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ROLE OF INSULIN RESISTANCE IN THE DEVELOPMENT OF ALZHEIMER-LIKE SYMPTOMS IN A TRANSGENIC MOUSE MODEL

by
TAREK ALI JENANI

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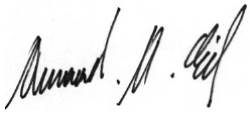

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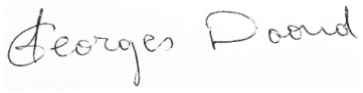

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ABSTRACT

OF THE THESIS OF

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Major: Physiology

Title: Role of Insulin Resistance in the Development of Alzheimer-like Symptoms in a Transgenic Mouse Model

The relationship between Alzheimer's disease (AD) and type 2 Diabetes has been demonstrated in many studies but remains to be thoroughly examined. Poorly controlled hyperglycemia has been shown to increase the risk of developing AD. This association is so strong that some have called AD the "neuro-endocrine disorder", or "type 3 diabetes (T3D)". Type 3 Diabetes mellitus (T3DM) consists of a chronic insulin resistance in nerve cells accompanied by insulin deficiency. In this study, we aimed to further investigate the role of insulin resistance associated with type 2 diabetes in the development of AD-like symptoms and pathologies in rodents.

All experimental procedures 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). Male MKR transgenic mice and their control (FVB mice) were used in this study. Animals were divided into three groups. One group was fed normal diet and the other with high fat diet. A third group of FVB mice was injected with an amyloid beta solution into the lateral ventricle to assess the symptoms of AD. Sensory and cognitive functions were evaluated in all mice prior to and monthly for 3 months using a battery of behavioral and immunohistochemical tests.

Cognitive functions in all mice were evaluated using the T-maze test for spatial memory, and the cheese board maze test for memory recognition. On the other hand, sensitivity to thermal stimulation was measured using the heat hyperalgesia test. Immunohistochemical staining of brain tissues was performed to detect the deposition of β-amyloid peptides. Compared to the control group, both MKR mice and Amyloid-beta injected mice showed deposition of amyloid beta protein in their brain tissues, concomitant with impaired cognitive abilities in both T-maze and cheeseboard maze, and reduced sensory functions. Collectively, our data indicate that Type-2 diabetes and insulin resistance significantly contribute to the sensory and cognitive decline and development of symptoms characteristic of Alzheimer's disease.

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ABBREVIATIONS

T3D: Type 3 Diabetes
DM: Diabetes Mellitus
AD: Alzheimer's Disease
Hx: History
NFT: Neurofibrillary tangles
A-beta: Amyloid beta
APP: Amyloid precursor protein
PSEN1: Presenilin
IGF: Insulin-like growth factors
MCI: Mild cognitive impairment
CNS: Central nervous system
T2DM: Type 2 diabetes mellitus
AChE: Acetyl choline esterase
CEI: Choline esterase inhibitors
NMDA: N-methyl-D-aspartate receptor
HNE: 4-hydroxy-2-nonenal
GLAST: glutamate-aspartate transporter
GSK: Glycogen synthase kinase
IR: Insulin receptor
POMC: arcuate pro-opiomelanocortin
BBB: Blood brain barrier
TNF: Tumor necrosis factor
IRS: Insulin receptor substrate
HFD: High fat diet
IACUC: Institutional animal care use committee

CHAPTER I

INTRODUCTION

A. Type 3 Diabetes

1. Definition

Nowadays, many people are familiar with type 1 or type 2 diabetes mellitus, however, there is another form of diabetes that has just recently been identified, known as type 3 diabetes (T3DM) (Nguyen et al., 2020). This lesser-known type manifests as insulin resistance within the brain and has major potential to impact cognition and contributes to the etiology of Alzheimer's disease [AD] (Arnold et al., 2018). AD has already been identified as the sixth leading cause of death in the United States, and the fifth leading cause of mortality in people 65 and older. It has no current cure, but treatments for symptoms are available and research continues (Osborn & Saunders, 2010). Neurotransmitter deficits, degenerated neurons, synaptic dysfunction, extracellular buildup of β-amyloid (Aβ) and intracellular neurofibrillary tangles (NFT) are the major characteristics of AD (Forette & Hauw, 2010). To produce Aβ peptides of different lengths, the amyloid precursor protein (APP) cleaves at several sites within the membrane due to the active enzymatic component of the γ-secretase complex, presenilin 1 (PSEN1), and PSEN2 (Hillen, 2019). Unfortunately, diabetes is following right behind AD as the seventh leading cause of mortality and is projected to affect almost half a billion people by the year 2045 (Report, 2020). Both diseases have been recognized to have multifactorial interactions involving both the environment and to a lesser degree, genetics. Yet, insulin insensitivity has been linked to memory deficits, cognitive decline, and many of the characteristic symptoms that have been displayed in AD. At the same time, type 2 diabetes has remained one of the most adjustable risk

factors for the development of AD. DM may be classified into four clinical categories: type 1, type 2, type 3, and type 4. In 2005, after the post mortem study of the brains of Alzheimer's disease patients, a new hypothesis came up. It states that Alzheimer's disease is a brain-specific form of diabetes.

It has been demonstrated that compromised cerebral glucose use and energy metabolism are pathological modifications that appear prior to, or with the first stages of cognitive impairment (Herholz, 2010). In addition, the conjecture that insulin deficiency and insulin resistance are the basis of Alzheimer's disease pathological modifications, has led to the proposition of Alzheimer's disease as neuro-endocrine disorder. Thus, its classification as "type 3 diabetes".Type 3 diabetes mellitus (T3DM) consists of a chronic insulin resistance accompanied by an insulin deficiency that is mainly centered in the CNS. It has been proposed that T3DM is one the major causes of Alzheimer's disease pathogenesis (Nguyen et al., 2020).

Moreover, several hallmarks of Alzheimer's disease are influenced by insulin and its signaling pathway. For instance, the expression of the tau gene and the phosphorylation of tau proteins are regulated by the signaling pathways of insulin and the insulin-like growth factor (IGF) (Galle et al., 2020). In addition, the impairment of the insulin cascade causes the apparition of the same patterns of neurodegeneration that can be observed in Alzheimer's disease. The postmortem study of the brains of advanced Alzheimer's disease patients showed an alteration in the levels of insulin and insulin-like growth factor 1 (IGF1) and their receptors. All the pathways related to insulin and IGF1 that mediate cell survival, energy metabolism, tau phosphorylation and expression, and mitochondrial function were defective (Tanokashira et al., 2019).

Interestingly, the use of insulin sensitizers (metformin and thiazolidinedione: rosiglitazone and pioglitazone) as a possible treatment for Alzheimer's disease is being actively investigated (Galimberti & Scarpini, 2017). They may be effective in restoring the insulin pathway and in decreasing insulin resistance.

2. Signs and Symptoms

Researchers have proceeded to disentangle the complex brain changes involved in the onset and progression of Alzheimer's disease. It appears that damage to the brain begins a decade or more before memory and other cognitive issues show up. During this preclinical stage of AD, individuals appear to be symptom-free, but toxic changes are taking place in the brain (Hampel et al., 2004). Brain damage in patients with AD starts to appear in exceptionally early clinical signs and symptoms. For most individuals with Alzheimer's, those who have the late-onset variety- symptoms first show up in their mid-60's. Signs of early-onset Alzheimer's start between the age of 30 and mid-60's. (Jagust et al., 2007),

The first symptoms of Alzheimer's vary from person to person. Memory problems are typically one of the first signs of cognitive impairment related to Alzheimer's disease. Decline in non-memory aspects of cognition, such as word-finding, vision/spatial issues, and impaired reasoning or judgment, may also signal the very early stages of Alzheimer's disease. Some people may be diagnosed with mild cognitive impairment. As the disease progresses, people experience greater memory loss and other cognitive difficulties. (Tuszynski et al., 2005)

Patients can be diagnosed based on certain criteria, which can be divided into three primary stages. A mild preclinical stage (sometimes called early-stage), a

moderate-middle phase of mild cognitive impairment (MCI), and a last stage set apart by indications of severe dementia (Braak et al., 2011). However, these criteria, though determined by neuropsychological testing or diagnosis of exclusion, cannot identify the early phases of AD, the pre-symptomatic stage (Sperling et al., 2013).

Stages of Alzheimer Disease

	Folstein Mini-Mental State Examination (of 30)	Examples of Cognitive Loss	Examples of Functional Loss
Mild	20–24	Some short-term memory loss; word-finding problems	Loss of IADLs such as laundry, housekeeping, and managing medications; may get lost in familiar places
Moderate	10–19	Disorientation to time and place, inability to engage in activities and conversation	Needs assistance with ADLs such as bathing, dressing, and toileting
Severe	< 10	Loss of speech and ambulation, incontinence of bowel and bladder	Dependency in basic ADLs such as feeding oneself; often requires around-the-clock care

ADLs = activities of daily living; IADLs = instrumental activities of daily living.

