AMERICAN UNIVERSITY OF BEIRUT

DIMORPHISM IN THE ASSOCIATION BETWEEN DIABETES AND DEPRESSION: UNRAVELING NOVEL BIOLOGICAL PERSPECTIVES

by
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AN ABSTRACT OF THE THESIS OF

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Background: Diabetes and depression are two disorders that are among the leading non-communicable diseases currently on the horizon worldwide. Recent epidemiological studies highlight the rising occurrence of depression among diabetic patients; however, this phenomenon has not been well understood. While both disorders have distinct pathological entities, they are multifactorial with shared genetic, biological, and environmental features that are to be elucidated. In this work, we aim to comprehend how the underlying mechanistic alterations driving diabetes exacerbate the severity of depression in male and female type 2 diabetic mice (T2DM).

Aim: This study aims to investigate the mechanistic bidirectionality of both comorbidities in animals exhibiting a depressive, diabetic, and diabetic-depressive phenotype. We further assess whether the observed trends are reflected in a sexually dimorphic manner.

Methods: Five groups of male and female mice were used throughout the work: Controls, Diabetic, Depressed, Diabetic-depressed, and Depressed-diabetic. The animal model for depression was induced in this study via the Chronic Mild Stress procedure in C57-BL6/J and High Fat Diet-induced T2DM mice. Depression was assessed via the Sucrose test, Tail Suspension test, and Forced Swim test. Sensory and motor function in addition to nociception were assessed via a series of behavioral tests: Raised beam walking test, Electronic Von-Frey Test, and Heat Hyperalgesia Test. Western blots were then performed to assess protein expression levels of signaling cascade markers, and neurotrophic factors: BDNF, IRS1, AMPK, and GLP1 in brain tissue lysates (Hippocampi and Prefrontal Frontal Cortices).

Results: The results from behavioral testing reflect depressive-like symptoms in depressed animals in both male and female groups. However, the sexual dimorphism of depression was prominent in the female diabetic animals relative to the males. At the molecular levels, neurotrophic factor BDNF levels were significantly decreased in all groups relative to controls. These were further paralleled by alterations in metabolic homeostasis, reflected by reductions in protein expression of metabolic cascade markers AMPK, IRS1 and GLP1 in diabetic animals by contrast to controls. Interestingly, depressed, and diabetic-depressed animals exhibit signaling alterations that mimic the diabetic groups.

Conclusion: The findings from this work highlight pivotal and shared molecular changes that underlie both depression and diabetes induced brain injury. We underline for the first time the involvement of insulin signaling alterations that may serve as a common ground in the pathogenesis of both comorbidities. We further show that the effects of depression and diabetes may be more pronounced in the nervous system of female subgroups than males.

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ABBREVIATION

BW: Beam Walking

TST: Tail Suspension Test

FST: Forced Swim Test

PW: Paw Withdrawal

VF: Von Frey

SPT: Sucrose Preference Test

CMS: Chronic Mild Stress

CUMS: Chronic unpredictable mild stress

DM: Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

BBB: Blood Brain Barrier

MDD: Major Depressive Disorders

HPA: Hypothalamic pituitary adrenal

CMS: Chronic mild stress

CNS: Central nervous system

PNS: Peripheral nervous system

IR: Insulin resistance

SSRI: Selective serotonin reuptake inhibitor

BGL: Blood glucose levels

BDNF: Brain-derived neurotrophic factor

IRS-1: insulin receptor substrate 1

GLP-1: Glucagon-like peptide1

AMPK: AMP-activated protein kinase

CHAPTER I

INTRODUCTION:

Depressive disorder, otherwise known as clinical depression, is a psychological mood disorder predominantly characterized by symptoms portraying anxiety, with a persistent state of lowered mood affecting feelings, thoughts, and routine behavior like sleep, appetite, and social interactions. Depression, contrary to popular belief, is not grounded on sadness. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), this disorder also portrays signs of hopelessness, pessimism, emptiness, worthlessness, restlessness, guilt, loss of pleasure, and suicidal thoughts (Institute & Health, 2016). All these signs and symptoms must persist, on average, daily, for a minimum period of two weeks (Institute & Health, 2016). In order to diagnose subjects with depression, a brief questionnaire and clinical interviews are usually used by specialists to confirm the disorder's presence and evaluate its severity, allowing patients to seek professional help.

Notably, depression heterogeneously affects women compared to men (Jan Detka et al., 2019), where biological changes are compromising but not limited to hormonal factors, play a crucial role in aggravating mood swings in women causing a higher depression rate. Furthermore, men suffering from depression tend to depict substance abuse and irritable mood accompanied by a denial state more often witnessed in older adults.

Depression can be categorized into different types, including major depressive disorder (MDD), persistent depressive disorder, perinatal depression, seasonal affective disorder, and psychotic depression. Moreover, they are sought to coincide with various maladies such as cancer, cardiovascular diseases, and diabetes (Jan Detka et al., 2019).

In both depression and diabetes, metabolic disturbances in the central nervous system may occur observed in animal models of both these illnesses interrelating their frequent co-occurrence (Jan Detka et al., 2019).

Increased clinical data reported that depression, among other mood disorders, can be more commonly seen in diabetic patients compared to patients suffering from other chronic disorders. A growing body of evidence supports that comorbid diabetes and depression share biological vulnerabilities. Similar functional and structural abnormalities are seen in the brain of diabetic and depressed patients. These include a decrease in the volume of the hippocampus and cerebral atrophies (Eun Joo Kim, 1994; Fanselow & Dong, 2010; Lloyd et al., 2004; McReynolds, Anderson, Donowho, & McIntyre, 2014; Sheline, Wang, Gado, Csernansky, & Vannier, 1996). These shared mechanisms cause injury to the brain of depressed and diabetic patients. Moreover, injurious pathways are reported to be implicated in both diseases, and these include molecular mechanisms, increased immunological and inflammatory processes, dysregulated Hypothalamus-Pituitary-Adrenal cortex (HPA) axis, oxidative stress, and reduced antioxidative mechanisms(Khanzode, Dakhale, Khanzode, Saoji, & Palasodkar, 2003; Martín-Gallán, Carrascosa, Gussinyé, & Domínguez, 2007; Mylona-Karayanni, Gourgiotis, Bossios, & Kamper, 2006). Nevertheless, a growing body of evidence demonstrates that metabolic disturbances in the central nervous system (CNS) are a critical driving force in both disorders' pathogenesis, as observed in experimental animal models interrelating their frequent co-occurrence (Jan Detka et al., 2019).

Diabetes mellitus (DM) can be divided into many types. Type 2 diabetes mellitus (T2DM) is the most prevalent among the four types (Réus et al., 2017).). It is a chronic metabolic disease manifested mostly in adults and characterized by abnormally high plasma glucose levels, known

as hyperglycemia. Also, it is described by insulin resistance, having a decreased sensitivity of the peripheral insulin receptors toward the action of insulin (Liaw et al., 2015). Moreover, T2DM results from an interaction between environmental, behavioral, and genetic risk factors. In this way, a lousy lifestyle plays an essential role in developing T2DM like physical inactivity, sedentary lifestyle, cigarette smoking, overconsumption of alcohol, and obesity (Réus et al., 2017).

T2DM is related to a cluster of complications involving brain function, including cognitive decline and depression (Ho, Sommers, & Lucki, 2013a), cardiovascular disease, peripheral neuropathy, retinopathy, and nephropathy.

In treating T2DM, the patient undergoes many changes to his lifestyle where a strict alimentary diet is prescribed, depriving him of rudimentary delights, medication becomes a necessity and exercise. All these changes combined may provoke depressive symptoms, as cited in Egede et al. (Egede & Ellis, 2010). On the contrary, many studies showed that significant depressive symptoms favor the development of diabetes (James E. Gangwisch & Raz Gross, 2015), linking the two and disclosing their interrelationship. Mutually driven by shared underlying biological and behavioral mechanisms, such as hypothalamic-pituitary-adrenal axis activation, inflammation, sleep disturbance, inactive lifestyle, poor dietary habits, and environmental and cultural risk factors, deepens the link (Buchowski et al., 2017; Holt, de Groot, & Golden, 2014).

Although depression is considered as a symptom of diabetes, we believe that a biological link and a shared mechanism are underlying mood-related brain injuries underlying both disorders where changes are affecting the central nervous system. Their comorbidity puts the diabetic-depressed patient at a higher risk of developing complications. In this study, we try to shed light on the biological crosstalk underlying the mechanisms by which diabetes induces depression and causes more severe complications in a gender-specific way.

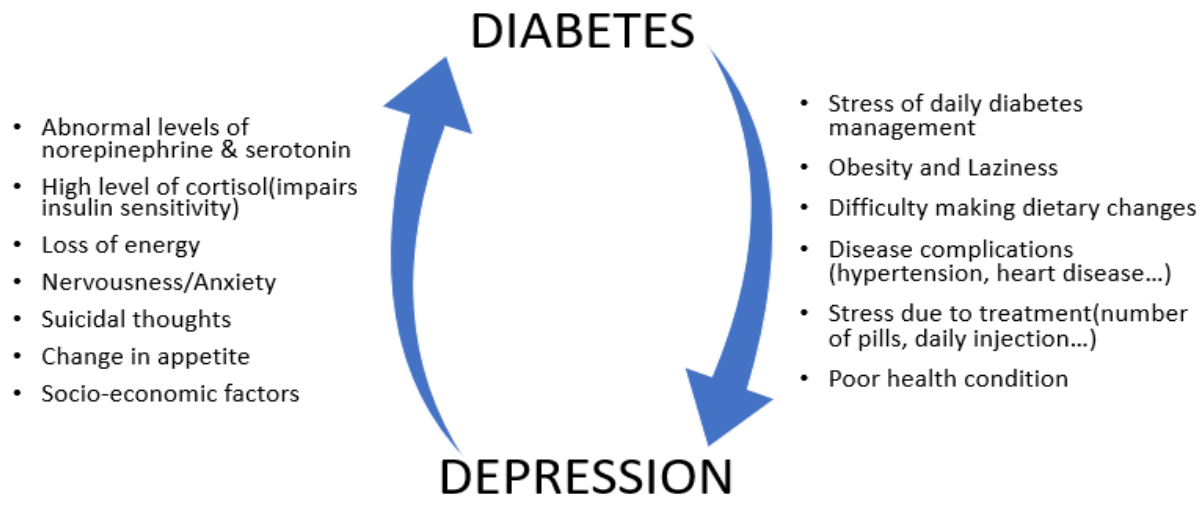


Figure 1: The causative link between depression and Diabetes

Epidemiology of Diabetes and Depression

Diabetes mellitus, described as a global disease (Ahmadieh et al., 2018), is a chronic debilitating disease that currently affects a large population section, disrupting the standard quality of life. According to the WHO, it is estimated that 463.0 million adults between the ages of 20 and 79 worldwide, comprising 9.3% of adults globally, have diabetes. Based on the last estimates, by 2030, a projected 578.4 million, and by 2045, 700.2 million adults aged between 20 and 79 will be living with diabetes (Rhys Williams, 2019).

Furthermore, an upward trend is prevalent in 2019 estimates showing diabetes and age affecting both women and men. In 2019, 17.2 million more men than women were diagnosed and live with diabetes. Women exhibit a slightly lower prevalence of diabetes than men estimated at 9.0% vs. 9.6%. Considering the limit of healthcare services in low-income countries, the later exhibits the highest proportion of undiagnosed diabetes consisting of 66.8% of the population. On the other hand, high-income countries indicate 38.3% of the population undiagnosed. Under those

circumstances, it is vital to understand how a population's risk for diabetes is changing over time by assessing the incidence of diabetes. The annual incidence, which is considered a more direct indication of the risk for diabetes in the general population than prevalence, measures the rate at which new cases of diabetes are occurring. Incidentally, expenditures committed to diabetes in the MENA region ranges to 15.2% of the regional total. Countries with the highest spending related to diabetes consist of Sudan (2.7%), Lebanon (20.4%), Pakistan (19.7%), and Oman (6.8%) being the lowest percentage of total health expenditure spent on diabetes in the region. Consequently, the MENA region suffers from a discrepancy in the amount per person dedicated to diabetes. The two highest estimates ranged between 1,751 USD, in Qatar, to 1,584 USD in Lebanon, while Pakistan ranked lowest with 83 USD (Rhys Williams, 2019).

At the same time, depression, on a global scale, impacts more than 322 million individuals of all age groups, while depressive symptoms are much more common. Multiple factors contribute to the occurrence of depression, with the most prevalent being age, marital status, social class, and social conditions (Lehtinen & Joukamaa, 1994). Prevalence of depression varies per age, peaking in the later stages of adulthood (above 7.5% among females aged 55-74 years, and above 5.5% among males). On the contrary, adolescents below the age of 15 depict a lower level of depression. Moreover, the clinically significant depressive disorder affects 4% of men in contrast to 8 % of women, while existing long-term medical conditions increase the risk of developing major depressive disorder from 2.8% to 4.0%. Depression imparts a severe effect on chronic medical conditions, with potentially adverse influences on self-care (Katon, 2008). One of the most threatening influences is suicidal thoughts. According to the World Health Organization (WHO), close to 800 000 are declared dead due to suicide every year (Organization, 2017).

Furthermore, depressed adults have a 37% increased risk of developing type 2 diabetes. T2DM is considered one of the significant elevating attributes of depression but is not limited to it. Depressed diabetic patients experience low income and poor socioeconomic status as principal factors for increasing depression levels. On the other hand, patients with supportive and continuous medical care display less depression while age and medication determine the level. As reported by Probst et al., residents in rural areas, in contrast to residents in urban areas, demonstrated higher depression levels due to low health status, chronic diseases, and poverty (Dong, 2009; Janice C. Probst, 2006). The study conducted in Lebanon showed the prevalence of depression to be 28.8% among the participants with T2DM (Ahmadieh et al., 2018). The study conducted in Lebanon showed the prevalence of depression to be 28.8% among the participants with T2DM (Katon, 2008).

Diabetes Mellitus and Insulin Resistance

DM is a metabolic disorder characterized by an increase in blood glucose for a prolonged period. More than 422 million adults worldwide suffer from DM (Farhadi, Vosough, Zhang, Tahamtani, & Shahpasand, 2019). Also, DM may be classified into type 1 DM, type 2 DM, and gestational diabetes. Type 1 diabetes mellitus (T1DM) is characterized by a defect in the pancreas' β cells, which results in insulin secretion deficiency. Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance (IR). The pancreas produces insulin, but the body does not respond to it. Individuals with excessive body weight and not enough physical activity are more likely to be diagnosed with T2DM. Gestational diabetes affects pregnant women with no previous history of diabetes (Tao, Shi, & Zhao, 2015).

Insulin is a hormone that regulates body glycemia. Insulin and insulin-like growth factor1 (IGF1) activate complex intracellular signaling pathways (Myers, Sun, & White, 1994; Sun,

Crimmins, Myers, Miralpeix, & White, 1993) affecting cell growth, survival, energy metabolism, cholinergic gene expression, oxidative stress, and apoptosis.

Insulin resistance can be defined as a state of reduced responsiveness to regular circulating insulin (Savage, Petersen, & Shulman, 2005). It is a hallmark of type 2 diabetes also uncontrolled type 1 diabetes, obesity, and metabolic syndrome (Zimmet, Alberti, & Shaw, 2001). Several conditions are associated with insulin resistance, such as cystic fibrosis, uremia, glucocorticoid excess, and polycystic ovary syndrome. When the body is resistant to insulin, the hormone fails to lower plasma glucose. Thus, tissues that absorb glucose in response to insulin are affected. Specifically, skeletal muscle, heart muscle, and adipocytes express the insulin-responsive glucose transporter GLUT4. Mainly, the primary defect is the inability of insulin to bind to its receptors (Buse, 2006). Insulin resistance is genetically determined most of the time, but it is affected by diet, exercise, and aging. The concept of "glucose toxicity" was introduced when high-blood glucose levels were maintained in humans (Yki-Jarvinen, Helve, & Koivisto, 1987) and animals (Rossetti, Giaccari, & DeFronzo, 1990), thus inducing insulin resistance. Studies have demonstrated that the lack of responsiveness to insulin could affect endothelial cells. Following acute hyperinsulinemia, subjects experienced hypertension (Tooke, 1995). Moreover, unbalanced insulin signaling through ERK-dependent pathways could lead to other vascular changes, thus affecting cell growth and differentiation, and increasing permeability and fibrogenesis (Jiang et al., 1999).

