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DIABETES, DEPRESSION, AND PERIPHERAL NEUROPATHY: THE ROLE OF NADPH OXIDASES-INDUCED REACTIVE OXYGEN SPECIES IN THIS VICIOUS CYCLE

by

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Submitted in partial fulfillment of the requirements
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DIABETES, DEPRESSION, & OXIDATIVE STRESS: UNCOVERING THE BIOLOGICAL BERMUDA

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"When a crew and a captain understand each other to the core, it takes a gale, and more than a gale to put their ship ashore"-Rudyard Kipling

Having said this, I'd like to express my gratitude to the captain of this ship, Dr. Assaad A. Eid, for teaching us how to follow first, and ultimately stir this ship in the right direction. The crew, on the other hand, forms the base of it all. Their hard work, team spirit, and diverse scientific and personal inputs further ensured smooth sailing. A deep appreciation also goes to the 'night-watch' of this ship, Dr. Frederic Harb. His constant 'on the look-out' approach has guaranteed an iceberg-free sailing.

I shall not adopt this cliché approach of thanking each and every person for adding an interesting perspective to my project, or more importantly for becoming a valuable asset throughout my academic journey here at AUB. I do want to say this though. All our lives we've been taught that there's light at the end of the tunnel. But, hey. What if the tunnel were an illusion all along?

AN ABSTRACT OF THE THESIS OF

Patil Vanig Kalenderian for Master of Science
Major: Neuroscience

Title: Diabetes, Depression, and Peripheral Neuropathy: The Role of NADPH oxidases-induced Reactive Oxygen Species in This Vicious Cycle

Background: Diabetes Mellitus is a metabolic disease affecting over 347 million people worldwide. This disease comes with complications such as diabetic peripheral neuropathy (DPN) that affect 50% of the patients. Its symptoms encompass sharp pains, and/or insensitivity to pain and temperature, demyelination, and impaired nerve conduction velocity. Depression is another complication of diabetes that occurs in some of the patients. The incidence of depression in the diabetic population is higher than that of the non-diabetic.
Reactive oxygen species (ROS) have been shown to cause myelin damage in the central and peripheral nervous systems in depression and DPN respectively. The longer the coexistence of diabetes and depression, the more severe the myelin injury state. However, the mechanisms by which myelin injury is caused by these two disorders need to be elucidated.

Aim: To assess the role of NADPH-induced ROS production in depression and diabetes-induced depressive-like behaviors in animal models. To examine whether depression can cause myelin alterations at the level of the peripheral nervous system. And to investigate whether the effect of comorbid diabetes and depression may further contribute to peripheral myelin alterations possibly exacerbating peripheral injury.

Methods: A chronic 28-day stress protocol is employed to induce depressive-like behaviors in both control and non-obese type 2 diabetic mice. Tail suspension, forced swim, sociability and sucrose preference tests are performed to assess depression in mice. The raised beam walking test allows the assessment of sensorimotor malfunction in diabetic and depressed animals. mRNA levels of Nox1, Nox4, PLP, MBP and PMP22 will be assessed using Reverse transcription polymerase chain reaction (RT-PCR). Moreover, NADPH oxidase activity determines the activation of Nox enzymes measuring superoxide anion production.

Results: Upon depression, altered molecular expressions of the aforementioned myelin proteins in both the central and peripheral nervous systems are observed, paralleled by an increase in motor behavior injury. Furthermore, the mRNA expressions of Nox1, and Nox4 are upregulated in the prefrontal lobes and hippocampi of both control/depressed and diabetic/depressed mice. Upon GKT treatment, NADPH-induced ROS production in the prefrontal lobes, hippocampi and sciatic nerves of diabetic, depressed, and diabetic-depressed mice decreases. Escitalopram shows to play a role in reversing not only the depressive-like symptoms in depressed and diabetic/depressed mice, but also reverse the myelin alterations observed centrally and peripherally.

Conclusion: This study may hold promising results for the treatment of depression and DPN in diabetic patients.

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ABBREVIATIONS

DM:	Diabetes mellitus
DN/DPN:	Diabetic neuropathy/Diabetic peripheral neuropathy
MDD:	Major depressive disorder
HPA:	Hypothalamic pituitary adrenal
CMS:	Chronic mild stress
CNS:	Central nervous system
PNS:	Peripheral nervous system
Il-6:	Interleukin-6
TNF-α:	Tumor necrosis factor-alpha
MBP:	Myelin basic protein
PLP:	Proteolipid protein
MPZ:	Myelin protein zero
PMP22:	Peripheral myelin protein 22
AGE:	Advanced glycated end products
NADPH:	Nicotinamide adenine dinucleotide oxidase
IR:	Insulin resistance
H_2O_2:	Hydrogen peroxide
SSRI:	Selective serotonin reuptake inhibitor
FST:	Forced swim test
TST:	Tail suspension test
SPT:	Sucrose preference test
PGL:	Plasma glucose levels