Fig1: Stages of Alzheimer's disease: Mild-Moderate-Severe. Folstein Mini-Mental State Examination

In mild Alzheimer's disease, a person may seem to be healthy but has more and more trouble making sense of the world around him or her. The realization that something is wrong often comes gradually to the person and his or her family. Problems can include: Memory loss, poor judgment leading to bad decisions, loss of spontaneity and sense of initiative, taking longer to complete normal daily tasks, repeating questions, trouble handling money and paying bills, wandering and getting lost, losing things or misplacing them in odd places, mood and personality changes, increased anxiety and/or aggression (Urbanowitsch et al., 2015). Alzheimer's disease is often diagnosed at this stage.

In moderate stage, symptoms may include: Increased memory loss and confusion, inability to learn new things, difficulty with language and problems with reading, writing, and working with numbers, difficulty organizing thoughts and thinking

logically, shortened attention span, and problems coping with new situations. Moreover, difficulty carrying out multistep tasks, problems recognizing family and friends, hallucinations, paranoia, impulsive behavior such as undressing at inappropriate times or places or using vulgar language, inappropriate outbursts of anger, restlessness, agitation, anxiety, tearfulness, wandering—especially in the late afternoon or evening, repetitive statements or movement, occasional muscle twitches are all possible symptoms of AD (Wei et al., 2017).

People with severe Alzheimer's cannot communicate and are completely dependent on others for their care. In the terminal stage of the disease, the person may be in bed most or all of the time as the body shuts down. Their symptoms often include: Inability to communicate, weight loss, seizures, skin infections, difficulty swallowing, groaning, moaning, or grunting, increased sleeping, and loss of bowel and bladder control (Bature et al., 2017).

A common cause of death for people with Alzheimer's disease is aspiration pneumonia. This type of pneumonia develops when a person cannot swallow properly and takes food or liquids into the lungs instead of air. There is currently no cure for Alzheimer, though there are medications that can treat the symptoms of the disease.

3. Causes and Mechanisms

a. Insulin Resistance/Impaired Glucose Tolerance:

Insulin is a peptide secreted by the pancreas and plays an important role in the regulation of glucose metabolism in peripheral tissues. Although the role of insulin in the periphery is well understood, less is known about its multifactorial role in the brain.

The CNS has been wrongly considered for too long to be insulin insensitive. However, emerging evidence from human and animal studies indicate that insulin influences cerebral bioenergetics, enhances synaptic viability and dendritic spine formation, and increases turnover of neurotransmitters, such as dopamine (McEwen & Reagan, 2004). Insulin also has a role in proteostasis, influencing clearance of the amyloid β peptide and phosphorylation of tau, which are hallmarks of Alzheimer's disease. Insulin also modulates vascular function through effects on vasoreactivity, lipid metabolism, and inflammation (Sedaghat, 2020). Through these multiple pathways, insulin dysregulation could contribute to neurodegeneration. Thus, new approaches to restore cerebral insulin function that could offer therapeutic benefit to adults with Alzheimer's disease, vascular cognitive impairment, or related disorders are being investigated.

Studies show the risk of developing Alzheimer's disease among people with diabetes is 65% higher than that of those without diabetes. With such a strong link, research has focused on explaining the connection between the two diseases. Researchers have attempted to characterize AD as a metabolic disorder, which will lead to abnormalities connected to progressive brain insulin resistance with resulting impedance of central insulin signaling forms, aggregation of neurotoxins, neuronal stress, and neurodegeneration.

Brain insulin resistance is a significant feature of (AD) (Wang & Michaelis, 2010). This phenomenon by itself can promote many of the neural and cognitive abnormalities of AD. Insulin resistance, which refers to the reduced sensitivity of target tissues to the favorable effects of insulin, is related to multiple chronic conditions known to impact cognition and increase dementia risk (Ekblad et al., 2017).

Rising information illustrates significant roles for brain insulin resistance and insulin deficiency as mediators of cognitive impairment and neurodegeneration, especially AD. Insulin and insulin-like growth factors (IGFs) control neuronal survival, energy metabolism, and plasticity, which are required for learning and memory. Consequently, endogenous brain-specific impairments in insulin and IGF signaling account for majority of AD-associated abnormalities (C.J. et al., 2012). However, a second major mechanism of cognitive impairment has been connected to obesity and T2DM. Human and experimental animal studies revealed that neurodegeneration related with peripheral insulin resistance is likely effectuated by means of a liver-brain hub whereby harmful lipids, counting ceramides, cross the blood brain barrier and cause brain insulin resistance, oxidative stress, neuro-inflammation and cell death (Folch et al., 2019). In essence, there are double mechanisms of brain insulin resistance driving to AD-type neurodegeneration: one mediated by endogenous, CNS factors, and the other mediated by peripheral insulin resistance associated with reduced hippocampal glucose metabolism, lower gray matter volume, and excessive cytotoxic inflammatory markers production (Blázquez et al., 2014).

b. Cholinergic Involvement:

Due to the cholinergic hypothesis of AD – where loss of acetylcholine neurons in the basal forebrain, and loss of enzymatic activity for acetylcholine synthesis is attributed to cognitive decline – it was proposed that Acetyl choline esterase inhibitors (CEI) could be used to inhibit the degradation of Acetylcholine by inactivating cholinesterase (Herholz, 2008). The loss of cholinergic neurons is the major disruptor of the neural circuitry that contributes to AD pathology. The U.S Food and Drug

Administration approved four acetylcholine esterase inhibitors prescribed for mild to moderate AD, and include donepezil, rivastigmine, galantamine and galantamine (It was withdrawn due to hepatotoxicity) (Loy & Schneider, 2006). Donepezil is highly selective for AChE (Seltzer et al., 2004). Rivastigmine inhibits AChE and blocks the part of the active site responsible for plaque deposition. Galantamine stimulates pre- and post-synaptic nicotinic receptors which produce increased levels of ACh (Hansen et al., 2008).

CEIs will not produce a long-lasting improvement in the cognitive function; rather they will slow the cognitive decline and maintain the ability to take care of one self. Comparing the 4 choline esterase inhibitors, it appears that they exhibit similar adverse effect profile, while the optimal duration of treatment is still unknown (Khare & Fatima, 2020). Any time CEI is stopped for more than 3 days, it should be restarted at a lower dose & titrated up (Richarz et al., 2011).

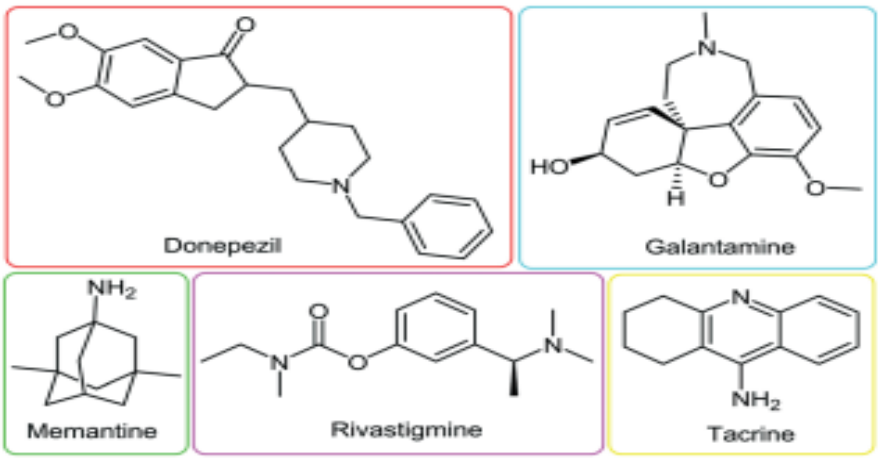


Fig 2: FDA Approved treatments for Alzheimer's disease. Acetylcholine esterase inhibitors: Donepezil, Galantamine, Rivastigmine, Tacrine. NMDA receptor antagonist Memantine

Targeting the cholinergic system continues to be the cornerstone treatment of choice. The function of the cholinergic neurons is critical in cognitive functions such as

learning, memory, and attention. Moreover, the loss of the activity of these neurons is attributed to the loss of acetylcholine, which activates cholinergic receptors. The knowledge of the cholinergic system as a pharmacological target is well established in the AD literature.

c. Glutamate Transporters:

Glutamate is the major excitatory neurotransmitter of the central nervous system and may induce cytotoxicity through persistent activation of glutamate receptors and oxidative stress. There is good evidence for an involvement of the glutamatergic system in the pathophysiology of dementia. The glutamatergic transmission machinery is quite complex and provides a gallery of possible drug targets.