Patients with type 2 diabetes have more prominent endothelial dysfunction than other patients. They are also diagnosed with microalbuminuria, which seems to be more severe due to insulin resistance (Y. Yu, Suo, Yu, Wang, & Tang, 2004). It has also been shown that patients with a higher insulin resistance level tend to suffer from advanced retinopathy (Tarnow et al., 2000). Insulin

resistance could also reduce nitric oxide (NO) synthesis or increase endothelin 1 (ET-1) synthesis, amplifying ischemic injury in the retina due to the failure of reactive vasodilation (Abiko et al., 2003). It also plays a role in neuropathy by contributing to endoneurial capillary microangiopathy (Sugimoto, Baba, Suda, Yasujima, & Yagihashi, 2003).

Moreover, insulin induces neurite outgrowth and regeneration of small myelinated fibers. Studies have shown that direct insulin stimulation of neurons reverses diabetic neuropathy, which leads to thinking that defective insulin signaling in peripheral neurons could contribute to diabetic neuropathy (Brussee, Cunningham, & Zochodne, 2004). Due to the importance of insulin resistance and its adverse effects on the body, the pathways leading to glucose uptake have been thoroughly studied.

Pathophysiology linking Diabetes and Depression

Brain metabolism and depression

Glucose, a simple sugar (monosaccharide), is the primary energy source for the adult brain. Its metabolism enables the proper synthesis and function of neurotransmitters, especially glutamate and γ aminobutyric acid (GABA), and the formation of correct levels of NADPH (Nicotinamide adenine dinucleotide phosphate) (Detka et al., 2015). In this context, glucose metabolism dysfunction is involved in many pathogeneses, such as depression in the CNS and diabetes mellitus (Detka et al., 2015). Also, glucose metabolism in the pentose cycle is a significant source of NADPH that can be used to remove the excess of reactive oxygen species, while oxidative stress plays a role in the pathogenesis of depression (Detka et al., 2015). As explained earlier in MDD, there is an increase in the HPA axis function, leading to a high cortisol expression level. Therefore, in peripheral tissues, glucocorticoids inhibit insulin-stimulated glucose uptake,

down-regulate glucose metabolism, mainly in glycolysis, and inhibit insulin signaling. Some studies have shown that these hormones may exert similar effects on glucose uptake and metabolism in the brain. Such action may, in turn, lead to a reduction in energy production and disrupt the normal function and plasticity of brain cells (Detka et al., 2015). This theory is supported by studies showing that chronic corticosterone treatment decreases glucose metabolism in the rat parietotemporal cortex and hippocampus. Also, adrenalectomy increases glucose metabolism in these brain regions (Hoyer & Lannert, 2008).

However, current evidence indicates that lactate is an important energy source for the brain and supports neuronal function and survival. It has been demonstrated that lactate released from astrocytes may be taken up by neurons and metabolized in the tricarboxylic acid cycle. Detka et al. have shown that lactate concentration is approximately 25 times higher than that of pyruvate. These data confirm the suggestion that the end product of glycolysis in the brain is lactate rather than pyruvate (Detka et al., 2015), meaning that there is a presence of anaerobic glycolysis in depressed patients' brains.

Moreover, insulin is a hormone implicated with varied roles in the central nervous system (CNS) like the modulation of feeding behavior, energy maintenance by the hypothalamus, and memory-related processes by the hippocampus (Rasgon & McEwen, 2016). Modulation of dopaminergic and serotonergic systems, which play a pivotal role in depression, is greatly affected by insulin signaling (A. Kleinridders, Ferris, Cai, & Kahn, 2014). Insulin receptors are expressed in most body tissues, like the liver, muscle, and fat tissues. They are also present throughout the brain, with distinct patterns of expression, in regions involved in mood regulation like nucleus accumbens (NAc), ventral tegmental area(VTA), amygdala, and raphe nuclei (Figlewicz, Evans,

Murphy, Hoen, & Baskin, 2003) and in other regions like the olfactory bulb, hypothalamus, and pituitary gland (Bruning, 2000). Their function in some of these regions remains unknown.

The insulin receptor, a member of the ligand-activated receptor and tyrosine kinase family of transmembrane signaling proteins, are fundamentally essential regulators of cell differentiation, growth, and metabolism. The insulin receptor has several unique physiological and biochemical properties that distinguish it from other sizeable, well-studied receptor families (Lee & Pilch, 1994). The insulin receptor's primary physiological role appears to be metabolic regulation while regulating cell growth and/or differentiation is the direct engagement of other receptor tyrosine kinases. Receptor tyrosine kinases, regulated by their cognate ligands, function as dimers. The insulin receptor and 2 closely related receptors are covalently maintained as functional dimers by disulfide bonds, while all other cases, these dimers are noncovalent. Ligand's initial response is receptor autophosphorylation for all receptor tyrosine kinases, resulting in receptor association of effector molecules that have unique recognition domains for phosphor-tyrosine residues, and that is binding to these results in a biological response. For the insulin receptor, this does not occur; rather, it phosphorylates a large substrate protein that, in turn, engages effector molecules (Lee & Pilch, 1994).

During ligand binding to the α subunits, IR and IGF-1R undergo a conformational change-inducing activation of the kinase activity in the b subunits. Thus, results in transphosphorylation among β subunits, further activating the kinase and allowing the recruitment of receptor substrates. The best-characterized substrates are members of the insulin receptor substrate (IRS) family of proteins, referred to as a range from IRS-1 to IRS-6, which act as scaffolds to organize and mediate signaling complexes (Boucher, Kleinridders, & Kahn, 2014). Although these substrates show similarities in the tyrosine phosphorylation motifs, they have different functions in vivo. Growth

retardation and impaired insulin action are exhibited in IRS-1 knockout (KO) mice (Boucher et al., 2014). While, IRS-2 KO mice only selective tissues display growth reduction, such as specific neurons and islet cells, and have defective insulin signaling in the liver, which, when combined with the loss of b cells, results in the development of diabetes (Boucher et al., 2014).

Insulin resistance is a modifiable metabolic pro-inflammatory state underlying mainly in T2DM and recently in cognitive impairment, and CNS linked diseases. T2D patients with defective brain insulin signaling showed an association with impaired transport of the hormone across the blood-brain barrier (Gray, Aylor, & Barrett, 2017).

IR, at the molecular level, is caused by a loss/downregulation of the insulin receptors and insulin receptor substrates (IRS-1 and IRS-2) and impairment of the insulin receptor's binding activity (Maciejczyk, Zebrowska, & Chabowski, 2019). Functionally, alterations in neurite outgrowth, impaired neuroplasticity, and disturbances in neurotransmitter's release and uptake can manifest due to reduced brain sensitivity to insulin. Bearing in mind the multi-component insulin transport system to the brain, such as lipo-toxicity, inflammation, oxidative stress, and glucotoxicity, systemic IR may affect the cerebral insulin signaling. Undeniably, it has been demonstrated that peripheral IR alters the blood-brain barrier (BBB) function by lessening the level of endothelial insulin receptors and diminishing the BBB permeability to insulin. Thus, it results in the impairment of physiological insulin functions and increased BBB permeability to many substances (Maciejczyk et al., 2019).

Moreover, the latest clinical studies have shown a strong relationship between whole-body IR and higher incidence of neurodegeneration, depression, dementia, and mild cognitive impairment having the key role attributed to the mitochondrial dysfunction and brain oxidative stress (Maciejczyk et al., 2019).

Under IR conditions, disruption in enzymatic and non-enzymatic antioxidants and the increased content of oxidative modification products have been reported in serum/plasma and liver, muscles, adipose tissue, and brain tissue. However, the brain is particularly sensitive to free radical attack. The brain, constituting less than 2% of the body, uses over 20% of its oxygen. Enriched in polyunsaturated fatty acids, phospholipids of cerebral cell membranes, consecutively with low activity of brain antioxidant enzymes, and high content of pro-oxidant metal ions makes the brain very vulnerable to oxidative stress. Therefore, ROS's overproduction in the IR brain causes damage (oxidative) associated with the cell membrane's higher permeability, ATP depletion, and accumulation of protein aggregates. One of the consequences of lipid and protein oxidation may also be pro-inflammatory enzymes, thus stimulating brain inflammation. The above mentioned may incline neurodegeneration and/or neuronal apoptosis (Maciejczyk et al., 2019).

Peripheral insulin resistance causes the increase of pro-inflammatory cytokines production like IL-1β, IL-6, tumor necrosis factor-α (TNF-α), which activates several serine-threonine kinases phosphorylating the serine residues of the insulin receptor substrate 1 (IRS-1), which in turn blocks the insulin signaling proteins, causing a decrease in AMPK (Maciejczyk et al., 2019).

Rasgon et al. findings suggest that in middle-aged adults, insulin resistance is linked with disrupted memory, an executive function corresponding to a metabolic decline in the medial prefrontal cortex, reductions in hippocampal volumes, and aberrant intrinsic connectivity between the hippocampus and medial prefrontal cortex (Rasgon & McEwen, 2016). Antisense inactivation of the hypothalamic insulin receptor creates systemic insulin resistance, dyslipidemia, insulin resistance in the hippocampus, and depressive-like behavior and cognitive impairment (Rasgon & McEwen, 2016).

Many studies discuss knockdown models of insulin receptors, Grillo et al., indicating that insulin receptors knockdown in rat hypothalamus triggered depressive and anxiety-like behaviors (Grillo et al., 2011). They developed a lentivirus vector packaged with an insulin receptor antisense sequence (IRAS) targeting the decrease in insulin receptors selectively in the hypothalamus (hypo-IRAS). These hypo-IRAS rats demonstrated increased body weight, adipose tissue level, plasma leptin, and triglyceride levels. This obesity phenotype in hypo-IRAS rats stimulated depressive and anxiety-like behaviors (Grillo et al., 2011). Cai et al. indicate that insulin receptors knockdown in astrocytes also generated anxiety and depressive-like behavior in mice through decreased purinergic signaling and altered dopamine release (Cai et al., 2018). Other studies indicate that rats subjected to chronic unpredictable mild stress (CUMS) model of depression exhibit insulin resistance in the arcuate nucleus of the hypothalamus (Pan, Hong, Zhang, & Kong, 2013).

Furthermore, insulin resistance in the brain induces mitochondrial and dopaminergic dysfunction leading to anxiety and depressive-like behaviors (Mansur et al., 2018). An association between gene expression of proteins involved with the dopaminergic system and insulin signaling has been observed in a recent post-mortem brain analysis of patients with mental illness (Mansur et al., 2018). Therefore, demonstrating a potential molecular link between central insulin resistance and behavioral disorders (Andre Kleinridders et al., 2015b). Mice with a brain-specific knockout of the insulin receptor (NIRKO mice) exhibit brain mitochondrial dysfunction with reduced mitochondrial oxidative activity, increased reactive oxygen species levels, and increased lipid levels and protein oxidation in the striatum and nucleus accumbens. NIRKO mice also exhibit increased monoamine oxidase A and B (MAO A and B), leading to increased dopamine turnover in these areas. Studies in cultured neurons and glia cells indicate that MAO A and B's changes

directly affect insulin signaling loss. As a result, NIRKO mice develop age-related anxiety and depressive-like behaviors (Andre Kleinridders et al., 2015b).

HPA Axis

The hypothalamic-pituitary-adrenal axis (HPA or HTPA axis), also known as the limbic-hypothalamic-pituitary-adrenal axis, involves complex interactions between the hypothalamus, pituitary gland, and the adrenal glands (Neurology, 2012). This axis is a critical player in our response to physical or social stressors. HPA axis transforms stressful input into hormonal messages, enabling the organism to adapt to environmental change and maintain its homeostasis. The HPA axis is tightly regulated by central mechanisms involving the limbic system and the hypothalamus. Signals reach the paraventricular nucleus (PVN) of the hypothalamus from limbic structures. The secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin from neurons of the PVN triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. In addition to CRH, ACTH, cortisol, and numerous other messengers are involved in the HPA axis. ACTH travels in the blood and stimulates the adrenal gland. HPA axis is a classic example of an endocrine feedback system (Neurology, 2012). ACTH increases the production of cortisol, and the latter decreases the production of ACTH. Cortisol and other glucocorticoids exert feedback inhibition of both CRF and ACTH principally through the glucocorticoid receptor. Negative feedback on secretion can occur very rapidly, within 5–10 min, and this provides real-time inhibition to limit the stress response and prevents over secretion of glucocorticoids. The HPA axis functions as a component of the fight-or-flight response and the release of hormones such as cortisol and involved in body temperature, digestion, the immune system, mood, sexuality, and energy usage. HPA axis hyperactivity is commonly seen in people with depression and is associated with increased glucocorticoid levels in both the central nervous

system (CNS) (Neurology, 2012) and peripherally. Dysregulation of HPA function is a common underlying feature in neurodevelopmental and psychiatric disorders. Hypo and/or hyperreactivity of the HPA axis hormones have been reported in schizophrenia, autism, and depression. The hypothalamus is a structure that plays a significant role in coordinating neuroendocrine processes and can better reflect the changes associated with excessive activity of the HPA axis (Jan Detka et al., 2019). This hyperactivity, most likely due to the weakened inhibitory feedback mechanism, often occurs in depression, and stress is a common trigger of a depressive episode. Stress or excess glucocorticoid in experimental animals produces many of the behavioral, biochemical, functional, and morphological characteristics of depression (Detka et al., 2015). Notably, some stress factors acting during the perinatal period cause permanent changes in the HPA axis activity and increase the risk of depression. Factors adversely affecting the maternal environment, by persistent stimulation of the HPA axis activity, increase the risk of mental and metabolic, and cardiovascular diseases (Detka et al., 2015). Numerous studies have validated hypercortisolemia in depression; however, a coupled relationship was expected, which proved more challenging to demonstrate. It is unclear whether the hypercortisolemia of depression is due to excessive drive with increased ACTH secretion or enhanced adrenal sensitivity to ACTH. Thus, HPA overactivity in depression could be due to one or a combination of factors: increased CRH drive decreased sensitivity to glucocorticoid feedback, altered secretory rhythms, and altered response to stress.

Utilizing dexamethasone suppression test (DST) to measure hyperactivity of the HPA axis showed the risk of suicide among patients with abnormal DST responses was estimated to be 26.8% while the risk was only 2.9% for those with normal DST responses(Neurology, 2012).

Many of the hormones involved in the maintenance of glucose homeostasis, such as insulin and glucagon-like peptide1 (GLP-1), are also known to exert neurotrophic action and enhance

neurotrophic processes. However, some of the factors involved in energy homeostasis regulation may also negatively affect neuronal plasticity. A typical example of such a two-way acting hormone is corticosterone, which at physiological concentrations exerts neuroprotective properties, whereas at high levels of chronic exposure may aggravate nerve damage. Adverse effects of excessive glucocorticoid action on neuronal plasticity are essential in the pathogenesis of depression; however, this action's mechanism is poorly explored. Some studies suggest that these effects may result from the inhibition of glucose and glutamate uptake or downregulation of growth factor synthesis. Thus far, little attention has been directed at the action of glucocorticoids on brain pathways involved in energy metabolism in the development of depression. They act in the hypothalamus to impair insulin signaling in an animal model of depression. In contrast to the well-defined peripheral metabolic effects of glucocorticoids, these hormones' central metabolic actions and metabolic disturbances in depression are still poorly understood.

Furthermore, the peripheral action of glucocorticoids is known to enhance gluconeogenesis and evoke peripheral insulin resistance. These hormones increase blood glucose levels in experimental animals and evoke peripheral insulin resistance at pharmacological doses and concentrations observed under stress.

HPA axis hyperactivity causes prolonged exposure to excess glucocorticoids that can cause respiratory chain dysfunction, increased ROS generation, mitochondrial structural abnormalities, apoptosis, and cell death in skeletal muscle cells and hippocampal neurons (Neurology, 2012).

