., *Sodium-glucose cotransporter inhibitors and oxidative stress: An update.* J Cell Physiol, 2019. **234**(4): p. 3231-3237.
140. Perna, S., et al., *12-month effects of incretins versus SGLT2-Inhibitors on cognitive performance and metabolic profile. A randomized clinical trial in the elderly with Type-2 diabetes mellitus.* Clinical pharmacology : advances and applications, 2018. **10**: p. 141-151.
141. Lin, B., et al., *Glycemic control with empagliflozin, a novel selective SGLT2 inhibitor, ameliorates cardiovascular injury and cognitive dysfunction in obese and type 2 diabetic mice.* Cardiovasc Diabetol, 2014. **13**: p. 148.
142. Sa-Nguanmoo, P., et al., *SGLT2-inhibitor and DPP-4 inhibitor improve brain function via attenuating mitochondrial dysfunction, insulin resistance, inflammation, and apoptosis in HFD-induced obese rats.* Toxicol Appl Pharmacol, 2017. **333**: p. 43-50.
143. Deacon, R.M. and J.N. Rawlins, *T-maze alternation in the rodent.* Nat Protoc, 2006. **1**(1): p. 7-12.
144. Hussein, A.M., M. Bezu, and V. Korz, *Evaluating Working Memory on a T-maze in Male Rats.* Bio-protocol, 2018. **8**(14): p. e2930.
145. Leger, M., et al., *Object recognition test in mice.* Nat Protoc, 2013. **8**(12): p. 2531-7.
146. Squire, L.R., J.T. Wixted, and R.E. Clark, *Recognition memory and the medial temporal lobe: a new perspective.* Nat Rev Neurosci, 2007. **8**(11): p. 872-83.
147. Grundke-Iqbal, I., et al., *Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology.* Proc Natl Acad Sci U S A, 1986. **83**(13): p. 4913-7.
148. Mondragón-Rodríguez, S., et al., *Phosphorylation of tau protein at sites Ser(396-404) is one of the earliest events in Alzheimer's disease and Down syndrome.* Neuropathol Appl Neurobiol, 2014. **40**(2): p. 121-35.
149. Callaghan, B.C., et al., *Diabetic neuropathy: clinical manifestations and current treatments.* Lancet Neurol, 2012. **11**(6): p. 521-34.
150. Lavin, D.P., M.F. White, and D.P. Brazil, *IRS proteins and diabetic complications.* Diabetologia, 2016. **59**(11): p. 2280-2291.
151. Biessels, G.J., et al., *Risk of dementia in diabetes mellitus: a systematic review.* Lancet Neurol, 2006. **5**(1): p. 64-74.
152. Brands, A.M., et al., *The effects of type 1 diabetes on cognitive performance: a meta-analysis.* Diabetes Care, 2005. **28**(3): p. 726-35.
153. Dingwell, J.B. and P.R. Cavanagh, *Increased variability of continuous overground walking in neuropathic patients is only indirectly related to sensory loss.* Gait Posture, 2001. **14**(1): p. 1-10.
154. Mustapa, A., et al., *Postural Control and Gait Performance in the Diabetic Peripheral Neuropathy: A Systematic Review.* Biomed Res Int, 2016. **2016**: p. 9305025.

155. O'Brien, P.D., et al., *Juvenile murine models of prediabetes and type 2 diabetes develop neuropathy.* Dis Model Mech, 2018. **11**(12).
156. Gonçalves, R.A., et al., *The Link Between Tau and Insulin Signaling: Implications for Alzheimer's Disease and Other Tauopathies.* Frontiers in cellular neuroscience, 2019. **13**: p. 17-17.
157. Cotman, C.W., et al., *The role of caspase cleavage of tau in Alzheimer disease neuropathology.* J Neuropathol Exp Neurol, 2005. **64**(2): p. 104-12.
158. Burgess, N., E.A. Maguire, and J. O'Keefe, *The human hippocampus and spatial and episodic memory.* Neuron, 2002. **35**(4): p. 625-41.
159. Scoville, W.B. and B. Milner, *Loss of recent memory after bilateral hippocampal lesions.* J Neurol Neurosurg Psychiatry, 1957. **20**(1): p. 11-21.
160. Lueptow, L.M., *Novel Object Recognition Test for the Investigation of Learning and Memory in Mice.* J Vis Exp, 2017(126).
161. Kleinridders, A., et al., *Insulin action in brain regulates systemic metabolism and brain function.* Diabetes, 2014. **63**(7): p. 2232-43.
162. Lein, E.S., et al., *Genome-wide atlas of gene expression in the adult mouse brain.* Nature, 2007. **445**(7124): p. 168-76.
163. Waseda, N., et al., *Effects of SGLT2 Inhibitors on Insulin Secretion and Insulin Resistance—Results from a Cross-Sectional Study.* Diabetes, 2018. **67**: p. 1187-P.
164. Xu, L. and T. Ota, *Emerging roles of SGLT2 inhibitors in obesity and insulin resistance: Focus on fat browning and macrophage polarization.* Adipocyte, 2018. **7**(2): p. 121-128.
165. Mizutani, T., et al., *Senile dementia of Alzheimer type characterized by laminar neuronal loss exclusively in the hippocampus, parahippocampus and medial occipitotemporal cortex.* Acta Neuropathol, 1990. **80**(6): p. 575-80.
166. Barua, S., et al., *The role of NOX inhibitors in neurodegenerative diseases.* IBRO Reports, 2019. **7**: p. 59-69.
167. Bruce-Keller, A.J., et al., *NOX activity is increased in mild cognitive impairment.* Antioxidants & redox signaling, 2010. **12**(12): p. 1371-1382.
168. Ibi, M., et al., *Depressive-Like Behaviors Are Regulated by NOX1/NADPH Oxidase by Redox Modification of NMDA Receptor 1.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2017. **37**(15): p. 4200-4212.
169. Chen, Y.Y., et al., *Dapagliflozin Prevents NOX- and SGLT2-Dependent Oxidative Stress in Lens Cells Exposed to Fructose-Induced Diabetes Mellitus.* Int J Mol Sci, 2019. **20**(18).
170. Fabbri, E., et al., *Insulin Resistance Is Associated With Reduced Mitochondrial Oxidative Capacity Measured by 31P-Magnetic Resonance Spectroscopy in Participants Without Diabetes From the Baltimore Longitudinal Study of Aging.* Diabetes, 2017. **66**(1): p. 170-176.
171. Lowell, B.B. and G.I. Shulman, *Mitochondrial dysfunction and type 2 diabetes.* Science, 2005. **307**(5708): p. 384-7.
172. Kleinridders, A., et al., *Insulin resistance in brain alters dopamine turnover and causes behavioral disorders.* Proc Natl Acad Sci U S A, 2015. **112**(11): p. 3463-8.
173. Houstis, N., E.D. Rosen, and E.S. Lander, *Reactive oxygen species have a causal role in multiple forms of insulin resistance.* Nature, 2006. **440**(7086): p. 944-8.
174. Tangvarasittichai, S., *Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus.* World journal of diabetes, 2015. **6**(3): p. 456-480.