Neuronal toxicity could result from excessive synthesis or release of glutamate or a glutamate-like substance, faulty glutamate reuptake, decreased glutamate degradation, or decreased inhibition of excitatory neurons (Scott et al., 2011).

Excitatory glutamatergic neurotransmission via N-methyl-d-aspartate receptor (NMDAR) is critical for synaptic plasticity and survival of neurons. However, excessive NMDAR activity causes excitotoxicity and promotes cell death, and is considered a potential mechanism underlying neurodegeneration in AD (Liu et al., 2019). The activation of synaptic NMDARs initiates plasticity and stimulates cell survival. In contrast, the activation of extra synaptic NMDARs promotes cell death and thus contributes to the etiology of AD, which can be blocked by an AD drug - memantine, an NMDAR antagonist that selectively blocks the function of extra synaptic NMDARs (McShane et al., 2019).

The synaptic glutamate concentrations are maintained by glutamate transporters. Because of its potential neurotoxicity, clearance of glutamate from the synaptic cleft may be critical for neuronal survival. Inhibition of glutamate uptake from the synapse has been implicated in several neurodegenerative disorders, particularly, in AD; however, the mechanism of decreased transporter activity is unknown. Glutamate transporters are inhibited by oxidative damage from reactive oxygen species and lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) (Scott et al., 2011).

The astrocytic glutamate transporters glutamate-aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) and their human homologs excitatory amino acid transporter 1 (EAAT1) and 2 (EAAT2), respectively, are the major transporters which take up synaptic glutamate to maintain optimal extracellular glutamic levels, thus preventing accumulation in the synaptic cleft and ensuing excitotoxicity. The dysregulation of GLAST/GLT-1 may play a significant role in excitotoxicity and associated neuropathogenesis (Pajarillo et al., 2019).

d. <u>GSK3 Hypothesis:</u>

Glycogen synthase kinase 3 (GSK3) is a constitutively active, proline-directed serine/threonine kinase that plays a part in a number of physiological processes ranging from glycogen metabolism to gene transcription. GSK3 also plays a pivotal and central role in the pathogenesis of both sporadic and familial forms of Alzheimer's disease (AD), an observation that has led us to coin the ‘GSK3 hypothesis of AD’. According to this hypothesis, over-activity of GSK3 accounts for memory impairment, tau hyper-phosphorylation, increased β-amyloid production and local plaque-associated microglial-mediated inflammatory responses; all of which are hallmark characteristics

of AD. If our ‘GSK3 hypothesis of AD’ is substantiated and GSK3 is indeed a causal mediator of AD then inhibitors of GSK3 would provide a novel avenue for therapeutic intervention in this devastating disorder (De Simone et al., 2021).

Glycogen synthase kinase (GSK)-3 has been proposed as the link between the two histopathological hallmarks of AD, the extracellular senile plaques made of beta-amyloid and the intracellular neurofibrillary tangles made of hyperphosphorylated tau. GSK-3 is one of the main tau kinases that modifies several sites of tau protein present in neurofibrillary tangles. It is also able to modulate the generation of beta-amyloid as well as to respond to this peptide. The use of several transgenic models overexpressing GSK-3 has been associated with neuronal death, tau hyperphosphorylation and a decline in cognitive performance. Lithium, a widely used drug for affective disorders, inhibits GSK-3 at therapeutically relevant concentrations and has been demonstrated to prevent tau phosphorylation (De Simone et al., 2021).

4. Relationship Between Diabetes and Dementia:

Diabetes is a major public health burden. Even a modest effect of diabetes on cognitive function has significant public health implications. Several lines of mechanistic evidence implicate a role of insulin and glucose metabolism in the development of dementia, including Alzheimer's disease. Population-based studies have shown that those with type 2 diabetes mellitus have an increased risk of cognitive impairment, dementia, and neurodegeneration. There are many mechanisms through which diabetes could increase risk of dementia, including glycemia, insulin resistance, oxidative stress, advanced glycation end products, inflammatory cytokines, and microvascular and macrovascular disease (Yilin Shen et al., 2018).

Diabetes conferred a 1.25- to 1.91-fold excess risk for cognitive disorders (cognitive impairment and dementia). Subjects with pre-diabetes also had higher risk for dementia. The connection between obesity, diabetes and dementia represents a public health challenge, but also an opportunity to further understand these conditions. The key intersection among them is insulin resistance, which has been classically described to occur in peripheral tissues in type 2 diabetes and obesity. Recently, it has been shown to develop centrally in Alzheimer's disease (AD) brains. Accordingly, experimental observations are identifying that markers of metabolic dysregulation are also present in AD, the most remarkable being insulin resistance. Over the past 10 years, research showed that the brain is an insulin-sensitive organ. The insulin receptor (IR) and related insulin-like growth factor receptors 1 and 2 (IGF1-R/IGF2-R) are expressed not only in hypothalamus, but also in cortex, hippocampus, thalamus, olfactory bulb and other regions (Folch et al., 2018).

It's important to say that insulin modifies neuronal activity by improving the memory function and promoting synaptic plasticity. Synaptic plasticity is the ability of synapses to strengthen or weaken over time, in response to increases or decreases in their activity. Insulin modulates proinflammatory cytokine secretion in microglia and astrocytes *in vitro.* Hypothalamic astrocyte IRs control glucose-induced activation of POMC neurons. The arcuate pro-opiomelanocortin (POMC) neurons in particular have been shown to be critical regulators of metabolism and reproduction because of their projections to several brain areas both in and outside of the hypothalamus, such as autonomic regions of the brain stem and spinal cord (Shen et al., 2016).

Moreover, the glucose transport through the BBB (Blood Brain Barrier), the central and peripheral response to glucose availability are controlled by hypothalamic astrocyte IRs (Hendrix et al., 2021)**.**

Many studies show that insulin signaling is impaired in the brains of AD patients and AD experimental models. In the primary cultures of hippocampal neurons, the Aβ-oligomers can induce neuronal insulin resistance. Moreover, the intracerebroventricular injection of Aβs in mice can induce neuronal insulin resistance. It is mediated by TNF-α activation and IRS inhibition and has major impact on synaptic dysfunction, impaired synaptic plasticity, and synapse loss (Townsend et al., 2007; De Felice et al., 2009). Remarkably, ICV injection of Aβ oligomers also induce peripheral glucose intolerance with classic hallmarks of peripheral insulin resistance, a process also observed in transgenic AD mice models, and that may underlie increased risk for diabetes in AD. Additionally, anti-diabetic drugs exerts beneficial effects on cognition, synapse protection, insulin signaling deficits, and other AD-related pathological mechanisms, such as endoplasmic reticulum stress and chronic inflammation (Balbaa et al., 2017).

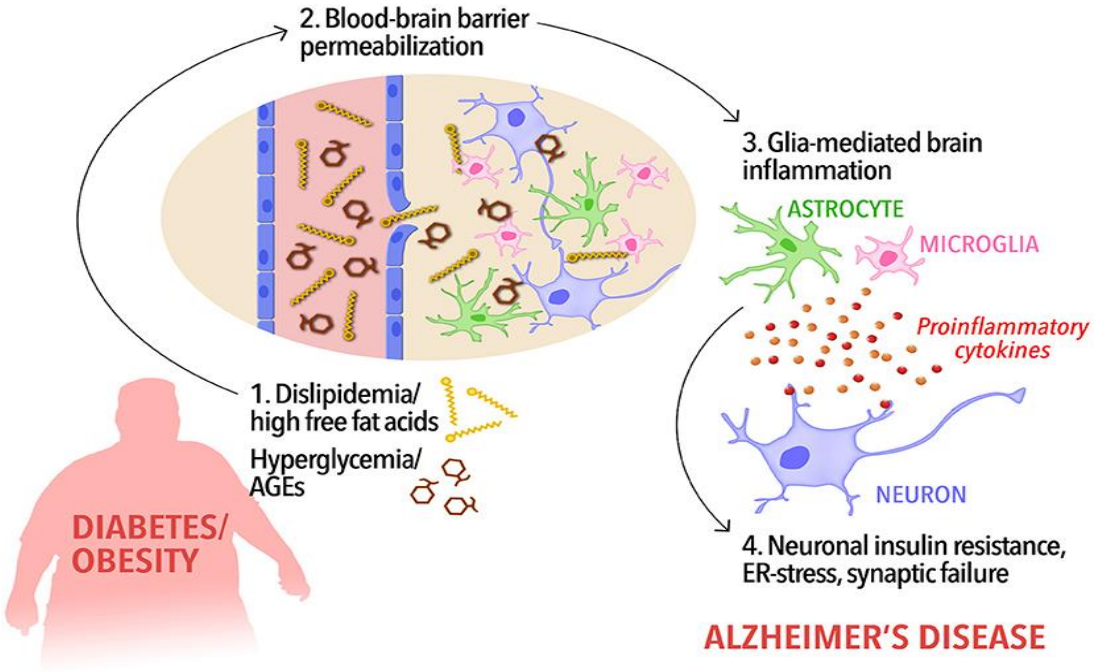


Fig.3: A possible cascade of events connecting peripheral metabolic dysregulation to dementia. In diabetic and/or obese subjects, dyslipidemia, and increased circulating free fat acids as well as hyperglycemia and elevated peripheral AGEs levels (1) may increase blood-brain barrier permeability, allowing the influx of FFAs into the brain (2). Disrupted BBB along with high levels of brain FFAs and AGEs, in turn, would cause activation of microglia and astrocytes and the release of pro-inflammatory cytokines (3). Low-grade, chronic brain inflammation leads to detrimental events in neurons, including insulin resistance (4), priming the brain to cognitive impairment.