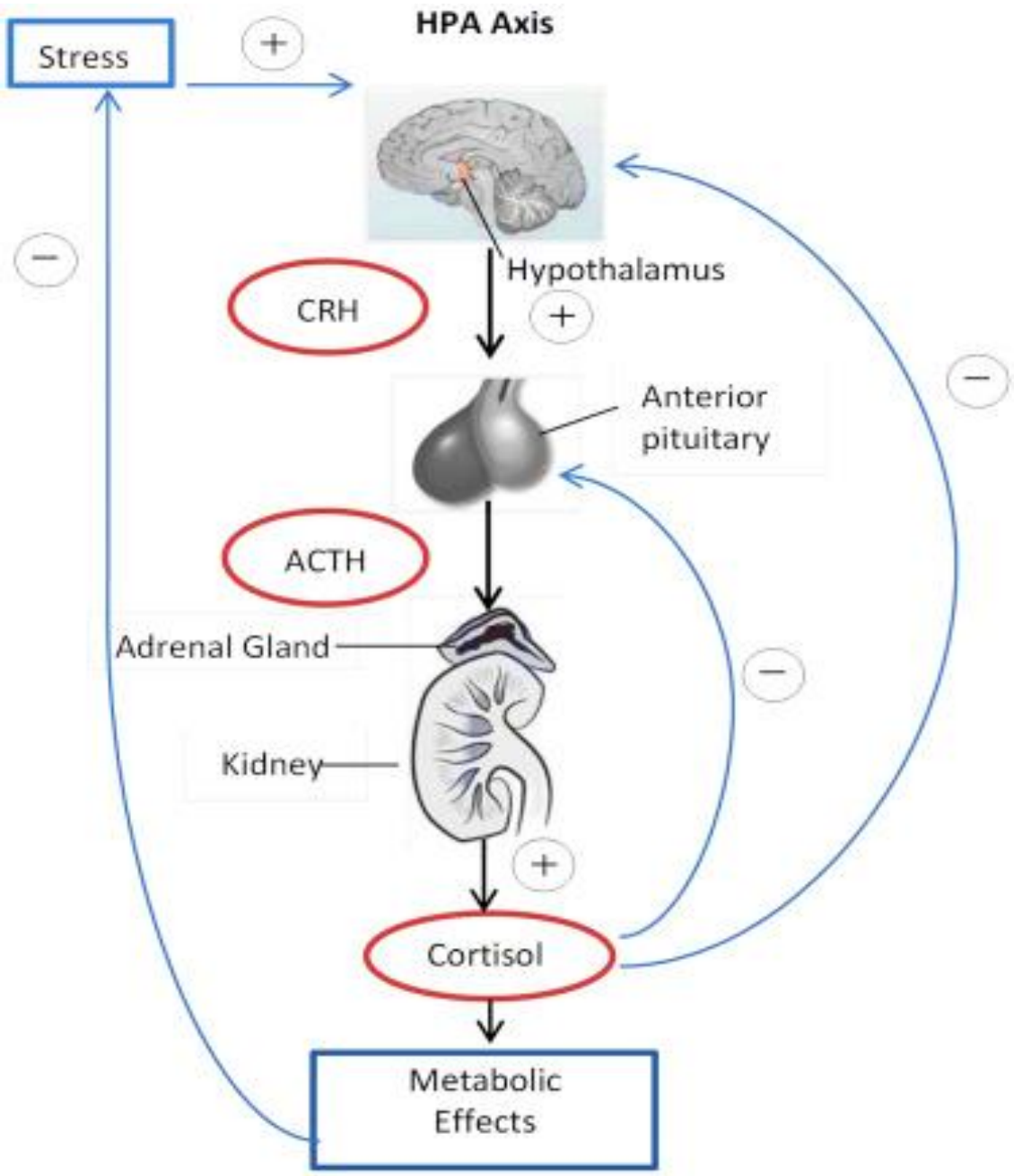


Figure 2: HPA axis interactions and feedback regulations(Kulkarni, Gavrilidis, & Worsley, 2016)

Inflammation

Metabolic and immune pathways are highly integrated and interdependent. They are critical requirements for survival and highly conserved throughout evolution. It became apparent that this crossing point plays a critical role in the pathogenesis linking chronic metabolic diseases like obesity, type 2 diabetes, with inflammation. Notably, the inflammatory component in obesity and diabetes is now firmly established. Causal links between inflammatory mediators, such as tumor necrosis factor (TNF) overexpressed in the adipose tissues from rodent models of obesity as an inflammatory cytokine, disrupt insulin receptor signaling action cells and whole animals (K. Teoman Uysal*‡ & Hotamisligil*, 1997). Besides, inflammation is the body's response to deal with a broad range of injuries. Other studies indicate that chronic inflammation may also bear the association of cytokines and other inflammatory markers, such as increased C-reactive protein that rises in diabetes and the metabolic syndrome and is associated with causing sickness behavior in animal models and depression in humans (Holt et al., 2014).

Obesity, a significant contributor to insulin resistance, shows an overproduction of TNF-α in adipose tissue, a feature seen in experimental models (Buchowski et al., 2017). Obesity inducing hyperlipidemia is responsible, in part, for inducing peripheral tissue insulin resistance, dyslipidemia, and contributes to the development of atherosclerosis. Lipids achieve a complicated role in metabolic disease. Hyperlipidemia leads to elevated uptake of fatty acids by muscle cells and the production of fatty acid metabolites stimulating inflammatory cascades and inhibiting proper insulin signaling (Zick, 2003). Interestingly, metabolic changes characterized in the acute-phase response are also proatherogenic; thus, altered lipid metabolism, which has short-term benefits in fighting against infection, is harmful if maintained chronically.

Insulin affects cells by binding to its receptor on the surface of insulin-responsive cells, causing the receptor and several substrates' phosphorylation, including members of the insulin receptor substrate (IRS) family, initiating downstream signaling events. Signaling downstream inhibition of the insulin receptor is a fundamental mechanism where inflammatory signaling leads to insulin resistance. Exposure to TNF-α or elevated levels of free fatty acids stimulates inhibitory phosphorylation of serine residues of IRS-1, thus reducing tyrosine phosphorylation of IRS-1 in response to insulin and the IRS-1's ability to associate with the insulin receptor, inhibiting downstream signaling and insulin action (Hotamisligil et al., 1996; M.F.White, 1997). It has been demonstrated that overloading the functional capacity of the endoplasmic reticulum (ER) is mainly due to obesity and that this ER stress leads to the activation of inflammatory signaling pathways and thus contributes to insulin resistance (Nakatani et al., 2005). Therefore, obesity is associated with a state of chronic, low-grade inflammation, particularly in white adipose tissue.

Inflammatory pathways adipocytes or macrophages can be initiated by extracellular mediators such as cytokines and lipids or intracellular stress such as ER stress or excess ROS production by mitochondria. Signals from all these mediators converge on inflammatory signaling pathways, including the kinases JNK and IKK. These pathways lead to additional inflammatory mediators' production through transcriptional regulation and insulin signaling's direct inhibition. TNFα promotes insulin resistance by the phosphorylation of insulin receptor substrate 1 (IRS1) on serine residues (Felger, Haroon, Woolwine, Raison, & Miller, 2016).

Stress also increases the levels of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, and TNF-α, which can suppress mitochondrial activity and induce mitochondrial damage (Neurology, 2012). Analysis of the mixed-gender cohort showed that depressive symptoms were positively associated with higher leptin and CRP(C reactive protein) levels (Buchowski et al.,

2017).). Inflammatory and stress-response genes, revealed in transcriptional profiling studies, are among the most abundantly regulated gene sets in adipose tissue of obese animals (Buchowski et al., 2017). Leptin is one such hormone that plays essential roles in both adaptive and innate immunity, and both mice and humans lacking leptin function exhibit impaired immunity(Alexander Soukas, 2000). Inflammatory or stressful stimuli activate several serine/threonine kinases and contribute to insulin signaling inhibition, including JNK and IKK (Zick, 2003).

Furthermore, TNFα contributes to depressive states by modifying the serotonin system. For instance, this cytokine can activate the enzyme indoleamine 2,3-dioxygenase degrading the precursor molecule tryptophan and indirectly decreasing brain serotonin levels (Cheng et al., 2018). This cytokine also promotes blood-brain barrier (BBB) disruption in patients with T2D and in a mouse model of depression, which could ultimately lead to loss of the BBB transport regulation of other inflammatory signals, and exacerbate allostatic load to the HPA axis, leading to its dysregulation (Felger et al., 2016).

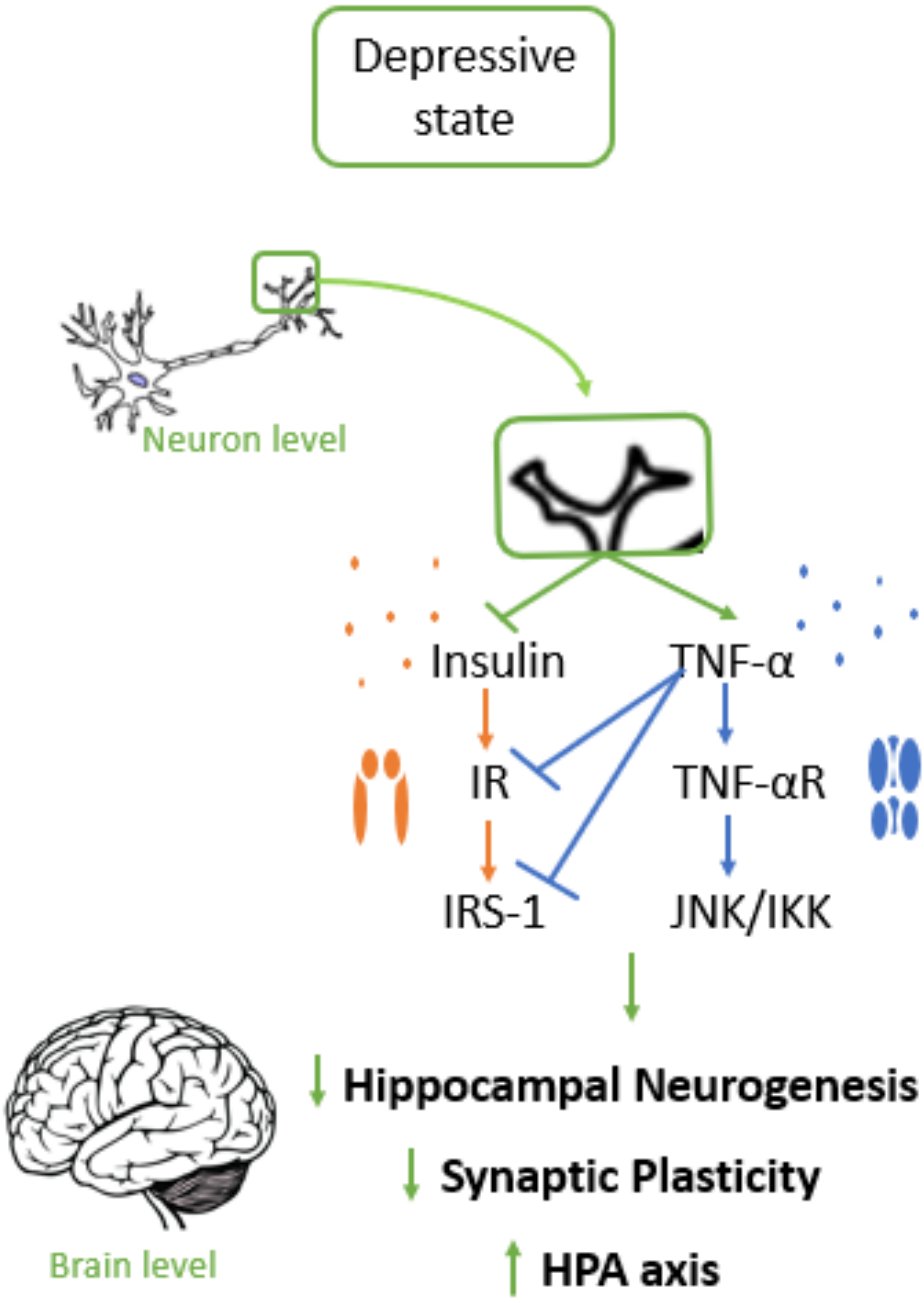


Figure 2: Inflammation overtaking in the depressive state causing an alteration in normal brain activity

Oxidative Stress

The literature establishes that both vulnerabilities, hyperglycemia, and depression are associated with increased cellular and systemic oxidative stress. Today, it is believed that oxidative stress milieu is considered a common pathway of injury leading to diabetes and depression (Arent et al., 2012; de Morais et al., 2014).

Antioxidant drugs such as N-acetyl-L-cysteine, deferoxamine, and vitamin E were reported to reduce oxidative stress, depressive behavior, and hyperglycemia in animals (Arent et al., 2012; de Morais et al., 2014). Other antioxidant treatments prevent or slow the development of depression in diabetic animals (Cameron, Cotter, Archibald, Dines, & Maxfield, 1994; Coppey, Gellett, Davidson, & Yorek, 2003; Sayyed, Kumar, & Sharma, 2006).

Added together, ROS may have a significant pathogenic role in the pathology of depression and diabetic complications. Several mechanistic pathways involve ROS production in the CNS and PNS (Edwards, Vincent, Cheng, & Feldman, 2008; Korczak, Pereira, Koulajian, Matejcek, & Giacca, 2011). Likewise, various sources of ROS alterations are reported in diabetes. Recent studies indicate a critical role for the NADPH oxidase (Nox) family as a ROS source in diabetic nephropathy, retinopathy, and cardiomyopathy However, NADPH oxidase involvement in depression and DPN remains unknown. The NADPH oxidases are a family of enzymes whose primary role is to produce ROS. The NADPH oxidase family includes Nox1, Nox2, Nox3, Nox4, and Nox5, DUOX1, and DUOX2. These isoforms contain a FAD-binding region near the COOH-terminus, an NADPH binding domain at the COOH-terminus of the oxidases, six transmembrane domains, and four highly conserved heme-binding histidines located on the third and fifth transmembrane domains (Bedard & Krause, 2007). The expression of these molecules differs across tissues. In the nervous system, Nox2 is the main isoform present, made of two membrane-

bound subunits, gp91phox (also known as Nox2) and p22phox. The interaction between p67phox, p47phox, p40phox, and Rac leads to ROS production, including oxidative stress (Bedard & Krause, 2007; Brandes, Weissmann, & Schröder, 2014; Touyz, Briones, Sedeek, Burger, & Montezano, 2011).

Neuroplasticity and Neurogenesis

The neural plasticity is the ability of the nervous system to adapt in response to intrinsic and extrinsic stimuli (Jan Detka et al., 2019). The process of plasticity involves neurogenesis, that is, proliferation and differentiation of neural stem cells (NSCs) and changes in differentiated neurons' morphology and activity. Hippocampal neurogenesis, which involves neural progenitors from the sub-granular zone to differentiate into new neurons in the dentate gyrus, is proposed to be involved with depression and be impaired in diabetes (Hill, Sahay, & Hen, 2015). Adult hippocampal neurogenesis a process where new granule cells are added to the dentate gyrus. These cells are produced from progenitors located in the dentate gyrus' sub-granular zone, and their maturation, rates of proliferation, and survival are impacted by environmental conditions such as age, stress, exercise, and antidepressants (Hill et al., 2015).

These adjustments are essential to various peripheral organs' brain organization to adapt energy expenditure to nutrient availability (Mainardi, Fusco, & Grassi, 2015).

Mediators of brain structural plasticity include glucocorticoids, excitatory amino acids, growth factors such as brain-derived neurotrophic factor (BDNF), circulating hormones such as insulin, insulin-like growth factor-1, leptin, and ghrelin (Rasgon & McEwen, 2016). Defects in this process may be linked with the decreased level of the glucagon-like peptide-1 receptor (Jan Detka et al., 2019). Defects in synaptic plasticity may lead to impairment of stress adaptation, prompting the onset of depression. In the food reward circuitry, synaptic plasticity is modulated due to insulin

actions, depending on concentration, time, and brain region-dependent manner. Equally, insulin promotes long-term depression of glutamatergic afferent connections into the VTA while increasing the striatal cholinergic interneurons' activity, elevating dopamine release into the NAc (Ferrario & Reagan, 2018; Labouebe et al., 2013). Downregulation of insulin receptors in rats' hippocampus impaired proper long-term potentiation response mediated by high-frequency stimulation and decreased glutamate receptors levels. This approach also worsened learning behavior, like what is observed in T2D rodent models. The acquired data indicates that brain insulin resistance can impair reward and learning physiological mechanisms that would elicit depressive symptoms (Grillo et al., 2015).

The hypothalamus is only one of the brain areas sensitive to hormones and metabolic signals. Indeed, food-seeking during fasting is a complex activity that involves information processing to identify or remember the location of resources necessary for survival (Mainardi et al., 2015). The astrocytic energy sources glycogen and lactate seem to be directly relevant for learning and memory, although the underlying mechanisms have not yet been elucidated. The glucose transporters (GLUT1 and GLUT3) mediate glucose uptake from the extracellular fluid into glial and neuronal cells. These transporters are insulin-independent, suggesting that the impact of insulin and related signals on brain plasticity should be independent of glucose uptake (Mainardi et al., 2015).

Following this, it has been demonstrated that in animal models of both diabetes and depression, there are similar changes in the structure and function of the hippocampus, including inhibition of neurogenesis in the dentate gyrus, reorganization of the apical dendrites of pyramidal cells in the CA3 region and disturbance of hippocampal-related spatial memory (Hoyer & Lannert, 2008).

Additionally, cAMP response element-binding protein (CREB), CREB, acts as a major transcription factor in brain development and neurogenesis. CREB is activated in a phosphorylated form, and multiple protein kinases phosphorylate this transcription factor and convert CREB to its active form (Keshavarzi et al., 2019). CREB acts on DNA and prompts BDNF proteins' production, essential in neurogenesis and neurons' development. Many previous works indicated that phosphatidylinositol 3-kinase (PI3K) could activate Akt in brain cells, and by activation of this protein, glycogen synthase kinase 3 (GSK3), which is involved in neurodegeneration, will be inhibited, protecting cells from neurodegenerative effects of GSK3 (Keshavarzi et al., 2019).