B. Aim of the Study:

The similarity of the pathological mechanisms underlying diabetes mellitus and Alzheimer's disease is indubitable. Based on that, the present study aims to assess the role of insulin resistance observed in type-2 diabetes in triggering the pathophysiological changes associated with AD. It is hypothesized that insulin resistance leads to the deposition of senile plaques, followed by behavioral and cognitive changes mimicking all the pathological modifications observed in AD.

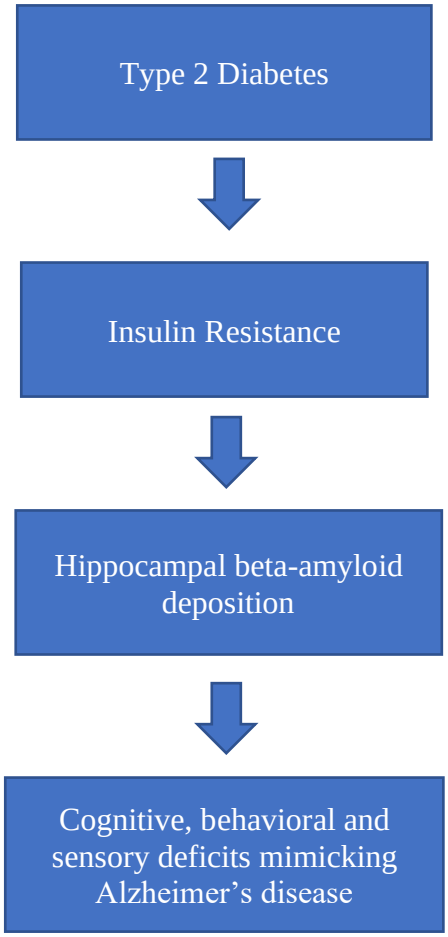


Fig. 4: **Hypothesis of the study.** Neuronal insulin resistance leads to deposition of senile plaques made of amyloid β and results in cognitive and sensory decline. These changes will mimic the symptoms observed in Alzheimer's disease.

CHAPTER II

METHODS

A. Animal Models:

Male mice of the FVB genetic background were used to conduct this study. This strain was named FVB due to its sensitivity to the Friend leukemia Virus B. FVB-NJ mice were used as control in all the study. In addition, genetically modified mice from the same background were used to study insulin resistance and its effects. The FVB-Tg (Ckm-IGF1R*K1003R) 1Dlr/J are also called MKR mice. The MKR mice develop decreased glucose uptake, insulin resistance, pancreatic β-cells dysfunctions, and diabetes (The Jackson Laboratory, 2019). Additionally, Alzheimer disease was chemically induced in FVB-NJ mice. This was achieved by injecting β -amyloid solution in the right lateral ventricle. Prior to the β -amyloid, the mice were on a high-fat diet (HFD) for three weeks, and they were kept on the HFD until the end of the experimental period.

All experimental procedures 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). The laboratory animals were received at 8 weeks of age. They were housed in standard environmental conditions with a controlled temperature range (20°C to 22°C), and a 12 hours light/dark cycle (light period from 7:00 am to 7:00 pm). The animals were provided with standard rodent chow (normal diet) or high-fat diet chow, depending on the group, and tap water ad libitum. Health assessments and checkups were performed daily.

B. Experimental Design:

Different groups and time points were used in this study in order to assess the behavioral, cellular, and histological changes that accompany insulin resistance. (Details concerning the groups of animals can be found in table 1).

Group	Type	Diet	Number
1	MKR transgenic	ND	4
	MKR transgenic	HFD	4
2	Control-FVB	ND	4
	Control-FVB	HFD	4
3	β-amyloid injected	ND	4
	β-amyloid injected	HFD	4
4	DDW injected	ND	4
	DDW injected	HFD	4

Table 1 Distribution of animals.

For all of these groups, body weights and glucose levels were assessed weekly. Glucose levels were measured using an Accu-Check Performa® glucometer and droplets of blood were obtained by a tail-prick. In addition, the animals were subjected to cognitive, and sensory behavioral tests on different time points. At the end of each experiment, animals were euthanized by cervical dislocation, and organs were collected and stored at -80 °C for further studies.

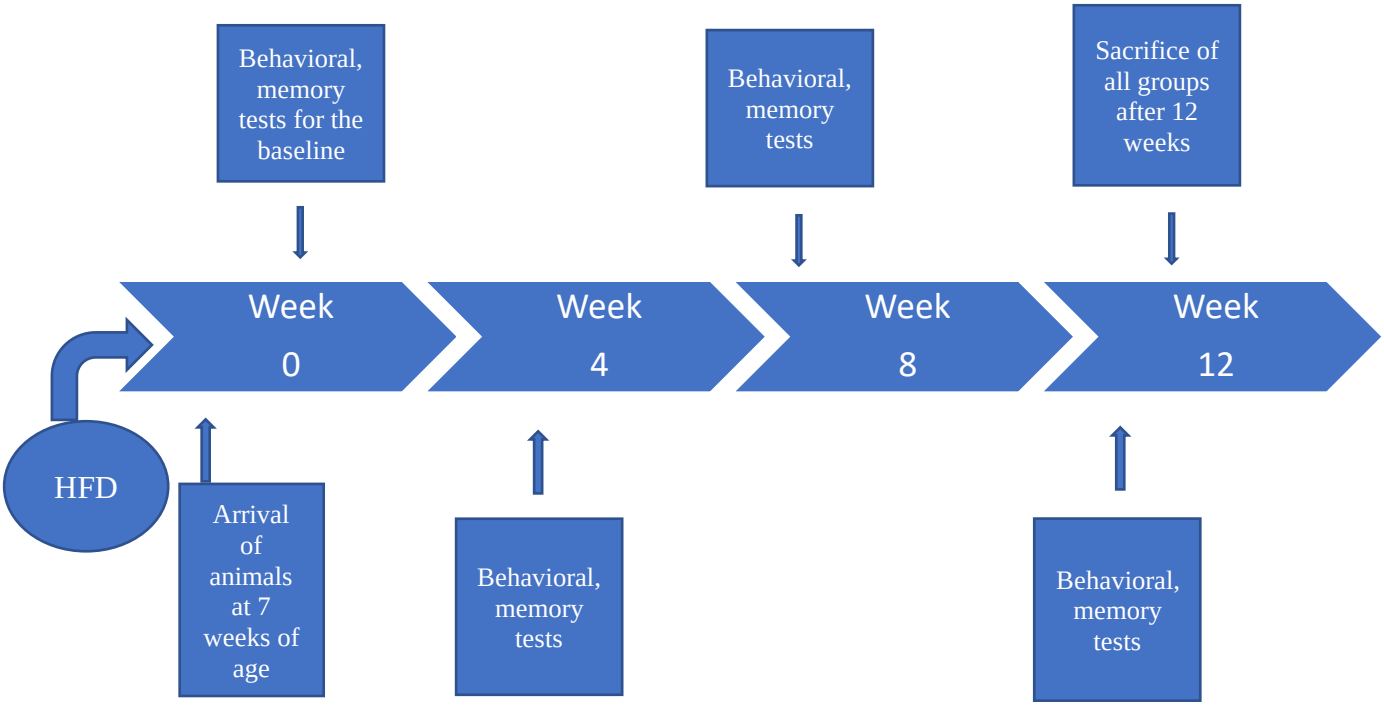


Fig. 5: Timeline of the entire experiment

C. Behavioral Tests:

1. Cognitive Behavioral Test: Spontaneous Alteration in T-maze

The T-maze alternation test was used to assess the working memory of the mice. The septal hippocampal system is critically involved in the spontaneous alternation but other brain structures, like the cerebellum, thalamus, and the substantia innominate are also involved (Lalonde, 2002). The T-maze is a T-shaped elevated apparatus, with one starting arm and two goal arms. A central partition that extends in the central arm was used to separate the two goal arms. If two trials are performed successively, on the second trial, the mice tend to choose the arm not visited on the first trial, and that indicates memory of the first arm chosen. Animals were placed in the testing room 30 min prior to the testing session. During the habituation period, the animals were accustomed to the

experimenter's touch to minimize their stress and anxiety level. (Deacon & Rawlins, 2006).