However, Insulin-like growth factor I (IGF-I) and leptin activate Akt and GSK3β pathway and mediate hippocampal neurogenesis (Papazoglou, Jean, Gertler, Taouis, & Vacher, 2015). Interestingly, the downregulation of those hormones is observed in the hippocampus of rodent models of T2D, leading to other possible targets to the link between T2D and depression (Garza, Guo, Zhang, & Lu, 2012). Mitochondrial dysfunction in T2D patients proposes an impairment of neurogenesis. Also, HFD impairs cell proliferation, insulin signaling, and neurogenesis (Mori et al., 2004).

Hypothesis and Aim of the Study

A growing body of research supports the increased prevalence of comorbid diabetes and depression. Evidence supporting that diabetes and depression share biological mechanisms is growing. These origins stem from a dysregulation of the HPA axis, and overactivation of innate immunity and inflammation, and oxidative stress. They cause insulin resistance, atrophy of the prefrontal cortex and hippocampus, β-cell apoptosis, circadian rhythm disturbance, endothelial dysfunction, and myelin injury. Besides, several studies indicated the presence of biological variation between female and male mice. However, the mechanisms causing a difference between gender in the nervous system in diabetes and depression are still unclear. Moreover, understanding if any difference is present between the onset of depression in diabetes will improve the outcomes and simultaneously provide a potential treatment of diabetes and depression.

Our hypothesis states that both disorders, through brain insulin resistance, contribute to the onset of one another and that their comorbidity worsens and speeds up the progression of pathological changes. This project will allow the study of the effect of gender on depression onset in diabetic mice. Also, to determine the disparity between diabetes-induced depression and vice versa.

It will allow the identification of novel molecular mechanisms involved in depression and diabetes from a fundamental perspective. This study will pave the way to examine potential tailoring of sex-specific diabetes and depression treatment from a clinical perspective.

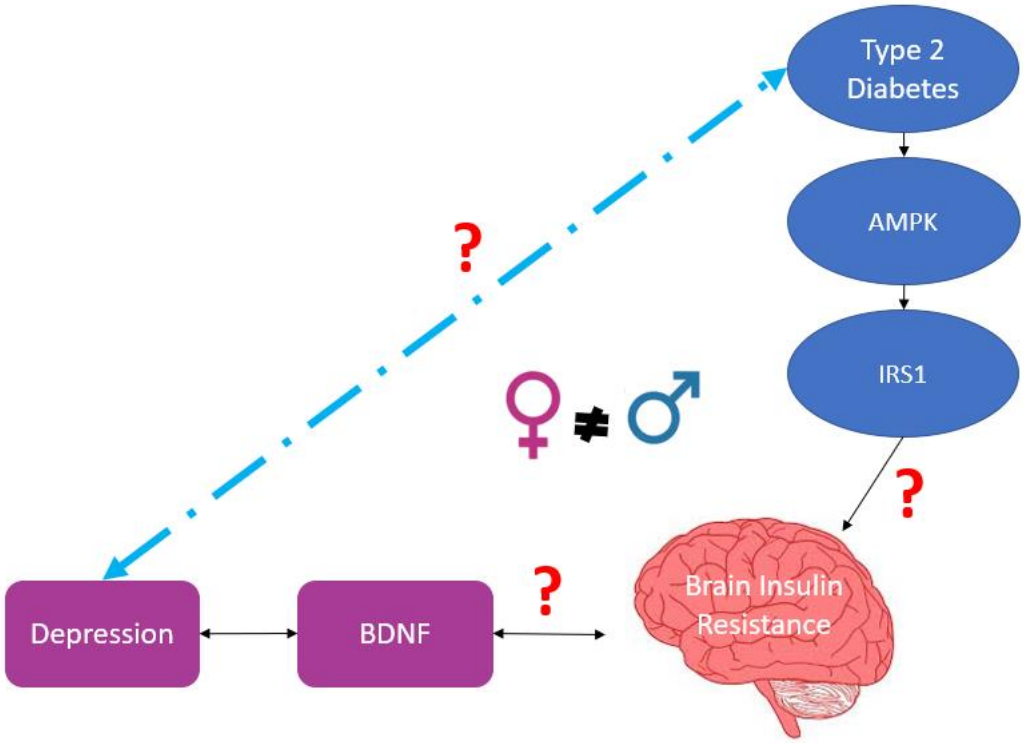


Figure 4: Brain insulin Resistance as the biological link between Depression and Type 2 Diabetes, and its sex specificity

CHAPTER II

MATERIALS AND METHODS:

Animal Studies

C57BL/6J mice, the type of mice utilized in this study, were bred at the American University of Beirut. Mice were randomly divided eight weeks of age into 5 groups each of 8 animals. At week 9 two of these groups where randomly selected to be exposed to a 28-day chronic mild stress (CMS) protocol, and two other groups where exposed to five consecutive weeks of high fat diet then STZ injection intraperitoneally to develop T2DM. These rodents were then reassessed for behavioral changes including Beam Walking (BW), Von Frey (VF), Paw Withdrawal (PW), Forced Swim Test (FST), Tail Suspension Test (TST) and Sucrose Preference Test (SPT). Behavioral testing was established to assess sensorimotor dysfunction and depressive-like symptoms prior to any modification. At week 22 control, diabetic, and depressed groups were sacrificed and at week 30 the two resting groups were sacrificed for molecular testing. Details pertaining to each section are provided below.

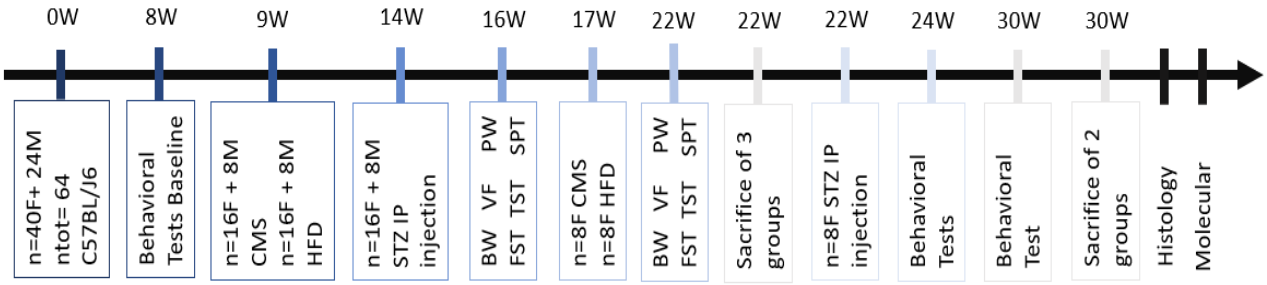


Figure 5: The Experimental Design of this Study

The total number of animals used is 57 divided to 37 female mice and 20 male mice, all animals are from the same background C57BL/J6. An obese type 2 diabetic mice model was used where diabetes is developed approximately after 1 week of STZ injection causing a selective destruction

of the β cells of the pancreas by oxidative stress. Therefore, insulin level in blood is decreased causing hyperglycemia and leading to sever symptoms. C57BL/J6 mice belonging to the same age group were used as control. These eight-week-old mice were divided into five groups as illustrated in table 1.

1. Control n=8F n=6M	
2. Control where depressive-like behavior was induced (depressed) n=8F n=8M	3. Control mice where diabetes was induced (diabetic) n=7F n=6M
4. Control where depressive-like behavior was induced and then diabetes (depressed + diabetic) n=8F	5. Control where diabetes was induced and then depressive-like behavior (diabetic + depressed) n=6F

Table 1: Grouping of 37 Female Mice and 20 Male Mice into Five Subsets

All animals were kept in a temperature-controlled room and on a 12/12 dark/light cycle and had standard chow and water access ad libitum. All experimental protocols were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee of the American University of Beirut. Body weights (BW) of these animals were taken on a weekly basis using a digital balance. Blood glucose levels (BGL) were monitored weekly via tail vein punctures using a glucometer (CONTOUR PLUS, Ascensia Diabetes Care Holdings AG)(Eid et al., 2009).

Depression Protocol

The protocol used is a modified version given by literature(Kumar, Kuhad, & Chopra, 2010; Molina et al., 1990; Murua, Gomez, Andrea, & Molina, 1991) where food deprivation was replaced with other stressors since the presence of diabetic mice. This protocol, spanning for 28 consecutive days, aimed to cause depressive-like symptoms in mice. Everyday a different stressor was introduced to the mice having a stress-free day per week(Franceschelli, Herchick, Thelen, Papadopoulou-Daifoti, & Pitychoutis, 2014; Katz, Roth, & Carroll, 1981; Kompagne et al., 2008;

Zhu, Shi, Wang, Wang, & Li, 2014). This way probability of habituation was lowered with a high unpredictability.

In this study, the following stressors were used. The change of mate stressor involved placing an 'intruder' mouse from one of the cages into another for 2 hours. Katz et al have reported an increase in plasma corticosteroid levels of both the 'intruder' mouse and the 'host' due to their exposure to different pheromones and odors(Katz et al., 1981). Titling the cage at an angle of 45 degrees made it difficult for mice to reach for their normal chow and bottled water. This was shown to trigger anhedonia behavior. In addition, pre-exposure to 95 dB of white noise for 6-8 hours has been demonstrated to initiate a highly active behavioral profile in mice. Wetting the bedding of the cages further induced discomfort, causing mice to urinate and defecate as a direct response to stress. Also, small pads of cotton dipped in freshly collected rat urine were placed in each cage for a period of 4 hours. Shivering symptoms and increased locomotor activity were observed in these mice. More recently, it was suggested that manipulation of the light/dark cycle could characterize a new model of depression.

In this study, these nocturnal animals were also exposed to room light for 48 consecutive hours. According to Abelaira et al light/dark cycle manipulation further contributes to anhedonia behavior, alongside increased corticosterone and decreased brain-derived neurotrophic factor levels in the hippocampus(Abelaira, Reus, & Quevedo, 2013).

Chronic Mild Stress (28 days)	
Day 1: Change of mate (2h) (change number 1)	**Day 14&15:** Sleep deprivation (46h) (lights off)
Day 2&3: Sleep deprivation (lights off)	**Day 16:** Cage tilt 45º (8h) + Change of mate (2h) (change number 4)
Day 4: Wet cage (8h)	**Day 17:** White noise (8h)
Day 5: Rat urine (4h)	**Day 18:** Rat urine (4h)
Day 6: Cage tilt 45º (8h)	**Day 19:** Wet cage (8h)
Day 7: Stress free	**Day 20&21:** Sleep deprivation (46h) (lights on)
Day 8&9: Sleep deprivation (46h) (lights on)	**Day 22:** Stress free (sucrose measurement)
Day 10: White noise (8h)	**Day 23:** Cage tilt 45º (8h)
Day 11: Wet cage (8h)	**Day 24:** Change of mate (2h) (change number 1 and 2) + Rat urine (4h)
Day 12: Cage tilt 45º (8h)	**Day 25:** Wet cage (8h)
Day 13: Stress free	**Day 26&27:** Sleep deprivation (46h) (lights off)
Day 14: Change of mate (2h) (change 2 mates' number 2 and 3)	**Day 28:** Cage tilt 45º+ White noise (8h)

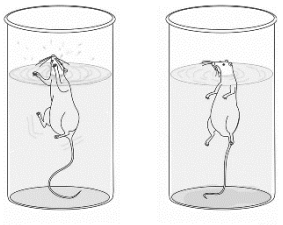
Table 2: Chronic Mild Stress Protocol used

Behavioral Tests

Prior to sacrifice, sucrose preference, forced swim, and tail suspension tests were performed for depression assessment. Sensorimotor dysfunction was assessed using the raised beam walking test, Von Frey and paw withdrawal test.

Depression Assessment

Forced Swim Test

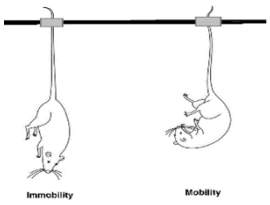


The Forced Swim Test (FST) is utilized for assessment of depressive-like behavior in rodents. When mice are forced to swim, after initial vigorous escape-directed behavior, such as swimming and climbing, they stop struggling and adopt an immobile position. This immobility is interpreted either as failure to persist in escape-directed behavior after stress, or passive behavior development that prevents the rodent from coping with stress. According to multiple studies, a test session without a pre-swim exposure is sufficient to ensure a stable immobility recording in mice for unknown reasons(Porsolt, Le Pichon, & Jalfre, 1977).

In this model the mice are put for 5 minutes each in a Plexiglas tank (diameter 20cm, height 30cm) without having its limbs encountering the bottom of the container. The tank is loaded with water (15cm of height with a temperature 25 ± 1°C) and the behavior of the mice to escape the tank is scored. The behavioral parameter scored in this test is called immobility, resembling a behavioral state of misery, as seen in human depression.

Non depressed mice will spend time more having a “mobile” behavioral. On the contrary depressed rodents will spend time more having an “immobile” state.

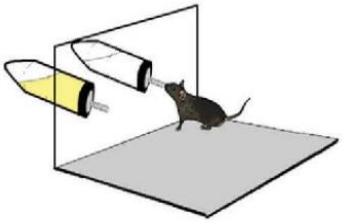
Tail suspension Test



The tail-suspension test is utilized for assessment of depression-like behavior in rodents and help in screening for potential antidepressant drugs. In this model the mice are suspended for 6 minutes by their tails with tape on a thin rod beam (50 cm of height), in such a position that it cannot escape or hold on to nearby surfaces. During this test, the resulting immobility behaviors are quantified resembling a behavioral state of misery, as seen in human depression(Can et al., 2012).

Non depressed mice will spend time more having a "mobile" behavioral. On the contrary depressed rodents will spend time more having an "immobile" state.

Sucrose Preference Test



The sucrose preference test, a reward based test, is utilized for assessment of depression-like behavior in rodents specifically to indicate anhedonia(Huynh, Krigbaum, Hanna, & Conrad, 2011). Anhedonia, defined as the inability to experience pleasure from enjoyable tasks, is a core symptom of depression. In this model the mice have access to 2 bottles (500ml) one containing water and the other containing 1% sucrose dissolved in water. This test takes one week where the bottles are changed after four days of start time with a randomized placement of the bottles to eliminate side preference artifact. The latter are weighed before and after placement in that way calculation will be made to know mice preference(Gross & Pinhasov, 2016; Kompagne et al., 2008).

Non depressed mice will have a higher consumption of sucrose water, since rodents are born with an innate preference for sweet tastes. Sucrose consumption below 65% is indicative of

anhedonia(Strekalova et al., 2011). On the contrary depressed rodents will have a higher consumption of regular water(Vogel, Neill, Hagler, & Kors, 1990; Willner, 2016).

Sucrose preference was calculated as the percentage of total liquid intake ascribed to 1% sucrose solution, according to the equation:

$$Sucrose\ Preference = \left(\frac{sucrose\ intake\ (g)}{sucrose\ intake\ (g) + water\ intake\ (g)}\right) * 100$$

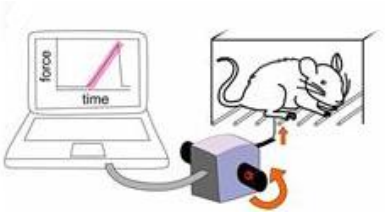
Motor Function Assessment

Raised Beam Walking Test Assay

Motor coordination and balance are assessed through the Raised Beam Walking test(Luong, Carlisle, Southwell, & Patterson, 2011). This task is useful for the detection of subtle deficits in motor skills and balance. The test was performed on all the animals at the age of 8 weeks (baseline) and at the age of 32 weeks. The mice were placed on a platform with a rod of 1.2 cm diameter, 70 cm length, and 50 cm above a flat surface. For housing purposes, we set a secure platform at the end of the rod. The animals were given time to adapt and were trained three times to cross to arrive to the secure platform. The time taken to cross, the speed, the number of stops and the number of faults/slips were recorded and analyzed.

Sensory Function Assessment

Mechanical Allodynia Test (electronic Von Frey 2g)



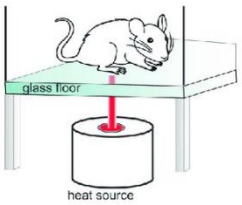
The Electronic von Frey instrument uses the Dynamic Plantar Aesthesiometer (DPA) (Ugo Basile Biological Apparatus) to automate the assessment of touch sensitivity on the plantar

surface of mice. At each paw withdrawal, the DPA automatically detects and records latency time, and the actual force at the time of paw withdrawal reflex. The animals were placed in clear plastic over an elevated horizontal wire mesh stand over 30 minutes before the test to acclimatize. They were not able to see each other due to opaque surfaces separating them. A movable force actuator was positioned under the plantar surface of the animals. The tip of a 2g force Von Frey filament was applied perpendicularly to the middle of the mouse's hind paw. The time taken to elicit a withdrawal response was noted. Five trials per animal were used, separated by a 5 minutes' interval. It is important to note that the withdrawal time should be measured in both hind paws. But in the case of polyneuropathy (diabetes), the measurement of withdrawal time in one hind paw is enough(Ferrier et al., 2015).

Mechanical Hyperalgesia (electronic Von Frey 15g)

The Electronic von Frey instrument uses the Dynamic Plantar Aesthesiometer (DPA) (Ugo Basile Biological Apparatus) to automate the assessment of touch sensitivity on the plantar surface of mice. At each paw withdrawal, the DPA automatically detects and records latency time, and the actual force at the time of paw withdrawal reflex. The animals were placed in clear plastic cages over an elevated horizontal wire mesh stand over 30 minutes before the test to acclimatize. They were not able to see each other due to opaque surfaces separating them. A movable force actuator was positioned under the plantar surface of the animals. The tip of a 15g force Von Frey filament was applied perpendicularly to the middle of the mouse's hind paw. The time taken and the required force to elicit a withdrawal response were noted. Five trials per animal were used, separated by a 5 minutes' interval.

Heat Hyperalgesia Test (Paw withdrawal)



The plantar test apparatus (Ugo Basile Biological Apparatus) was used to measure the withdrawal latency to noxious radiant heat application of an infrared light (IR 40) to the plantar surface of the hind paw of the animals. The mice were placed in clear plastic cages on an elevated thick glass plate for 30 minutes to acclimatize. They were not able to see each other due to opaque surfaces separating them. The time taken to elicit a withdrawal reflex was noted. Five trials per animal were used with a 5 minutes' interval to avoid damaging the tissues.

Histological

Tissue Removal (Prefrontal lobe and Hippocampus)

The mice were deeply anesthetized with cotton pads soaked in isoflurane to eliminate perception of pain. After cervical dislocation, these mice were then decapitated using a surgical scissor with a cut posterior from the ears. After pulling the scalp gently to lateral sides, the scalp skin of each head was cut from between the rodent's eyes down the midline using a razor blade. The tip of one of the scissors was placed into the foramen magnum and cut laterally into the skull. The same step was repeated for the other side, making cuts as superficial as possible not to perturb the brain. Small cuts were made from the midline incision near the eyes laterally. Forceps were used to apply gentle tangential pressure to either of the newly formed skull flaps. Properly applying this force allows the skull to be fully removed, exposing the brain. The brain was then transferred onto a petri dish filled with cold PBS solution placed on ice, with its ventral side facing the dish. Using large-curved forceps, the cortical halves were slowly opened. The initial white-colored part encountered is the corpus callosum, bearing the hippocampus underneath. Peeling the cortical

hemisphere laterally in a gentle manner required anchoring one spatula tip over the cerebellum near the junction with the cortex, and the other near the same junction. Once the cortical hemisphere is fully peeled laterally, the hippocampus should be exposed.

To obtain the prefrontal cortex, the brain was then flipped having the dorsal side facing the petri dish. Using a sharp razor blade, a coronal section was made to cut off the olfactory bulb. The anterior commissure should be well visible at this point. The first section contains mainly the motor cortex. The subsequent section comprises the anterior forceps of the corpus callosum with a darker area in the middle representing the prefrontal cortex. A second cut allows the collection of the prefrontal cortex. Since the brain is a delicate, soft tissue, performing these steps within 2-3 minutes was crucial. These surgical procedures are explicitly illustrated by Sultan, and Chiu et al (Chiu, Lau, Lau, So, & Chang, 2007; Sultan, 2013).

Additionally, blood was collected in EDTA tubes for HbA1c test.

Molecular

After euthanizing the animals, the prefrontal cortices and hippocampi were collected for biochemical analyses.

Western Blot

Mouse prefrontal cortices and hippocampi were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 50 mM Trishydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors. The lysates were centrifuged at 13,600 rpm for 30 minutes at 4°C. Protein concentration in the supernatants was measured using the Lowry Protein Assay. For immunoblotting, 30-70 µg of proteins were separated on 12-15% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory,

CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% BSA in Tris-buffered saline and then incubated with rabbit monoclonal anti-pAMPKα antibody (1:1000,cell signaling), rabbit monoclonal anti-IRS1 antibody (1:500, millipore), rabbit monoclonal anti-BDNF antibody (1:1000, cusabio), or rabbit monoclonal anti-GLP1 antibody (1:600, abcam). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). Goat polyclonal anti-HSC70 (1:1000; Santa Cruz Biotechnology) was used as a loading control. Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software(Eid et al., 2009).

Statistical Analysis

Results are expressed as mean ± SD for behavioral tests and mean ± SEM for molecular experiments. For both behavioral and molecular assessments, normality tests were done to ensure that the sample distributions of both the groups of mice are normal. Statistical significance was assessed using both one-way and two-way ANOVA to test for the efficacy of the administered treatments across all groups. Also, student's unpaired t-test was performed. P-value <0.05 is considered as statistically significant.

CHAPTER III

RESULTS:

Metabolic Parameters of All Eight Groups

Table 4 summarizes the body weights (BW), blood glucose levels (BGL) and the hemoglobin A1C levels (HbA1C) at the 25th week prior to sacrifice. BGL and HbA1c levels were significantly higher in diabetic, depressed/diabetic, and diabetic/depressed animals relative to control littermates. These levels were measured to provide a critical parameter for assessing long-term glycemic control and predicting the incidence of diabetic complications in diabetic patients (Han et al., 2008).

Group	BW(g)	BGL (mg/dL)	HBA1C(mmol/ml)
Control F (n=7)	21.82 ± 1.41	150.25 ± 9.24	5.45 ± 0.39
Depressed F (n=8)	20.59 ± 1.78	147.26 ± 11.45	5.1 ± 0.26
Diabetic F (n=7)	19.99 ± 0.58	228.58* ± 87.95	8.875*± 0.44
Depressed/Diabetic F (n=8)	20.97 ± 1.28	199.89* ± 87.77	7.975* ± 0.33
Diabetic/Depressed F (n=6)	20.81 ± 1.43	309.77* ± 44.79	8.0125* ± 0.39
Control M (n=8)	25.64 ± 2.37	155.89 ± 13.10	5.575 ± 0.27
Depressed M (n=8)	26.08 ± 2.01	153.96 ± 14.10	4.9 ± 0.63
Diabetic M (n=6)	23.98 ± 1.01	314.408* ± 143.71	9.2875* ± 0.51

Table 3: The body weights (BW), blood glucose levels (BGL) and hemoglobin A1C levels (HbA1C) of all 36 female mice and 22 male mice at week 22 or 30, prior to sacrifice. *$p<0.05$, comparison with respect to Control.

Behavioral

Depression Assessment

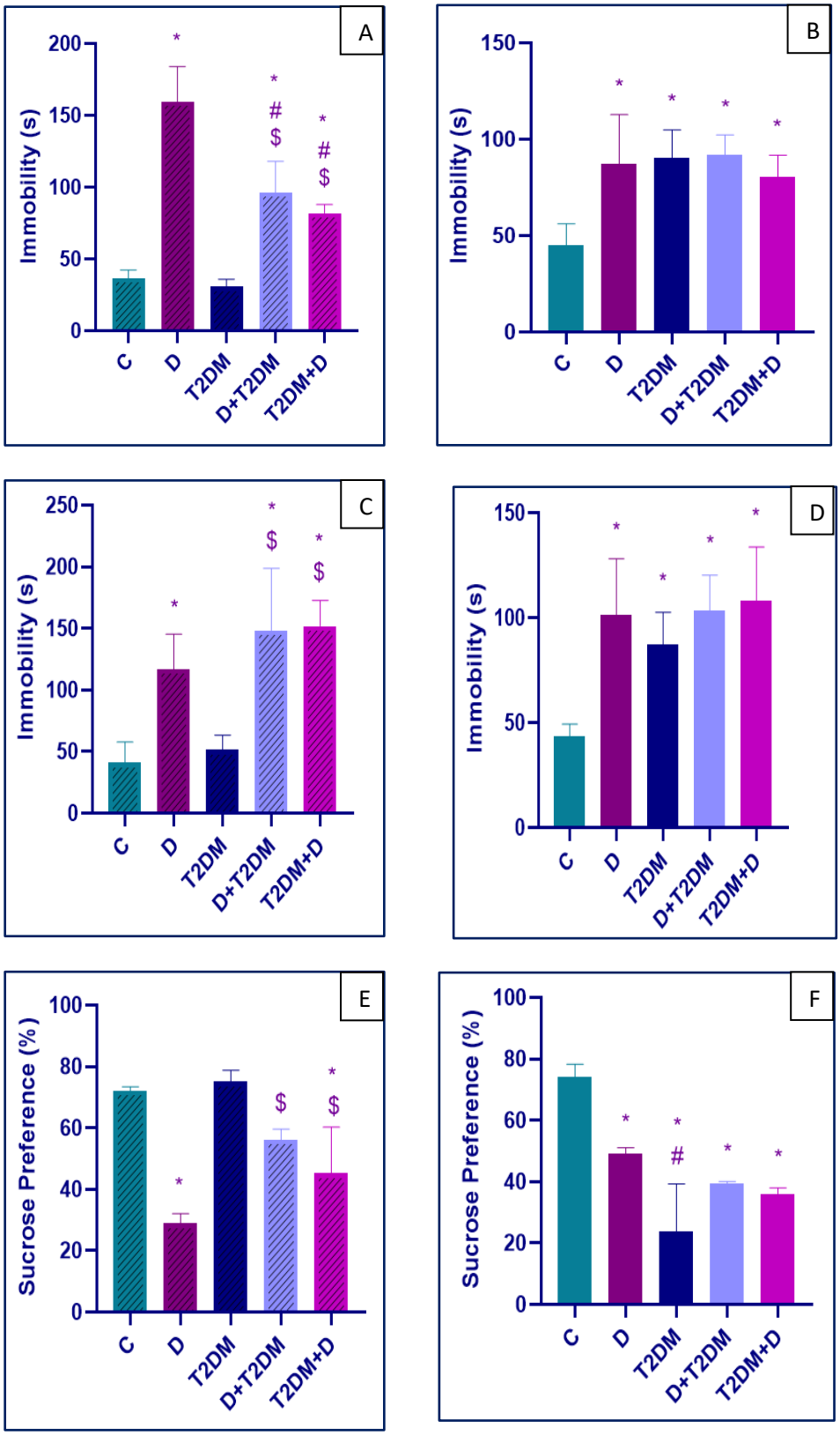
Female Mice with type 2 Diabetes Develop Depressive-like Symptoms

Using the FST (Figure 6A, B), the immobility time of the depressed, depressed/diabetic, and diabetic/depressed female mice is significantly greater than that of their control littermates after two weeks of disease induction. Also, the immobility time of the diabetic, the depressed/ diabetic and the diabetic/depressed animals is significantly greater than that of the diabetic group. Furthermore, a significant variance was present between diabetic/depressed and depressed/diabetic to the depressed group. However, after eight weeks, a similar trend is seen with a significant increase in the immobility time of the diabetic group than the control with no significant difference between depressed, diabetic/depressed, and depressed/diabetic.

In the TST (Figure 6C,D), a same drift is seen where the immobility time of the depressed, diabetic/depressed, and the depressed/diabetic is notably greater than that of the control group after two weeks of disease induction. Nevertheless, after at eight weeks no significant change was observed between diabetic, depressed, diabetic/depressed, and the depressed/diabetic groups.

Additionally, anhedonia is another major criterion for the diagnosis of depression. Moreover, we used the sucrose preference test or the sucrose consumption test to assess anhedonia. Consumption above 65% reflects that the mouse is feeling “pleasure”. The control group has a sucrose preference above 65% meaning that the animals are feeling “pleasure”. Our results show that in the SPT (Figure 6E,F) after two weeks of disease induction, depressed (28%) as well as the diabetic/depressed (45%) and depressed/diabetic (56%) have a sucrose preference below 65% when compared to the control mice (72%) and diabetic mice (75%). However, after eight weeks,

diabetic (23%), depressed (49%), diabetic/depressed (36%), and depressed/diabetic (39%) have a percentage lower than 65% with the control (74%) mice above it. Induction of depression in the diabetic animals revealed a more significant decrease in sucrose consumption after eight weeks of when compared to depressed animals (9% decrease).



* significance to the control group with p-value <0.05
# significance to the diabetic group with p-value <0.05
$ significance to the depressed group with p-value<0.05
& significance to the Diabetic Depressed group with p-value <0.05

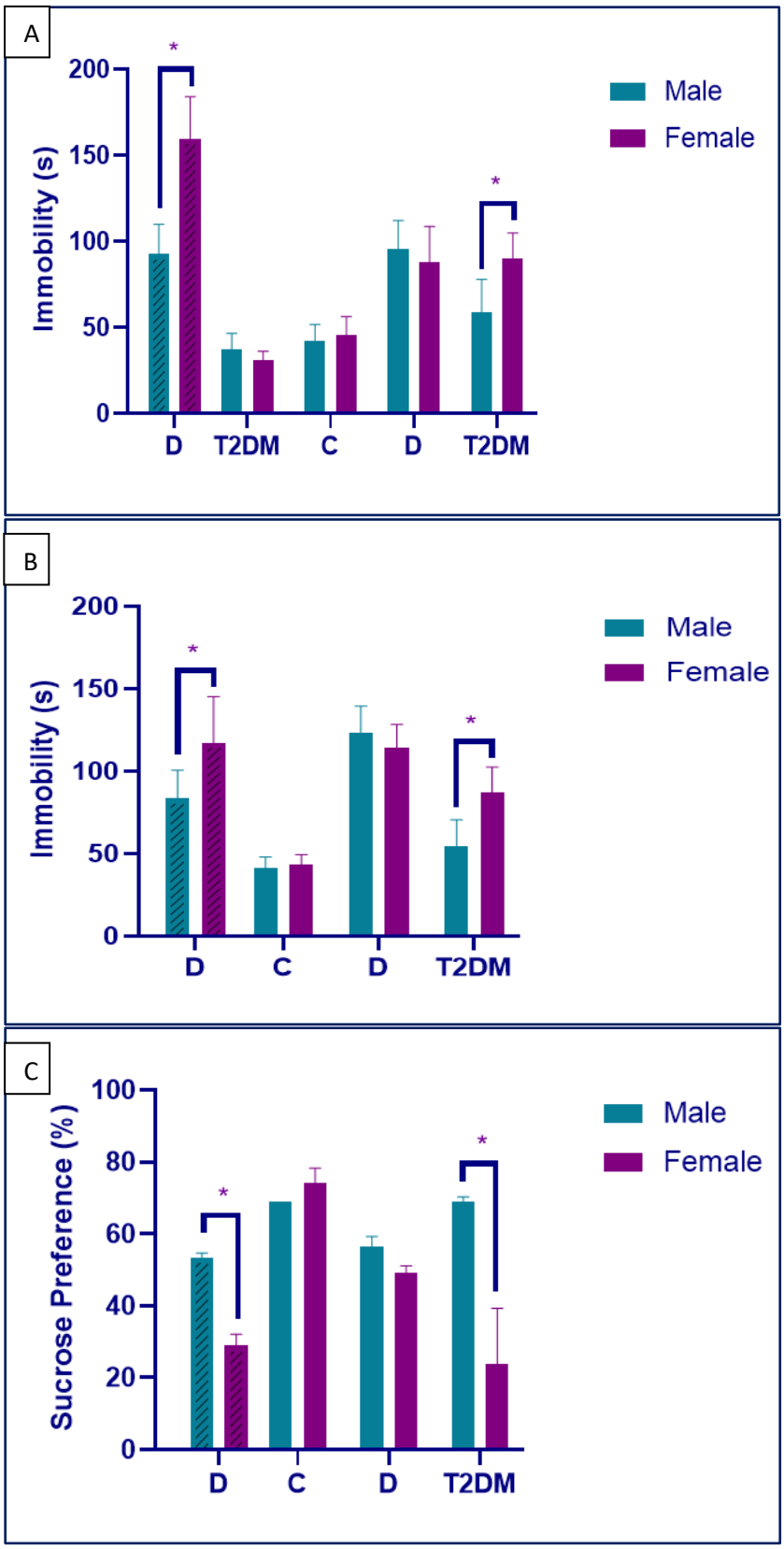
Figure 6: Assessment of Depressive-like Behaviors in female mice separated into Control (n=8), Diabetic (n=6), Depressed (n=8), Diabetic/Depressed (n=8), and Depressed /Diabetic (n=8) animals. Barograms represent the forced swim after 2 and 8 weeks of injury (Figure6 A, B), the tail suspension after 2 and 8 weeks of injury (Figure6 C, D), and the sucrose preference test after 2 and 8 weeks of injury (Figure6 E, F) tests. Values are the mean ± SD. * p

Sex dissimilarity in Depressive-like Symptoms

To start with the forced swim test, a significant difference is present between depressed females and depressed males. After two weeks of depression induction, depressed females show a large increase in immobility time. After eight weeks of diabetes initiation, a new trend is seen where diabetic females show a significant increase in immobility time than diabetic male mice (Figure 7 A).