The mouse was then placed in the starting arm facing the wall and allowed to explore freely. After its entry in one of the goal arms, it was entrapped inside the chosen arm by closing it using a plastic door. The animal was then allowed to explore the arm freely for 30 seconds. The criterion for the arm choice is the complete entry of the animal in the arm, including the tip of the tail. After a 30-second delay, the door was removed and the animal was placed back in the starting arm. (Deacon and Rawlins, 2006).

With healthy cognitive functions and an operating spatial working memory, the mice would instinctively choose the other arm (not the one explored in the first phase of the test). The motive to explore the unvisited arm is the potential chance to find food, water, a mate or shelter. If the other arm is chosen the animal is considered to have successfully completed the test. On the contrary, if the same arm is visited again, the animal is considered to have failed the experiment (Deacon and Rawlins, 2006).

Three trials per animal were performed, with a minimal interval of 5 minutes between each trial. The T-maze should be cleaned with 70% ethanol between each trial to eliminate the olfactory cues. The total time per trial was estimated to be 1 or 2 minutes. When an animal spends more than 90 seconds in the maze without completing the test, it was returned into its home cage, and the trial was performed again later. The percentage of successful trials was calculated, and the generated data were compared between the different groups.

2. Cognitive Behavioral Test: Cheeseboard Maze

The cheeseboard (CB) maze was constructed from a grey painted wooden circular board measuring 1.1m in diameter and 3 cm thick. It was elevated 75cm above the floor and positioned in a well-lit room. The same maze was used for both reference and working memory assessment, but its location within the testing room as well as the identity and spatial arrangement of the distal cues was completely changed for each task. Wells were symmetrically distributed on the CB, to minimize the risk of the mice using local cues to locate the target. The wells were spaced radially, resulting in a similar shape to the radial arm maze with wells distributed throughout the table (at 20–45cm from the center). A total of 32 wells were drilled (3.1cm in diameter and 1.3cm deep) forming a radial pattern. The rear side of the CB was not drilled with any wells and was used for habituation. A plastic bottle cap was fitted inside each well. Special caps equipped with a visual stimulus in the form of a flag (15cm high pole with a 3cm×3 cm flag attached to the end) were used in the non-spatial test of the experiment. The reward was l ml freshly prepared diluted (1:4 in water) condensed milk. To minimize the potential influence of olfactory cues, we applied a film of the reward to all wells with an artist paintbrush at the beginning of each day's testing. Furthermore, to remove the odor trails, the maze was cleaned with 10% ethanol between each trial. The latency to reach the reward was calculated; when an animal failed to locate the reward within 120 s, a maximal latency of 120 s was assigned.

All mice were introduced gradually to a food restriction regime over 5 days: initially with 12 h feeding which was progressively reduced to 2 h feeding per day. The animals were prevented from falling below their 90% free-feeding body weight with additional feeding whenever necessary. Over the first 5 days of food deprivation, the

animals were exposed to the undrilled side of the maze twice per day: on each exposure trial, the animal was first placed gently in the center of the CB and confined there by a semitransparent plastic beaker (diameter: 15 cm, height: 25 cm). The beaker was then lifted and the animal allowed 2 min to explore the surface. After an interval of 1 min, the animals were given a second identical 2 min exposure trial. These exposure trials served to familiarize the animals with the CB and the handling. On the 6th day, the animals were pre-trained on the CB using the visual cue for two consecutive trials. One random well was marked by the flagged plastic cup containing the reinforcer. Each trial began as described above, and ended when the animal reached the reward, which was operationally defined by an animal dipping its head into the baited food well and consuming the reward. If the animal failed to collect the reward within 2min, it was guided or placed next to it. All animals consumed the reward within 15 s after locating it.

Each animal had two trials per day with an inter-trial interval (ITI) of 1 min and the reward now placed in the same unmarked well throughout the 5 days. A different well was chosen for each animal. A trial began and ended as described above, with the animals allowed a maximum of 2 min to locate the reward. Reference memory acquisition was indexed by the reduction of either latency or distance moved to reach the reward, across test days and across trials, although the reduction during early testing might in addition reflect procedural learning, apart from (spatial) reference memory. However, the use of pre-training in the visual cue test had already familiarized the animal with some such procedural learning. On the 6th day, a probe test was conducted: the animals were allowed to search on the CB for 2min in the absence of any reward, thus allowing the assessment of spatial search bias. In this probe test, the use of a

different procedure measuring spatial bias, compared to the procedure used during acquisition, suggests that procedural learning was of minimal relevance to the performance. On the 7th day, another standard 2-trial acquisition procedure was performed. On the 8th day, another probe test was performed but with the CB rotated 180◦. This additional probe test allowed the possible distinction of spatial navigation guided by either invariant distal spatial cues or local cues on the CB.

Working memory: Working memory was assessed over the next 16 days, with the maze positioned in a new spatial environment. A different well was baited each day, remaining the same across the two trials on a given day. The baited well position was counterbalanced across days, delays and animals. Working memory function was indexed by the performance improvement from trial 1 (when the location of the reward was essentially unknown) to trial 2. On days 1–4 of working memory test, the ITI was minimal (1 min). The ITI delay was extended to 15 min on days 5–8, 1 h on days 9–12, and 2 h on days 13–16. The animals were kept in the transport boxes (opaque boxes with sawdust) during the 1 min and 15 min delays, but were returned to the home cages for the extended 1 h and 2 h delays.

3. Sensory Behavioral Test: Thermal Hyperalgesia

Thermal hyperalgesia is a state of altered perception of pain caused by a thermal stimulation. It is an increased responsiveness to noxious heat or cold. In this study the thermal hyperalgesia was assessed using the Plantar Test Instrument (Hargreave's Method) manufactured by Ugo Basile. This machine measures the response to infrared (IR) heat stimulus applied to the plantar surface. The animals were placed in their enclosures, on an elevated glass pane, and left 20 to 30 minutes to accommodate. They

were not able to see each other due to the opaque walls of the enclosures that separate them (only the walls separating animals are opaque).

The infrared red intensity was set to 40 ° C. After the accommodation period, the movable IR generator was placed below the glass pane. The IR source was focused underneath the plantar surface of each mouse's hindpaw, and the start button was pressed so the delivery of IR light can begin. The time latency to paw withdrawal was automatically recorded. Five trials per animal were conducted with an inter-session period of 5 minutes. The time latency to elicit a withdrawal reflex was recorded (Menghon Cheah et.al, 2017).

D. Immunohistochemical Studies

1. A-β Staining:

The detection of amyloid β plaques was done using immunohistochemical staining. Complete 7 µm fresh frozen brain sections were prepared and the Novolink polymer Kit (RE7150-K) was used in accordance to the instructions of the manufacturer.

The sections were fixed with 10% paraformaldehyde solution or ice-cold methanol. The washing steps were performed with 1X Phosphate Buffer Saline. The sections were incubated with rabbit polyclonal amyloid beta antibody (1:500). Sections were then counterstained with the haematoxylin solution provided by the kit and visualized using Zeiss light microscope where amyloid β plaques appeared in brown color.