Next, using the tail suspension test, a variation is seen after two weeks of depression induction where the females show an increase in immobility time. However, after eight weeks of disease introduction, diabetic female mice show a significance increase in immobility time than males (Figure 7 B).

Afterwards, sucrose preference test indicates analogous result where two weeks depressed females show a more depressed-like symptom having a lower sucrose consumption. Additionally, eight weeks diabetic females demonstrate a lower sucrose preference than diabetic male animals. Nevertheless, two weeks diabetic females indicated an increased anhedonia having a lower sucrose preference percentage (Figure 7 C).



* significance to the male group with p-value <0.05

Figure 7: Assessment of the difference in Depressive-like Behaviors between male/female mice separated into Control, Diabetic, Depressed, Diabetic/Depressed, and Depressed /Diabetic animals. Barograms represent the forced swim after 2 and 8 weeks of injury (Figure7 A), the tail suspension after 2 and 8 weeks of injury (Figure7 B), and the sucrose preference test after 2 and 8 weeks of injury (Figure7 C) tests. Values are the mean ± SD. * p

Sensory

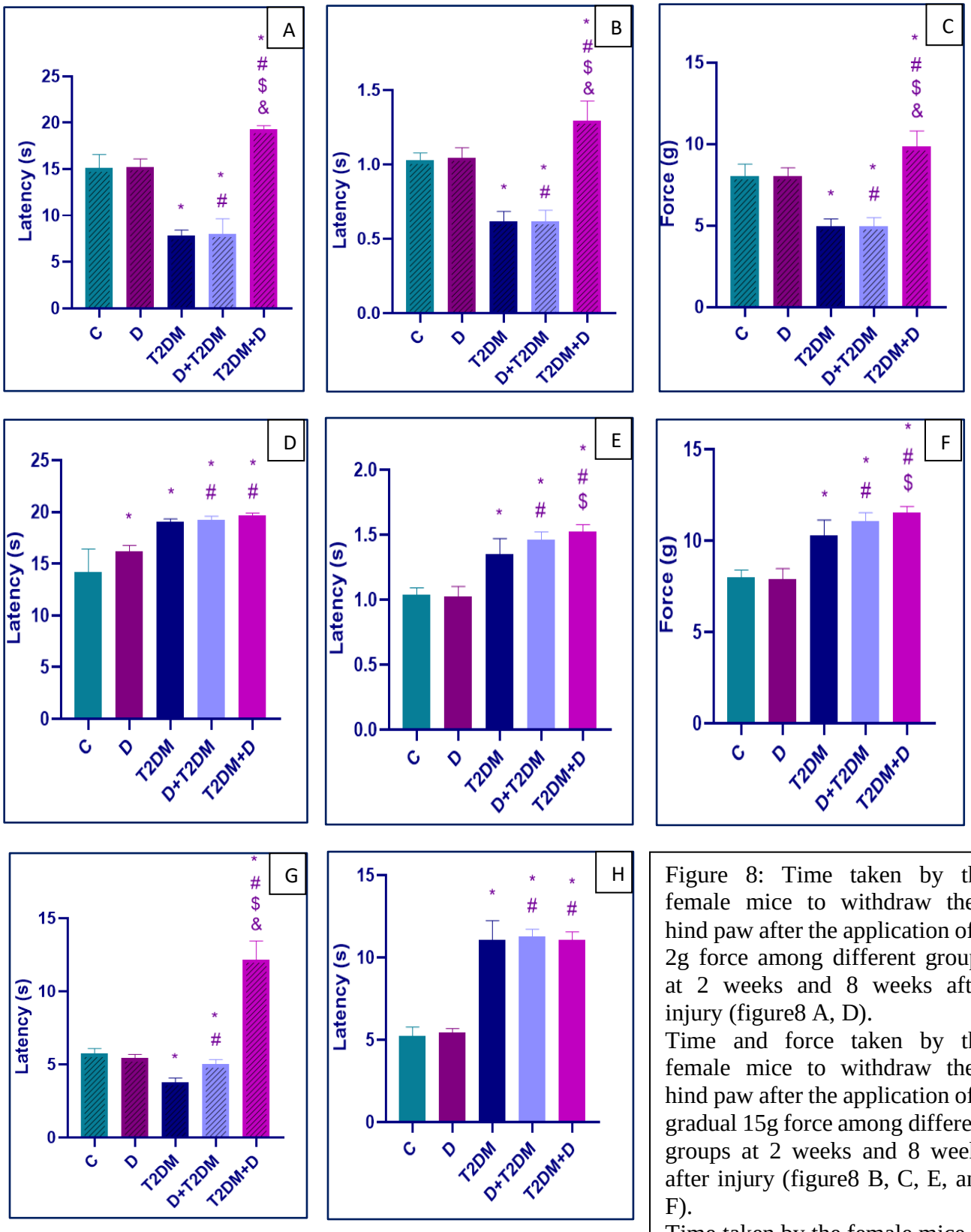
Type 2 diabetes affect the plantar sensitivity in female mice

The mechanical allodynia test was performed to assess the plantar sensitivity of the female mice hind paws by applying a 2g force. The C57BL/6 diabetic mice being 15 weeks of age, and the depressed/diabetic mice being 21 weeks of age showed a significant decrease changes in the time of hind paw withdrawal compared to their control littermates' mice; showing a hypersensitivity mode. Moreover, after 2 weeks, the diabetic mice, diabetic/depressed, and depressed/diabetic mice being showed a significant increase change in their values as shown in Figure 8 A, B.

To assess plantar touch sensitivity of the hind paw, we performed the mechanical hyperalgesia test by applying a 15 g force. We observe after two weeks of depression induction a significant increase in withdrawal time in the diabetic/depressed group (Figure 8 C). In contrast, the diabetic group, after two weeks of diabetes onset, displayed reduced withdrawal latency time reflecting hypersensitivity (Figure 8 C).

Moreover, significant increase in hind paw withdrawal time between the 8 weeks diabetic, diabetic depressed, and depressed/diabetic compared to their control littermates and the depressed group (Figure 8 D)**.** Similar trend is seen for the applied force results.

Next, to assess the sensitivity to radiant heat applied to the plantar surface of the hind paws, we performed the Heat Hyperalgesia Test (Figure 8 G, H). Our results indicate that the time taken for the diabetic group, after two weeks, is affected by a hypersensitivity causing a decrease in the latency time (Figure 8 G) but a significant increase in withdrawal time of the diabetic/depressed group is apparent compared to the control mice, the depressed group, and depressed/diabetic group. However, after eight weeks, all three groups affected by diabetes show a significant rise in the latency time compared to the control and depressed group (Figure 8 H).



* significance to the control group with p-value <0.05
\# significance to the depressed group with p-value <0.05
$ significance to the diabetic group with p-value<0.05
& significance to the depressed diabetic group with p-value <0.05

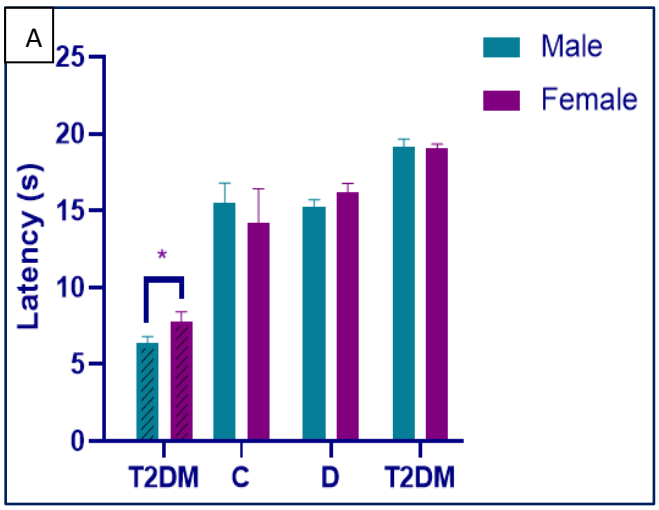
Figure 8: Time taken by the female mice to withdraw their hind paw after the application of a 2g force among different groups at 2 weeks and 8 weeks after injury (figure8 A, D).
Time and force taken by the female mice to withdraw their hind paw after the application of a gradual 15g force among different groups at 2 weeks and 8 weeks after injury (figure8 B, C, E, and F).
Time taken by the female mice to withdraw their hind paw after the application of a beam light among different groups at 2 weeks and 8 weeks after injury (figure8 G, H).

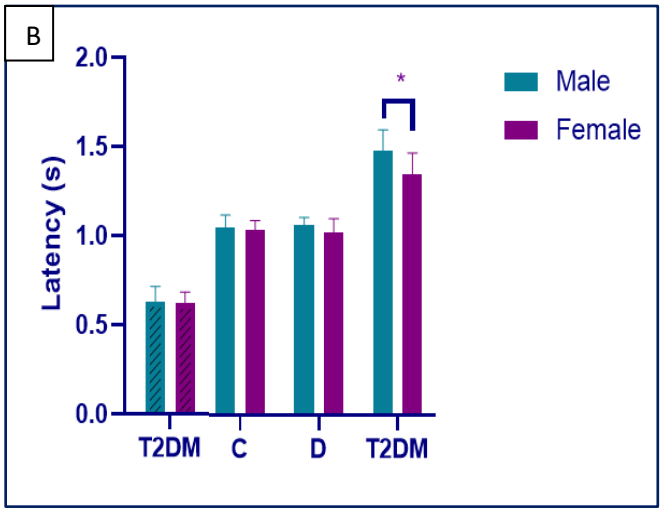
Sex variation in plantar sensitivity

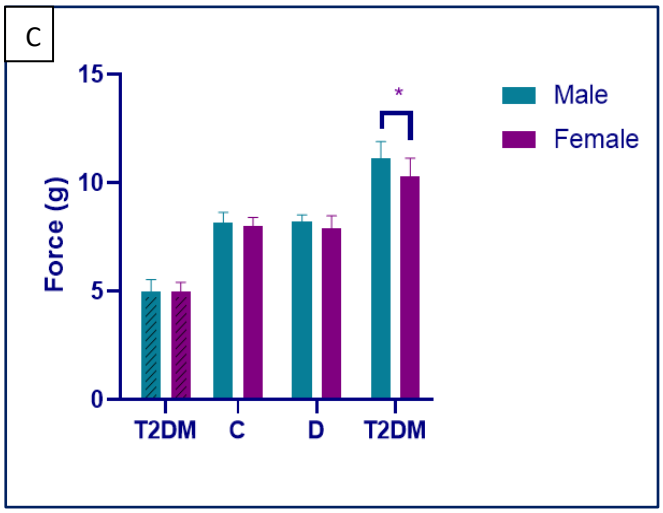
Using the mechanical allodynia test, no significance difference was seen between the groups after two weeks of injury. On the contrary, after eight weeks of disease induction, diabetic males had a more prone peripheral injury, where a significant increase in withdrawal time is seen (Figure 9A).

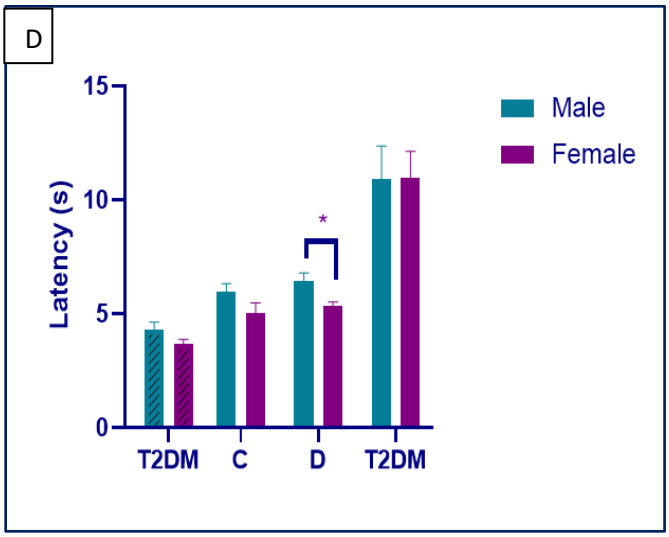
Additionally, using mechanical hyperalgesia test, a higher force was needed for the old diabetic males to withdraw their hind paw than the same age female animals. However, young diabetic females needed more time to withdraw their hind paw than young diabetic males (Figure 9B, C).

Furthermore, after two weeks, no significant difference was seen in the performance of male and female mice using the heat hyperalgesia test. After eight weeks depressed males show an increase in latency to withdraw their hind paw than female mice (Figure 9D).









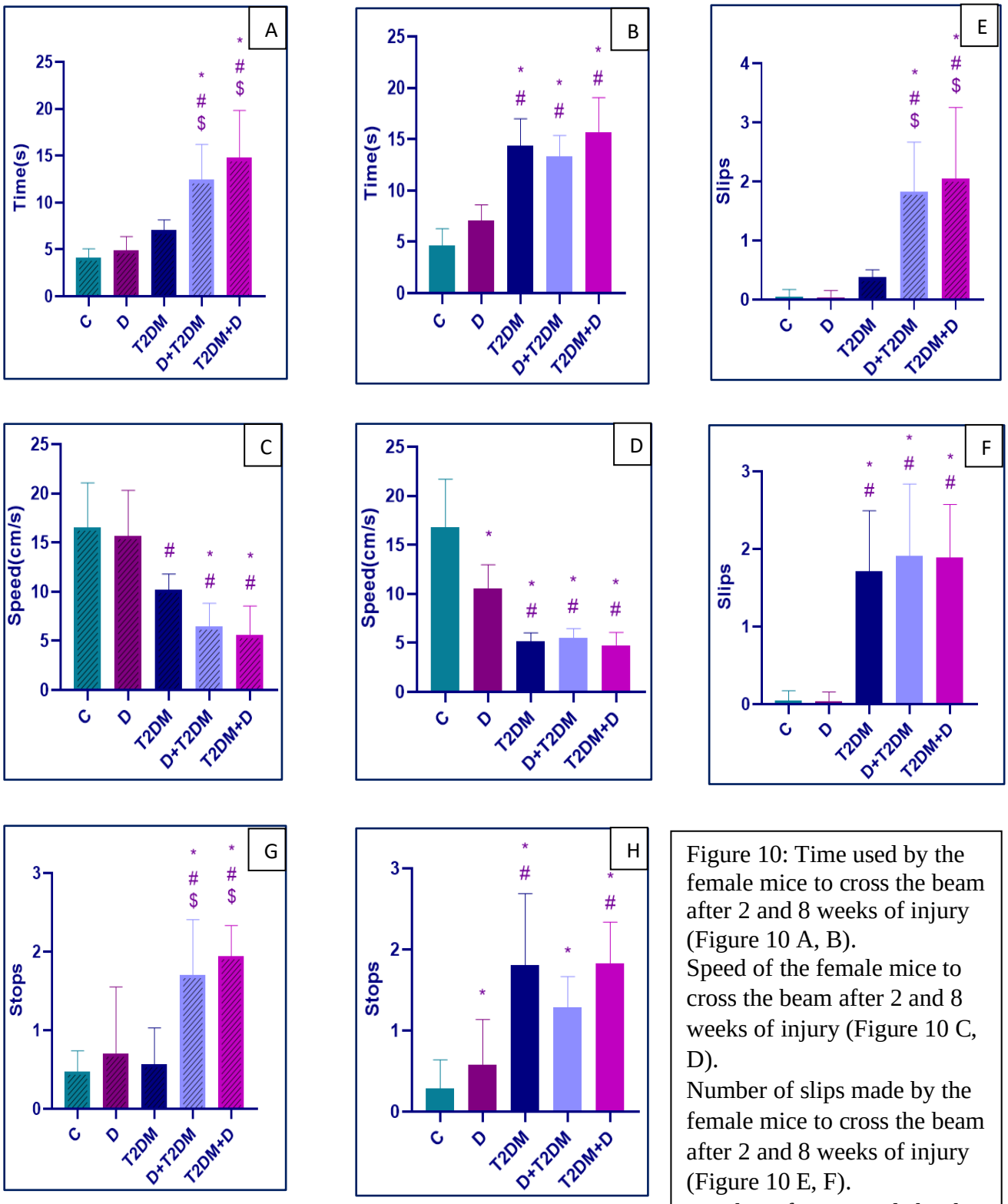
* significance to the male group with p-value <0.05

Figure 9: The difference in time taken between the male/female mice to withdraw their hind paw after the application of a 2g force among different groups at 2 weeks and 8 weeks after injury (figure9 A).
The difference in time taken between the male/female mice to withdraw their hind paw after the application of a gradual 15g force among different groups at 2 weeks and 8 weeks after injury (figure9 B, C).
The difference in time taken between the male/female mice to withdraw their hind paw after the application of a beam light among different groups at 2 weeks and 8 weeks after injury (figure9 D).