CHAPTER III

RESULTS

A. Behavioral Tests:

1. Cognitive Behavioral Test: Spontaneous Alternation in T-maze

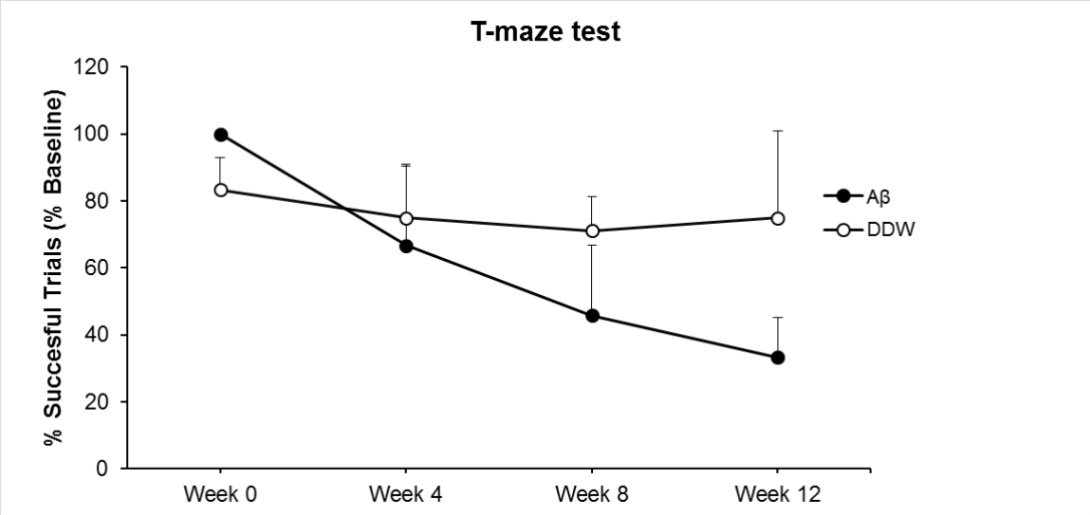


Figure 6: Effect of intraventricular Aβ injection on memory performance in the T-maze test

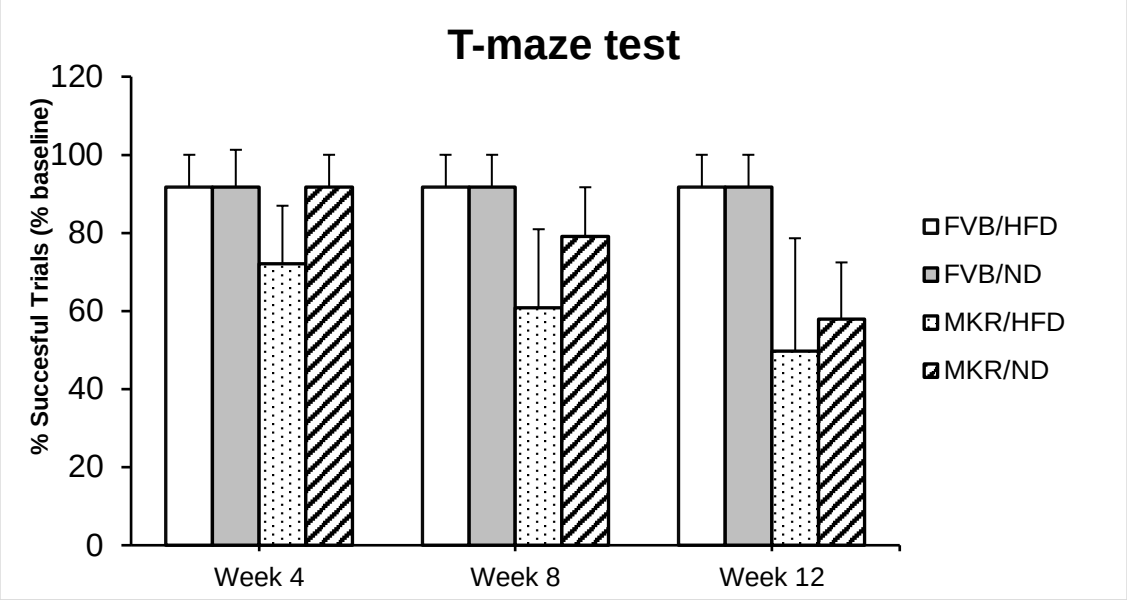


Figure 7: Bar graph showing the effect of insulin resistance and high fat diet (HFD) on memory performance in the T-maze test

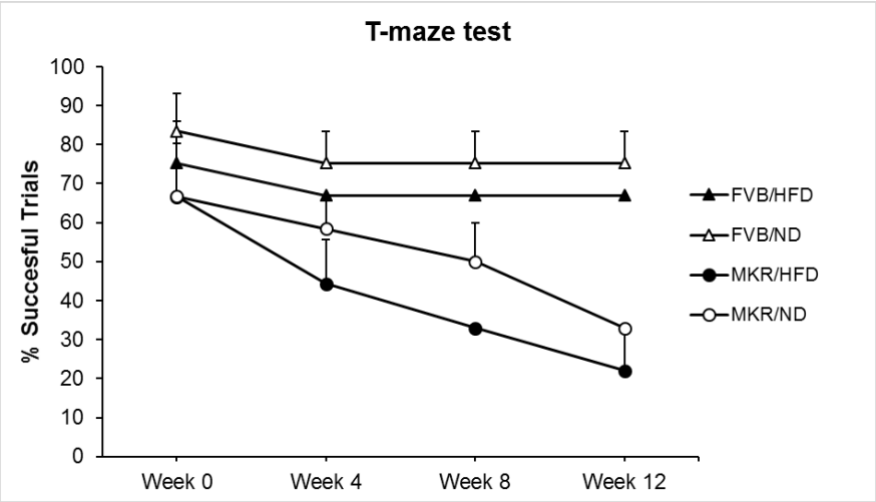


Figure 8: Line graph showing the effect of insulin resistance and high fat diet (HFD) on memory performance in the T-maze test

Mice of all groups were subjected to T-maze test prior to and at weeks 4, 8 and 12 after introduction of HFD. The insulin resistant groups (MKR), whether fed a normal diet or HFD, showed a statistically significant decrease in their cognitive performance at weeks 8 and 12 when compared to their baseline values (Figs. 7 and 8). A similar decrease in performance was noted in the Aβ group (Fig. 6) when tested at wks 8 and 12 and compared to its control group.

2. *Cognitive Behavioral Test: Cheeseboard Maze*

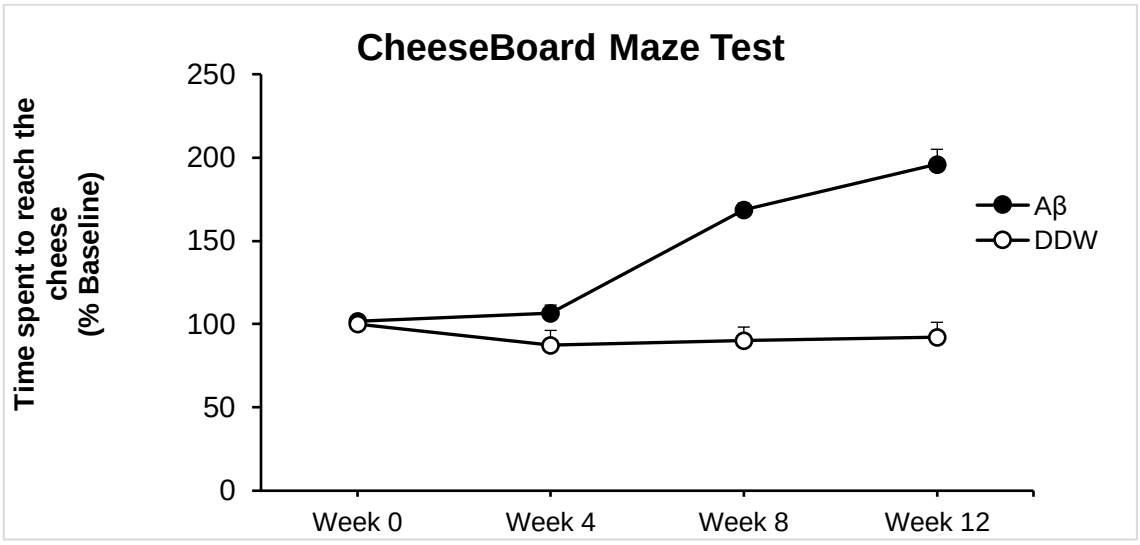


Figure 9: Effect of intraventricular Aβ injection on memory performance in the Cheese board test

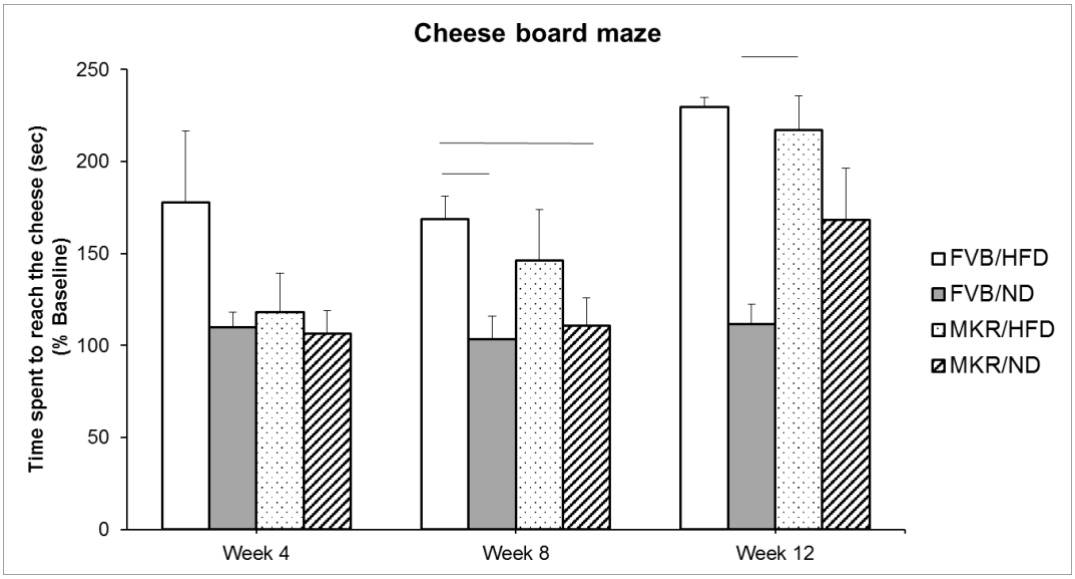


Figure 10: Bar graph showing the effect of insulin resistance and diet on memory performance in the Cheese board maze