Motor

Type 2 diabetes and depression affect fine motor coordination in female mice

Examining whether diabetes and/or depression alter motor coordination and balance of the animals was our next step. In addition, we studied the possibility of whether inducing depression in diabetic mice could exacerbate motor injury in comparison to diabetic and/or depressed mice. These experiments will allow us to highlight, if any, the correlation of depression with peripheral nerve injury. Deficit in fine motor coordination was assessed using the balance beam test or beam-walking test. Depressed female mice, after 8 weeks, showed a significant slower pattern of movement, more foot faults and made more stops then the control group (Fig 8 B, D, F, and H). Diabetic mice had a more aggravated pattern of decrease in locomotor coordination. Moreover, diabetic male mice were more prone to develop diabetic complications. Where after two weeks of diabetes induction, diabetic male mice have an increase in time needed (Figure9 A). Interestingly, the number of foot slips were more pronounced in diabetic/depressed and depressed/diabetic animals when compared to depressed or diabetic animals (Fig 8 E). Our results showed that the average speed of the diabetic, depressed, diabetic/depressed, and depressed/diabetic was significantly lower than that of the control (Figure 8 D). With respect to the number of foot stops, the depressed, the diabetic, the diabetic/depressed, and depressed/diabetic animals is significantly greater than that of the control (Figure 8 H). Interestingly, diabetic mice showed a greater number of foot faults when compared to the depressed group (Figure 8E).

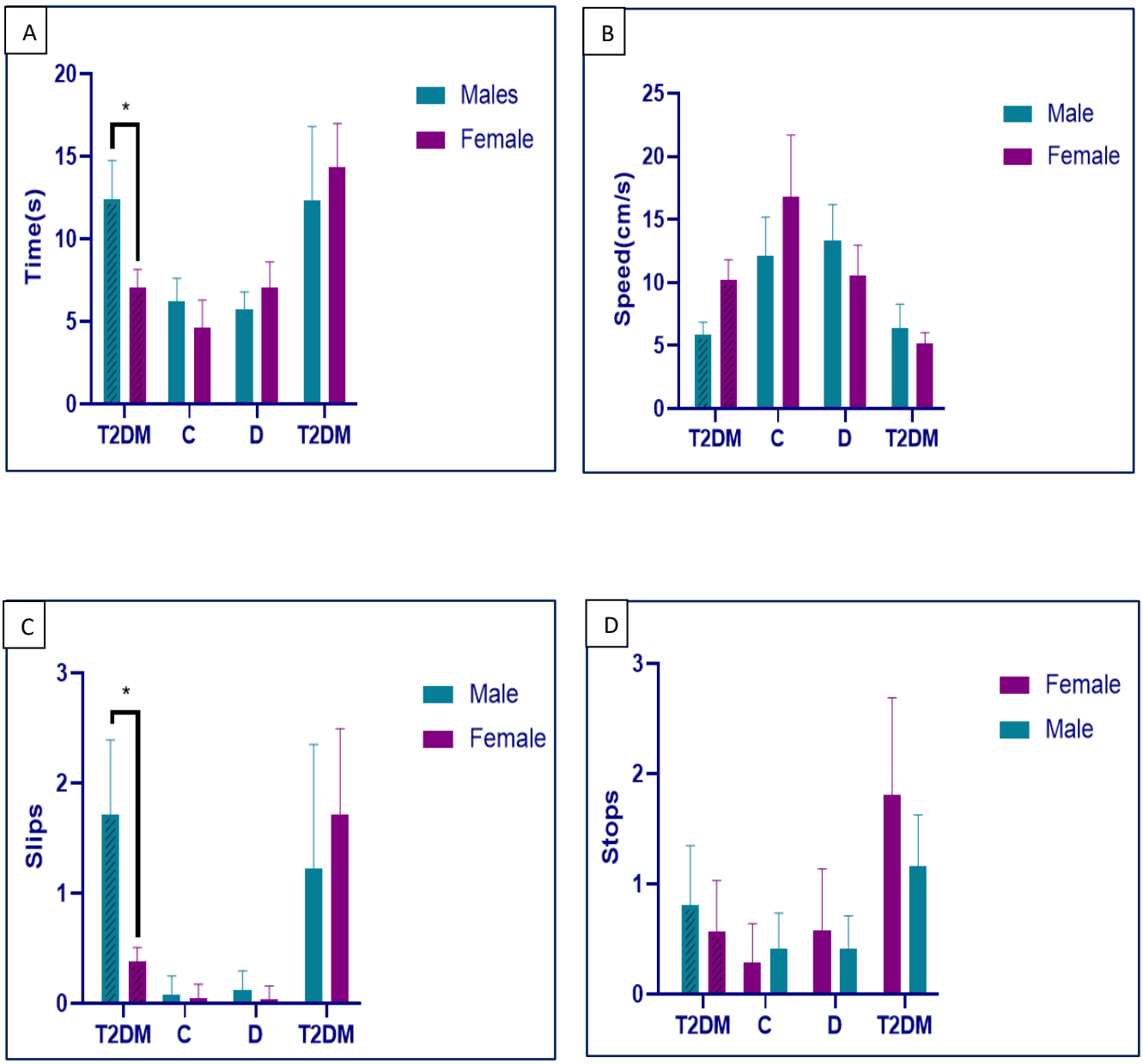


* significance to the control group with p-value <0.05
\# significance to the diabetic group with p-value <0.05
$ significance to the depressed group with p-value<0.05
& significance to the Diabetic Depressed group with p-value <0.05

Figure 10: Time used by the female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 A, B).
Speed of the female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 C, D).
Number of slips made by the female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 E, F).
Number of stops made by the female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 E, F).

Sex difference in fine motor coordination

Utilizing raised beam walking test, sex influence on motor function was assessed. After two weeks of diabetes induction, a significant increase in time and slips, and a tendency of decrease in speed was seen in male mice (Figure A, B, C). However, after eight weeks no difference in motor function was present between female and male mice. In addition, no difference was present in number of slips between female and male animals (Figure D).



* significance to the male group with p-value <0.05

Figure 11: The difference in time used between male/female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 A).
The difference in speed between male/female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 B).
The difference in number of slips made between male/female mice to cross the beam after 2 and 8 weeks of injury (Figure C).
The difference in number of stops made between male/female mice to cross the beam after 2 and 8 weeks of injury (Figure 10 D).

Molecular

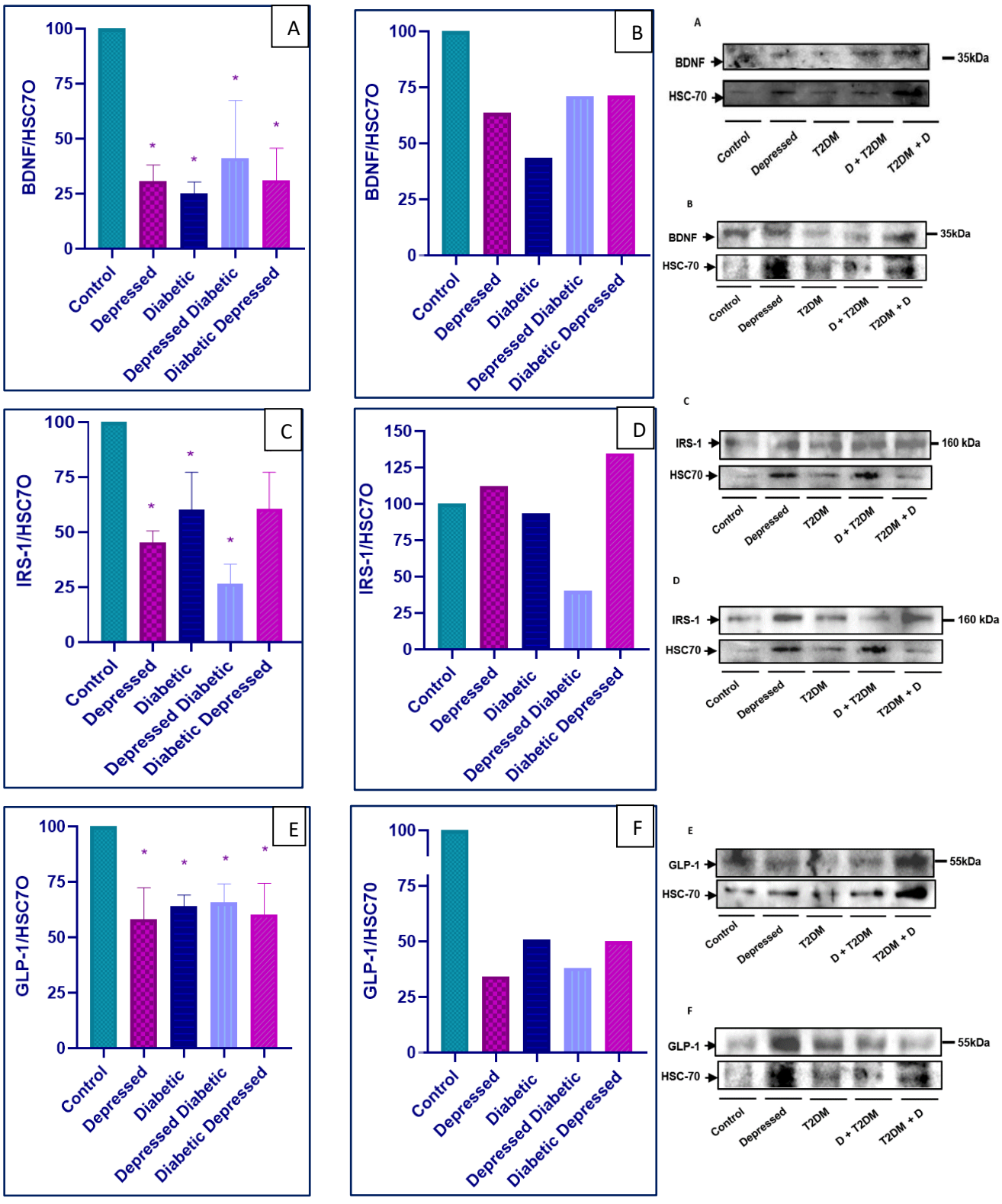
Western Blot – Diabetes, And Diabetes Paired with Depression, downregulate BDNF, IRS-1, GLP-1, p-AMPK, and AMPK in female mice brains

Within the prefrontal cortex, the BDNF protein levels of the diabetic, depressed, diabetic/depressed, and depressed/diabetic mice are significantly lower than those of the control (Figure12 A). In addition, the levels of IRS-1 protein for all four groups diabetic, depressed, diabetic/depressed, and depressed/diabetic animals are significantly lower in comparison to the control group (Figure12 C). Moreover, GLP-1 is significantly downregulated in the diabetic, depressed, diabetic/depressed, and depressed/diabetic groups when compared to the control (Figure 12 E). As shown in figure 12 G and I, p-AMPK and AMPK are significantly decreased, with a similar trend, for the diabetic, depressed, diabetic/depressed, and depressed/diabetic mice than those of the control. Furthermore, p-AMPK/AMPK ratio show a significant increased level in the diabetic, diabetic/depressed, and depressed/diabetic groups compared to the control. As for the depressed group, they showed a decrease in p-AMPK/AMPK ratio in their prefrontal cortexes when compared to the control group (Figure12 K).

Within the hippocampus, the BDNF protein levels of the diabetic, depressed, diabetic/depressed, and depressed/diabetic mice are lower than those of the control (Figure12 B). In addition, the level of IRS-1 protein for the diabetic group tends to be higher than the control group. On the contrary the level of IRS-1 protein for the depressed, diabetic/depressed, and depressed diabetic tends to decrease compared to the control (Figure12 D). As shown in figure 12 E the GLP-1 level within the hippocampi of the of the diabetic, depressed, diabetic/depressed, and depressed/diabetic animals are lower in comparison to the control animals. Moreover, when compared to the control

mice, all four groups tend to have an increase in p-AMPK level however no obvious change in AMPK level but to the depressed group. Likewise, p-AMPK/AMPK ratio show a decreased level in the diabetic, depressed, diabetic/depressed, and depressed/diabetic groups compared to the control group in the hippocampus area (Figure12 H) but in the prefrontal cortex diabetic, diabetic/depressed, depressed/diabetic have a significant increase but the depressed group shows a significant decrease compared to the control group(Figure 12G).

The values of the control groups in figure 12 have been standardized to 100.


A
BDNF/HSC7O
100
75
50
25
0
Control
Depressed
Diabetic
Depressed Diabetic
Diabetic Depressed
B
BDNF/HSC7O
C
IRS-1/HSC7O
D
IRS-1/HSC7O
150
125
E
GLP-1/HSC7O
F
GLP-1/HSC7O
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160 kDa
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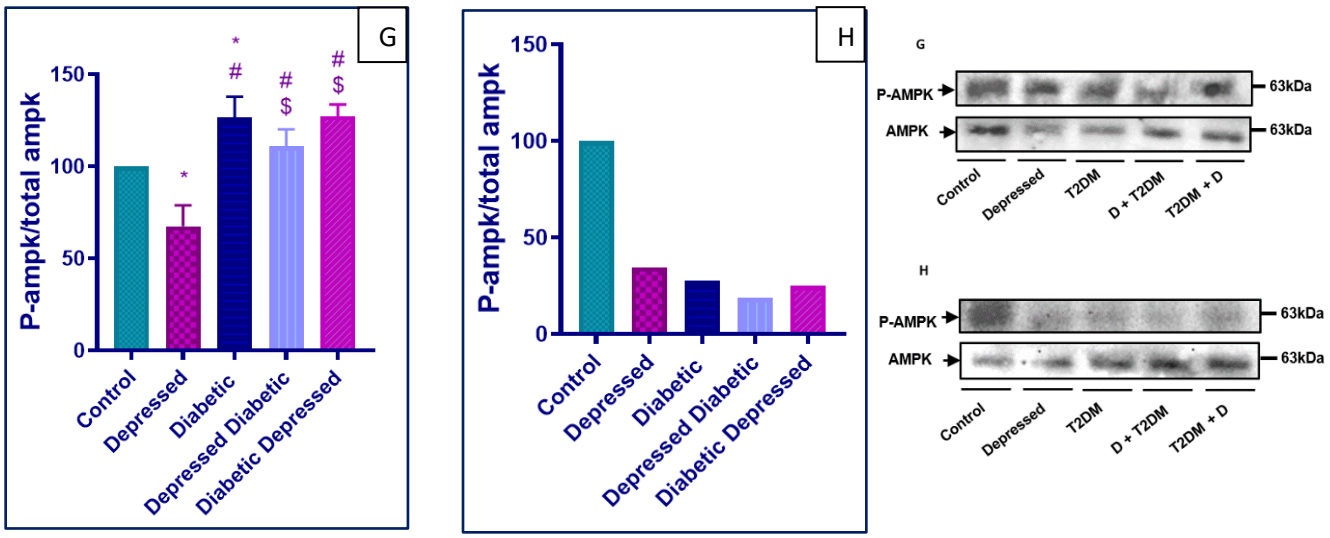
55kDa
T2DM
D + T2DM
T2DM + D



* significance to the control group with p-value <0.05
# significance to the diabetic group with p-value <0.05
$ significance to the depressed group with p-value<0.05
& significance to the Diabetic Depressed group with p-value <0.05

Figure 12: Quantitative results of BDNF, IRS-1, GLP-1, p-AMPK, and AMPK western blots, in the prefrontal cortex(n=3) and hippocampus(n=1) of control, diabetic, depressed, diabetic/depressed, and depressed/diabetic mice. Values are the mean ± SD.
HSC70 was used as the loading control.

CHAPTER IV

DISCUSSION

Diabetes, characterized by chronic hyperglycemia, has been implicated in fundamental structural and functional brain alterations. Depression, one of the chronic complications of diabetes, is significantly prevalent in diagnosed diabetic patients(Bădescu SV*, 2016; Moulton, Pickup, & Ismail, 2015). Consequently, a bidirectional relationship exists between diabetes and depression, where each disorder exacerbates the other(Ho, Sommers, & Lucki, 2013b; Semenkovich, Brown, Svrakic, & Lustman, 2015). Even though diabetes and depression share a wide range of biological mechanisms, including HPA axis dysregulation, aberrant ROS production, and increased synthesis of pro-inflammatory markers, the relation between these two conditions is not well characterized.

In this study, we show that depressive like behavior can be a direct consequence of diabetes by investigating whether diabetes induces depressive-like symptoms and pathophysiology using-obese type 2 diabetic animals. Theoretically, patients with diabetes develop depression from the first onset of the disease, due to the influence that it will have on their life either physically, socially, or economically. Nevertheless, the biological link(s) between diabetes and depression is still under investigation. Diabetes has been implicated in producing a depressive-like behavior in both humans and animal models of diabetes(Bădescu SV*, 2016; Andre Kleinridders et al., 2015a).

We performed a panel of behavioral tests on the mice described in Table1. Our TST results show that the immobility time of depressed, diabetic, diabetic/depressed, and depressed/diabetic mice was significantly greater than that of their control littermates after eight weeks of disease induction. Similar results were recorded using the FST. Interestingly, the TST and FST demonstrated a

significant increase in old diabetic female mice's immobility time compared to the males. Taking these data together, using non-obese type 2 diabetic mice, we demonstrated that depressive-like symptoms could be a direct result of T2DM, where diabetic mice exhibited emotional despair assessed by both tail suspension and forced swim tests.

Additionally, our results showed a sex specificity, where female diabetic mice were more prone to develop depression. These results showed that female mice tend to have a more severe depression than males.

These results are in sync with already published data, showing signs of “despair” and depressive-like behaviors in type 2 diabetic animals. According to Kleinridders et al mice with a brain-specific knockout of insulin receptor remained immobile for longer periods of time assessed by TST and FST(Andre Kleinridders et al., 2015a). Furthermore, Sharma et al reported that using the force swimming test obese type of mice require a long period of immobility (Sharma, Elased, Garrett, & Lucot, 2010).

Moreover, Yu et al. confirmed that type 2 diabetic patients recorded decreased sucrose preference, expressing their inability to experience pleasure from rewarding/pleasurable routine activities (C. Yu, Rouen, & Dobrowsky, 2008).

Anhedonia, another primary criterion for the diagnosis of depression, was assessed by the SPT, as previously stated. A depressed individual or animal would lose interest in rewarding stimuli and pleasurable activities. Sucrose consumption more significant than 65% indicates "pleasure" (Strekalova et al., 2011). The control group showed a sucrose preference value greater than 65%, reflecting the "pleasurable" state experienced by these mice. In contrast to the control group,

depressed, diabetic/depressed, depressed/diabetic, and eight-week diabetic mice, documented a sucrose consumption value less than 65%, presenting depressive-like symptoms.

Comparing females to males, two weeks depressed females had a more apparent anhedonia state having a decreased sucrose preference compared to two weeks depressed male mice. Additionally, after eight weeks, diabetic females showed a depressive-like state by achieving a percentage lower than 65% but diabetic male mice did not show any downfall.

These results correlated with already published data by other groups showing that type 2 diabetic mice present with signs of depression and anxiety and that females are more prone to develop depression rather than males(Hillerer, Slattery, & Pletzer, 2019; Labonté et al., 2017; Zhang et al., 2018).

Besides, diabetes has been implicated in peripheral nerve injury, which elicits depressive-like behaviors in human and animal models of diabetes(D'Amato et al., 2016; Gui et al., 2016; Hussam Murad, 2015). Diabetic peripheral neuropathy (DPN) usually presents as a distal degenerative polyneuropathy with sensory loss (Jolivalt et al., 2016). Chronic hyperglycemia contributes to nerve damage by triggering a range of metabolic abnormalities. Inflammation, dyslipidemia, mitochondrial reactive oxygen species, and endoplasmic reticulum stress firmly bring about peripheral nerve injury progression in both humans and experimental models of diabetes(O'Brien, Sakowski, & Feldman, 2014; Sima & Zhang, 2014).

Results from our groups and others show that diabetes induces peripheral nerve injury by affecting the sensory and motor function. However, no noticeable change in the peripheral nervous system in the depressed group is seen. Nevertheless, diabetic groups started after two weeks of injury by a hypersensitivity phase. Having a lower latency to remove the hind paw in von Frey and paw withdrawal tests and a tendency to decrease in the beam walking test speed. Intriguingly, after

eight weeks, injury in the peripheral nervous system took place, leading to a hyposensitivity, explained by an increase in the latency time in both von Frey and paw withdrawal tests. Also, peripheral nerve injury started in males before females since diabetes is more evident in males (Shepard, 2019).

Our results show a parallel significance in all three tests performed suggesting a sensory deficit induced by an decrease in the nociceptor threshold level and then an increase of this threshold, and a sensory deficit ultimately leading to a decreased sensitivity to heat and mechanical stimuli.

The next objective was to determine the existence of any protein alterations in diabetes-induced depression by showing insulin resistance in the brain.

Major depressive disorder (MDD) is one of the most common psychiatric disorder, but the underlying mechanisms are mostly unknown. Increasing evidence shows that brain-derived neurotrophic factor (BDNF) plays an important role in the structural plasticity induced by depression (Qiao, An, Xu, & Ma, 2017).

BDNF is a neurotrophic factor vital to the survival, growth, and maintenance of neurons in critical brain circuits involved in emotional and cognitive function. Accumulating evidence indicates that neuroplastic mechanisms involving BDNF are injuriously altered in MDD patients and animal models of depression. Herein, clinical and preclinical evidence provided that stress-induced depressive pathology contributes to altered BDNF level and function in persons with MDD and, thereby, disruptions in neuroplasticity (Phillips, 2017).

Chronic social isolation in animal models generally causes changes in hypothalamic-pituitary-adrenal axis functioning, associated with anxiety- and depressive-like behaviors. This chronic stress also causes downregulation of BDNF protein and mRNA in the hippocampus, a stress-

sensitive brain region closely related to the pathophysiology of depression (Zaletel, Filipović, & Puškaš, 2017).

In a study conducted by Qiao et. al., considering the opposite effects of BDNF and its precursor pro-BDNF on neural plasticity, they hypothesized that the balance of BDNF and pro-BDNF plays a critical role in CUMS-induced depressive-like behaviors and structural plasticity in the rodent hippocampus (Qiao et al., 2017). Interestingly, the pro-domain of BDNF (BDNF pro-peptide) is localized at presynaptic termini, where it facilitates long-term depression, implicating it as a novel synaptic modulator. The BDNF pro-peptide is present in human cerebrospinal fluid (CSF), and levels were significantly lower in patients with major depressive disorder (MDD) than in controls. Notably, male MDD patients exhibit significantly lower levels of CSF pro-peptide than females. These findings demonstrate that the BDNF pro-peptide is a biologically crucial synaptic modulator associated with MDD with a gender specific expression(Kojima, Matsui, & Mizui, 2019).

On the other hand, BDNF is a neurotrophic factor that plays an indispensable role in the central nervous system and systemic or peripheral inflammatory conditions, such as type 2 diabetes mellitus (T2DM). BDNF is also expressed in several non-neuronal tissues, and platelets are the principal source of peripheral BDNF. There are pieces of evidence of the antidiabetic effect of BDNF and the association with circulating inflammatory cytokines in T2DM (Eyileten, Kaplon-Cieslicka, Mirowska-Guzel, Malek, & Postula, 2017).

As seen in our results, BDNF is significantly downregulated in the prefrontal cortex in depressed, diabetic/depressed, and depressed/diabetic mice, showing that the depression protocol affected the mice's brains. Remarkably, diabetic mice were prone to develop depression, where BDNF is significantly decreased. This decrease was more apparent in females than male mice. A similar trend was seen in the hippocampus of these animals.

The results also suggest that STZ may not only cytotoxic to pancreatic beta cells but also hypothalamic and hippocampal neurons by inducing oxidative stress. As mentioned by Bathina et. al., STZ ability to suppress BDNF production by pancreas, liver, and brain tissues suggests that impaired memory, learning, and cognitive dysfunction seen in diabetes mellitus could be due to BDNF deficiency (Bathina, Srinivas, & Das, 2017).

Furthermore, Ling et. al. used dihydromyricetin (DHM) that could significantly ameliorate CI and reverse aberrant glucose and lipid metabolism in T2DM mice, likely through the suppression of oxidative stress and enhancement of BDNF-mediated neuroprotection. In conclusion, their results suggest that when BDNF is increased, diabetic state could be reversed(Ling et al., 2018).

Next, getting into another protein, IRS-1, that is downregulated in all four groups diabetic, depressed, diabetic/depressed, and depressed/diabetic in the prefrontal cortex compared to the control group. A parallel trend is seen in the hippocampus, where all groups have a decrease in IRS-1 level compared to the control.

Our results are compatible with other studies, where a growing body of evidence supports the involvement of disturbances in the brain insulin pathway in the pathogenesis of depression. Diminished insulin receptor phosphorylation evoked by prenatal stress procedure was present. Insulin receptor phosphorylation dysregulation in the frontal cortex was mainly related to serine312 and tyrosine IRS-1 phosphorylation, while in the hippocampus, it was related to the adaptor proteins Shc1/Grb2 (Głombik et al., 2017).

Additionally, animal studies have demonstrated that insulin and its signaling cascade typically control cell growth, metabolism, and survival through the activation of MAPKs and activation of phosphatidylinositide-3-kinase (PI3K), in which the activation of PI3K associated with insulin

receptor substrate 1 (IRS1) and IRS2 having a central role in the control of nutrient homeostasis and organ survival. The suppression of IRS1 and IRS2 in different organs following hyperinsulinemia, metabolic inflammation, and overnutrition may act as the underlying mechanisms for humans' metabolic syndrome(Guo, 2014).

After That, our results showed that glucagon-like peptide-1 (GLP-1) was downregulated in the prefrontal cortex of diabetic, depressed, depressed/diabetic, and diabetic/depressed animals. A similar result was present in the hippocampus showing a decrease in GLP-1 level. This outcome shows that stress and insulin resistance contributed to a perturbation in the normal brain function.

In depression, excessive glucocorticoid action may cause maladaptive brain changes, including in the pathways controlling energy metabolism. Insulin and GLP-1, besides regulation of glucose homeostasis, also possess neurotrophic properties. Detka et al. study aim at investigating the influence of prenatal stress on insulin, GLP-1, and their receptor (IR and GLP-1R) levels in the hypothalamus. GLP-1 and GLP-1R were also assayed in the hippocampus and frontal cortex - brain regions mainly affected in depression. Prenatal stress has reduced GLP-1 and GLP-1R levels, attenuated glucose-induced increase in insulin concentration, and increased phosphorylated IR in the hypothalamus of animals subjected to additional stress stimuli, and also decreased the GLP-1R level in the hippocampus. Prenatal stress may act as a preconditioning factor, affecting the concentrations of hormones such as insulin and GLP-1 in the hypothalamus in response to adverse stimuli. The decreased GLP-1R level in the hippocampus could be linked to disturbances in neuronal plasticity(J. Detka et al., 2019).

In Addition, GLP-1 released from enteroendocrine gut cells controls meal-related glycemic excursions by augmentation of insulin and glucagon secretion inhibition. GLP-1 also inhibits

gastric emptying and food intake, maximizing nutrient absorption while limiting weight gain(Drucker, 2018).

Further, GLP-1 is also associated with protective effects on pancreatic β-cells and the cardiovascular system, decreased appetite, and weight loss, making GLP-1 derivatives an exciting treatment for type 2 diabetes and obesity(Cheang & Moyle, 2018). Meaning that GLP-1 level affect T2DM and depressive disorders. Since recent studies indicate that metabolic disorders such as diabetes and obesity are a significant risk factor of psychiatric diseases. This relationship opens the opportunity to develop new antidepressant drugs by repurposing antidiabetic drugs. Previous research has demonstrated that GLP-1 analogs are neuroprotective in several neurological disease models. In addition, the GLP-1 analog liraglutide has been shown to promote neurogenesis, which is seen to play essential roles in memory formation and cognitive and emotional processing. However, whether liraglutide is an effective antidepressant remains unknown. Behavioral studies showed that liraglutide administration attenuated depressive and anxiety-like behaviors in the depression mouse model and attenuated the stress hormone's hyperactivity. Together, liraglutide can act as a therapeutic treatment of depression(Weina et al., 2018).

On the other hand, investigating the possible involvement of energy metabolism in the brain in diabetes-induced-depressive-like behaviors has also been conducted.

AMPK and P-AMPK are significantly downregulated in the prefrontal cortex of diabetic, depressed, diabetic/depressed, and depressed/diabetic mice compared to the control group, which means that insulin resistance and depression caused a dysregulation in energy metabolism and availability in the prefrontal cortex, an area involved in cognitive and emotional behavior. However, in the hippocampus, p-AMP tend to decrease, and AMPK to increase. A higher number of "n" is needed to confirm these results.

5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric protein with α, β, and γ subunits(Joshi et al., 2019). It plays a significant role in regulating cellular energy balance in eukaryotic cells(Carling, 2017). Also, AMPK is an evolutionarily conserved serine/threonine kinase initially identified as the key player in maintaining cellular energy homeostasis(Jeon, 2016). AMPK responds to changes in intracellular adenine nucleotide levels, activated by an increase in AMP/ADP relative to ATP. Activation of AMPK increases the rate of catabolic (ATP-generating) pathways and decreases the rate of anabolic (ATP-utilizing) pathways. Intensive research has identified diverse molecular mechanisms and physiological conditions that regulate AMPK activity over the last decade. In addition to its role in maintaining intracellular energy balance, AMPK regulates whole-body energy metabolism. Given its vital role in controlling energy homeostasis, AMPK has attracted widespread interest as a potential therapeutic target for metabolic diseases, including type 2 diabetes and, more recently, cancer(Carling, 2017; Jeon, 2016).

AMPK is ubiquitously expressed in various living system tissues, such as the heart, kidney, liver, brain, and skeletal muscles. Thus, the malfunctioning of AMPK is expected to harbor several human pathologies. The significant impact of AMPK modulation ensures healthy functioning of mitochondria and energy homeostasis in addition to maintaining a strict check on inflammatory processes, autophagy, and apoptosis(Madhavi et al., 2019).

Analogous to our results, Sambuceti et. al. state that diabetes Mellitus is correlated with a reduced phosphorylated AMP-activated protein kinase (p-AMPK)(Sambuceti et al., 2009). Where type 2 diabetes, a condition marked by both mitochondrial degeneration and dysregulated GSIS, was associated with a remarkable reversion of the standard AMPK-dependent adult β-cell signature to a more neonatal one characterized by mTORC1 activation. Manipulating how cellular nutrient

signaling pathways regulate β-cell metabolism may offer new targets to improve β-cell function in diabetes(Jaafar et al., 2019).

A target pathway to control diabetes is AMPK signaling pathway. In several studies, AMPK activation enhanced glucose uptake into cells and inhibited intracellular glucose production. Interestingly, drugs used in the treatment of diabetes, including metformin, are also known to act through the regulation of AMPK. Thus, drugs that activate and regulate AMPK are potential candidates for the treatment of diabetes(Joshi et al., 2019).

Considering a different perspective, little is known about the pathophysiology of depression and the therapeutic strategy for anti-depression. Wong et al. investigated irisin's role, a regulator of energy metabolism, in the modulation of depressive-like behaviors in chronic unpredictable stress (CUS) exposed rats. Irisin significantly increased glucose transport and phosphorylation levels, as reflected by the increased ATP level in vivo and in vitro. Further studies indicated that the AMPK pathway was involved in regulating irisin on depressive-like behaviors in CUS rats. In conclusion, they demonstrated that irisin has a crucial role in inducing antidepressant-like effects in CUS rats by regulating energy metabolism in the brain's prefrontal cortex, which may provide new insight into the biological mechanism of depression(Wang & Pan, 2016).

To conclude, we provide through this work evidence of a causal link between diabetes and depression with manifestations of nerve dysfunction in the CNS and PNS. Depression was assessed in diabetic mice using the forced swim test, tail suspension test, and sucrose preference test. In the periphery, diabetic neuropathy was assessed using the mechanical allodynia, mechanical hyperalgesia, heat hyperalgesia, and beam walking tests where depression did not affect the sensory or motor function. Moreover, protein expression was altered in the brain,

affecting the depression marker BDNF, insulin resistance markers GLP-1 and IRS-1, energy metabolism markers P-AMPK, and AMPK.

The following study thus lends support to the significance of approaching diabetes and depression from many angles, proving on a molecular level that diabetic patients might express depression markers and that gender contributes to a biological and behavioral difference.

Limitations of the Study

This study does not exclude itself from limitations. Since time was a limiting factor, no histological techniques were performed in this study. This would have made the prefrontal lobes and hippocampi of mice taken from each of the eight conditions accessible to observation, further distinguishing normal tissues from pathological ones. Additionally, further significant bioanalytical studies could be carried out by increasing the number of "n" used to compare the different groups for the western blots' analysis made for the hippocampus part. Including other experiments, such as the real-time RT-PCR, for assessing insulin resistance in the brain, and immunofluorescent, to evaluate the level of inflammatory markers in the brain would have further complimented our results. Screening for the combined therapeutic effect of SSRI and GLP-1 agonists was missing.

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