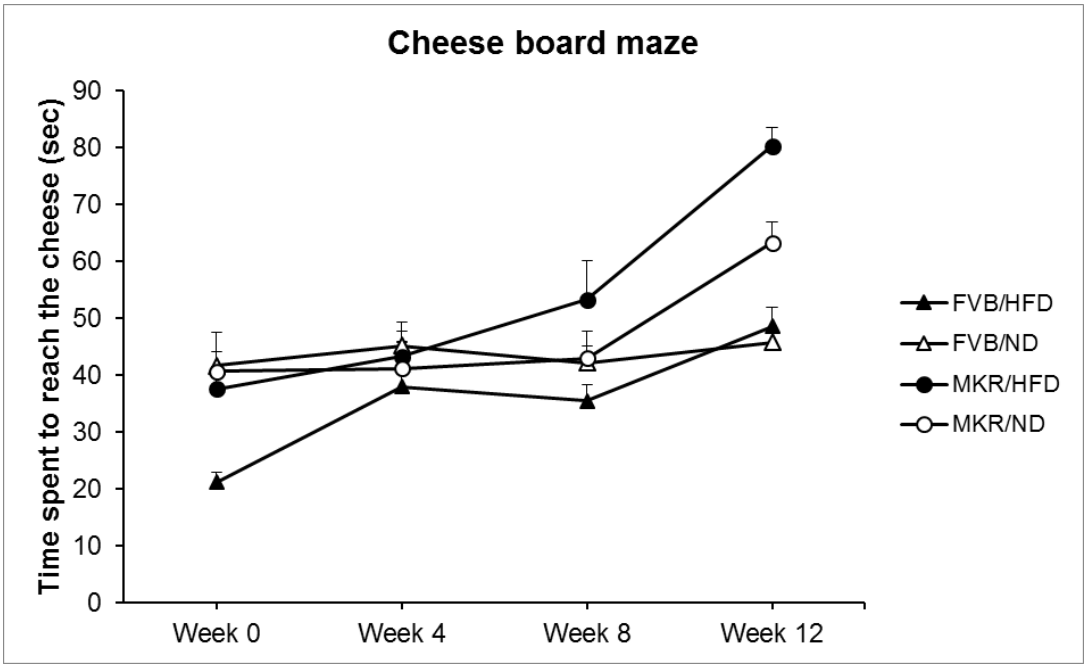


Figure 11: Line graph showing the effect of insulin resistance and diet on memory performance in the Cheese board maze

All mice were tested for cognitive functions with the cheeseboard maze prior to and at weeks 4, 8 and 12 post-HFD. The insulin resistant groups (MKR), whether fed a normal diet or HFD, showed a statistically significant increase in the time spent finding the reward at weeks 8 and 12, compared to their baseline values and to the control groups, indicating a decline in cognitive performance (Figs. 10 and 11). By comparison, a cognitive decline was also noted in the Aβ group (Fig. 9) suggesting that similar pathological changes in the Aβ and MKR mice might have occurred.

3. Sensory Behavioral Test: Thermal Hyperalgesia

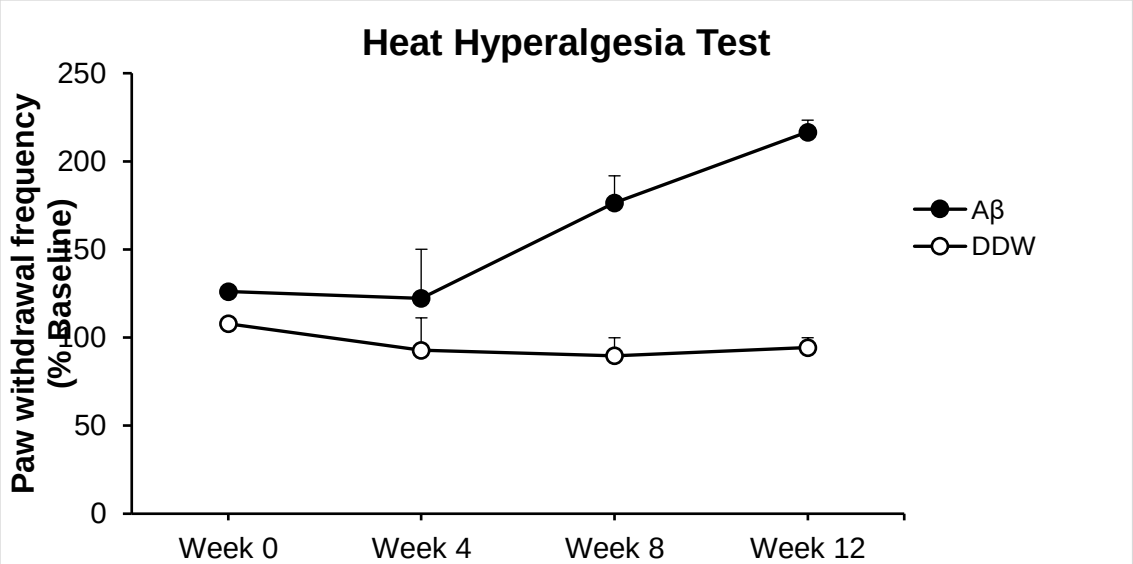


Figure 12: **Thermal hyperalgesia in mice injected with Aβ.** This diagram shows the paw withdrawal latencies of mice in response to painful heat stimulation applied to the plantar surface of the hind paws.

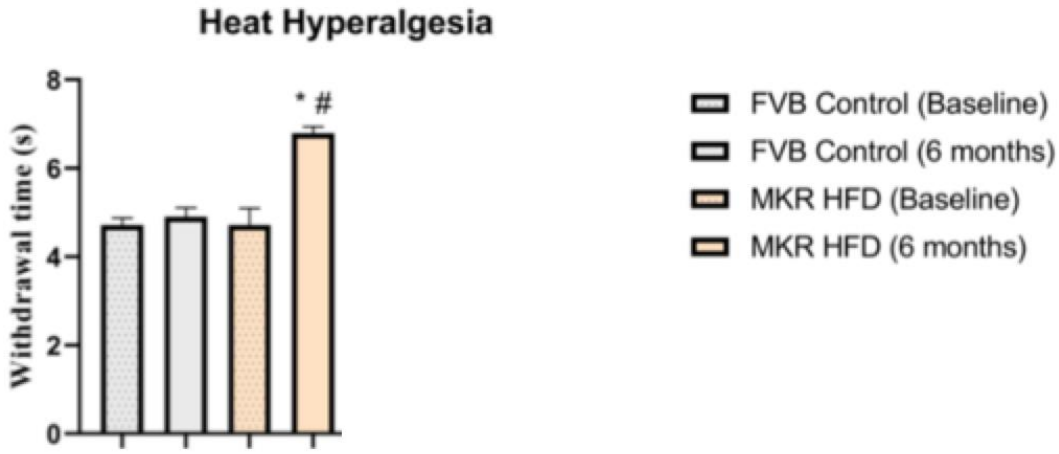


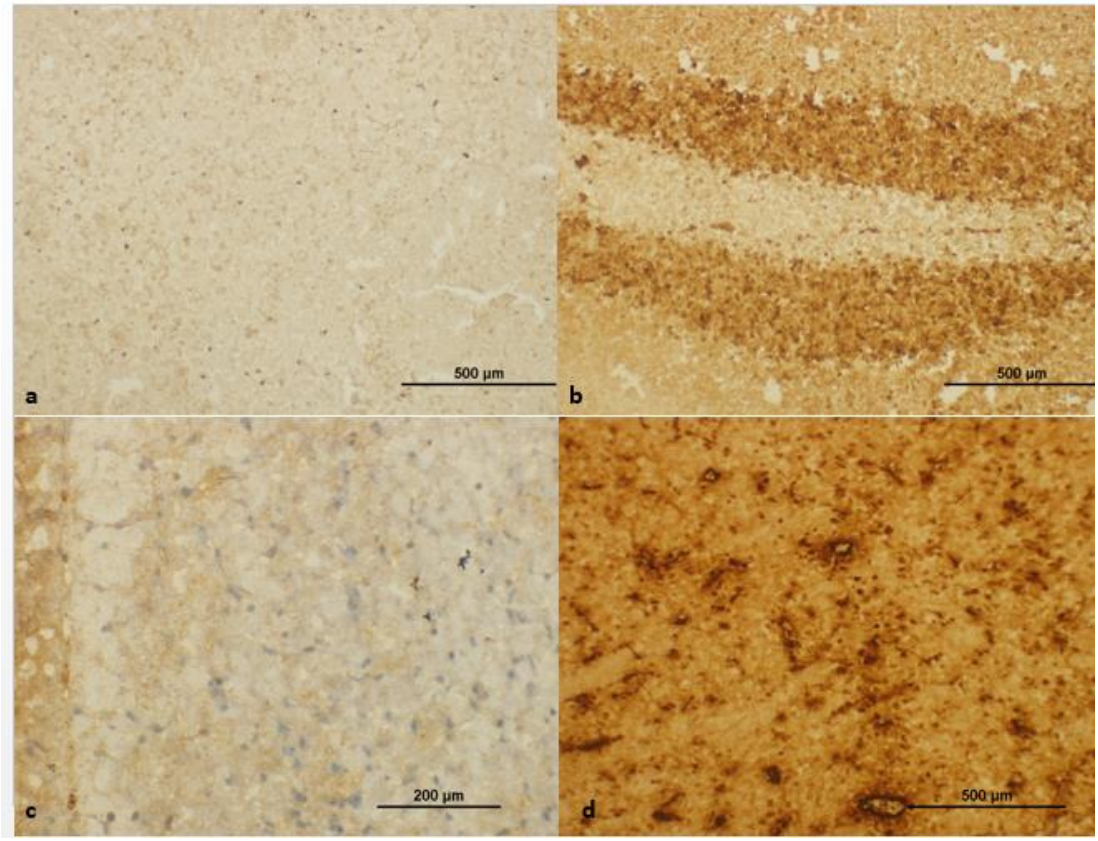
Figure 13: **Thermal hyperalgesia in MKR vs. FVB mice.** This diagram shows the paw withdrawal latencies of mice in response to painful heat stimulation applied to the plantar surface of the hind paws (This test was done in Dr. Lawand's lab by Guy Khalaf, 2019).

MKR and Aβ injected mice, subjected to the thermal hyperalgesia test, showed a similar increase in paw withdrawal latency when tested at wks 8 and 12 and compared to their corresponding control groups (Figs. 12 and 13). The increased latencies indicate a reduction in pain sensitivity.

B. Immunohistochemical Experiments:

1. Amyloid Beta Staining:

Amyloid β deposition was detected by immunohistochemical staining, in the brains of the diabetic MKR and beta-amyloid injected groups.



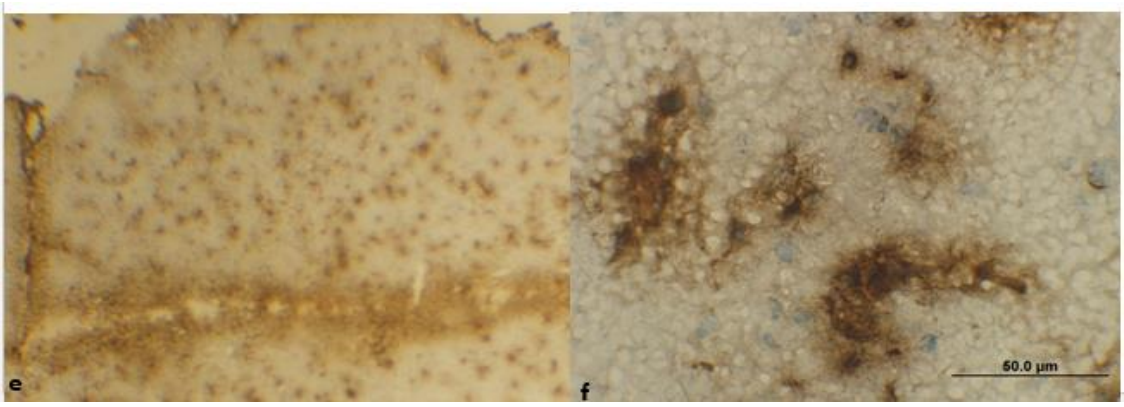


Figure 14: **Brain sections stained for Amyloid Beta deposition**. (a) brain section of a control mouse showing no deposition. (b and c) brain section of a mouse in an early stage of diabetes showing no amyloid deposition. (d & e) brain section from MKR mice showing amyloid deposition. (f) enlarged photo showing the amyloid beta plaques in the brain tissue of an MKR mouse (Adapted from Guy Khalaf, 2019).

CHAPTER IV

DISCUSSION

The results of the present study highlight the role of insulin resistance in the development of Alzheimer-like symptoms in diabetic mice. A decrease in sensory and cognitive functions was noted in the Aβ-injected group and MKR mice. The two groups followed a similar pattern of behavioral changes when tested at wks 8 and 12. Additionally, both displayed amyloid-β deposition in the hippocampus suggesting that insulin resistance in the MKR diabetic group might have contributed to the accumulation of amyloid proteins in the brain and triggered the reduced behavioral responses that are commonly seen in AD. Our findings are in accordance with the results of previous pre-clinical studies demonstrating a disturbance in cognitive functions in transgenic type 2 diabetic mice (*MKR* mice) when compared to their controls (Blázquez et al., 2014).

The diabetic and beta-amyloid injected groups of mice showed increased withdrawal latencies to noxious thermal stimulation. The reduced sensory functions in these mice suggest the development of peripheral neuropathy, a phenomenon that is typically observed in diabetic animals and patients. Many studies have shown that increased glucose level in the blood weakens the walls of capillaries that supply crucial molecules to peripheral nerves and leads to nerve damage and disruption of neural signals. Thus, the increased latency observed in the diabetic and amyloid-injected mice could be due to nerve injury; however, this remains to be determined since no evidence of myelin degeneration, loss of nerve fibers or reduced nerve conduction was provided in this study. Although previous reports have shown that MKR mice had no significant

loss of peripheral myelin and no alteration in the total sciatic nerve sulfatide and cerebroside levels even at advanced diabetic stages (Palavicini, et al., 2020), it is still possible that disruption of nerve activity at the molecular level may have occurred.

Many clinical studies that reported sensory deficits and altered force production in diabetic patients are in agreement with our findings. The prevalence of peripheral neuropathy among type 2 diabetics strongly links insulin resistance to altered sensory transmission and myelin abnormalities in peripheral nerves (Ochoa and Gorniak, 2014; Cermenati et al., 2012).

In addition to its involvement in the sensory system, insulin resistance has been shown to trigger a slew of events in the brain that might lead to the development of cognitive impairment (Kothari et al., 2017). Insulin resistance in nerve cells, associated with diabetes, disrupts the brain homeostasis and results in potentially harmful effects. Under normal conditions, insulin exerts significant impact on the brain by promoting the development of neurites, modulating the activity-dependent synaptic plasticity mediated by NMDA receptors and regulating trafficking of ligand-gated ion channels. However, under hyperglycemic conditions, the dynamics of insulin action in neural tissue shift leading to impaired signaling and acceleration of neurotoxic amyloid plaques and neurofibrillary tangles formation (Rad et al., 2018). Our results support the connection between insulin resistance and the development of AD-like manifestations in the brain. The poor performance of transgenic MKR mice, whether in T-maze test or cheeseboard maze, and the accumulation of Aβ protein in their brains and cerebral blood vessels, were similar to those exhibited by the Aβ-injected mice, suggesting a crucial role for insulin and its receptors in neurodegenerative diseases.

The increase in metabolic and oxidative stress, as a consequence of hyperglycemia and hyperinsulinemia, can be suggested as a possible mechanism underlying the formation of amyloid-β plaques in diabetic brains (Mullins, *et al*, 2017; De Felice *et al*., 2015*)*. Many studies have shown that insulin plays a major role in the physiology and pathophysiology of amyloid-β deposition and clearance, in the maintenance of the physiological state of tau, and in the normal functioning of the GSK3, an enzyme implicated in the pathogenesis of Alzheimer's disease. Therefore, it is likely that the insulin resistance in MKR mice played a key role in the manifestation of behavioral and histological changes observed in Alzheimer's disease. However, the results derived from this study are still at a preliminary stage. More experiments are still needed to determine the role of insulin receptors and transporters in the formation of amyloid plaques. Indubitably, the findings of this study shed more light on the involvement of insulin in neurodegenerative disease and pave the path for scientists to investigate further the intricate mechanisms underlying the pathogenesis of AD in diabetic patients.

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