CHAPTER I

INTRODUCTION

Diabetes Mellitus (DM) is a heterogeneous metabolic disorder of several etiologies marked by chronic hyperglycemia (American Diabetes Association, 2015). DM can be classified into two types based on impaired insulin secretion, insulin action or both. Type 1 DM is an autoimmune disease culminating in insulin deficiency due to pancreatic β-cell destruction. Type 2 DM is the more prevalent form of diabetes and is characterized by peripheral insulin resistance followed by compensatory insulin hypersecretion by the pancreatic islets, ultimately leading to decline in islet secretory function (Punthakee, Goldenberg and Katz, 2018). According to the World Health Organization, approximately 347 million people suffered from diabetes in 2008. This number is estimated to double by 2030. More importantly, DM is coupled with "conventional" microvascular and macrovascular complications affecting several organ systems. The former includes retinopathy, nephropathy and neuropathy, whereas the latter comprises accelerated cardiovascular impairments. Among these complications, diabetic neuropathy (DN) is the most common targeting 50-70% of diabetic patients (Pop-Busui et al., 2017). The peripheral nervous system (PNS) may be affected in DN depending on the duration and severity of the disease. Consequently, DN is classified according to the network affected and site of dysfunction.

Diabetic peripheral neuropathy (DPN) is the most common type of neuropathy among diabetic patients. DPN adopts a "stocking-glove" pattern, where loss of sensation progresses in a distal-to-proximal fashion. Clinical manifestations, including numbness, tingling, pain, or weakness, further disrupt quality of life (Pop-Busui et al.,

2017; Tankisi, H, 2017). DPN initially affects the small diameter thinly myelinated Aδ fibers alongside the unmyelinated small diameter C fibers, which transmit impulses pertaining to temperature and pain. With the progression of DPN, degeneration of larger myelinated nerve fibers results in a loss of ankle reflexes, sensory ataxia, and reduced proprioception. Ultimately, the risk of limb amputations increases as diseased tissue develops from ulcer formation and infection (O'Brien, Sakowski and Feldman, 2014). To date, therapeutic means for the management of DPN remain limited to analgesic agents for the alleviation of pain, and optimal glycemic control (Cooper and Forbes, 2013).

Among the "nonconventional" manifestations associated with diabetes, chronic complications include dementia, sexual dysfunction, and depression (Cooper and Forbes, 2013). According to the fifth edition of the American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders, major depressive disorder (MDD) is marked by either diminished/irritable mood, decreased interest/pleasure or both. For a proper MDD diagnosis, four of the following symptoms should be present: constant fatigue, loss of energy, feeling worthlessness or guilty, weight loss or gain, psychomotor retardation or activation, difficulties initiating and/or maintaining sleep, as well as suicidal thoughts (Bădescu et al., 2016; Gragnoli, 2014). A growing body of evidence confirms the existence of a bidirectional relationship between diabetes and depression. The rate of depression is doubled in patients with diabetes compared to normal individuals (Semenkovich et al., 2015; Moulton, Pickup and Ismail, 2015). Moreover, a meta-analysis of nine cohort studies reported a 37% increased risk of developing type 2 DM in adults with depression, after accounting for sex, poverty and body mass index (Holt, de Groot and Golden, 2014).

A multitude of neuroimaging, and neuropsychiatric studies find it difficult to determine the brain regions implicated in depression. One possible explanation lies in the simultaneous activation of multiple brain regions in depressed patients. The triune brain, an evolutionary model proposed by Maclean, attributes three different assemblies to the brain, each possessing different structural and functional properties. These regions include the prefrontal neocortex, the limbic system, and the brainstem, which process higher cognitive tasks, regulate emotions, and control the body's vital functions respectively. Interestingly, brain imaging studies successfully show the contribution of each of these three subdivisions to the multi-regional localization of depression. Decreased metabolism in the dorsolateral and dorsoventral prefrontal cortices, is a frequently replicated finding in MDD. In addition, both anatomical and functional abnormalities have been noted in the amygdala, hippocampi, and dorsomedial thalami of depressed patients, recording either reduced hippocampal or amygdala core volume (Pandya, Altinay, Malone & Anand, 2012). Moreover, the medial prefrontal cortex is a network of closely-related brain areas including the medial and caudo-lateral orbital cortex, amygdala, hippocampus, and others. Dysfunctions within and between the structures of this circuit have been implicated in subjects with recurrent depressive episodes (Drevets, Price & Furey, 2008).

Chronic stress is crucial for depression development. Response to stress occurs primarily within the hypothalamic pituitary adrenal (HPA) axis, ultimately, releasing glucocorticoids. Interestingly, the amygdala, which is the emotional storehouse of the brain, triggers the stress response. Initially, the rush of negative emotions activates the amygdala, which in turn stimulates the HPA axis through the hypothalamus. HPA axis activation causes glucocorticoid release reactivating the amygdala. Thus, chronic stress

results in a positive feedback loop between the HPA axis and the amygdala, prioritizing negative emotions while continuously producing glucocorticoids. Excess glucocorticoids lead to glial and neuronal apoptosis within the hippocampus and prefrontal cortex (Figure 1).

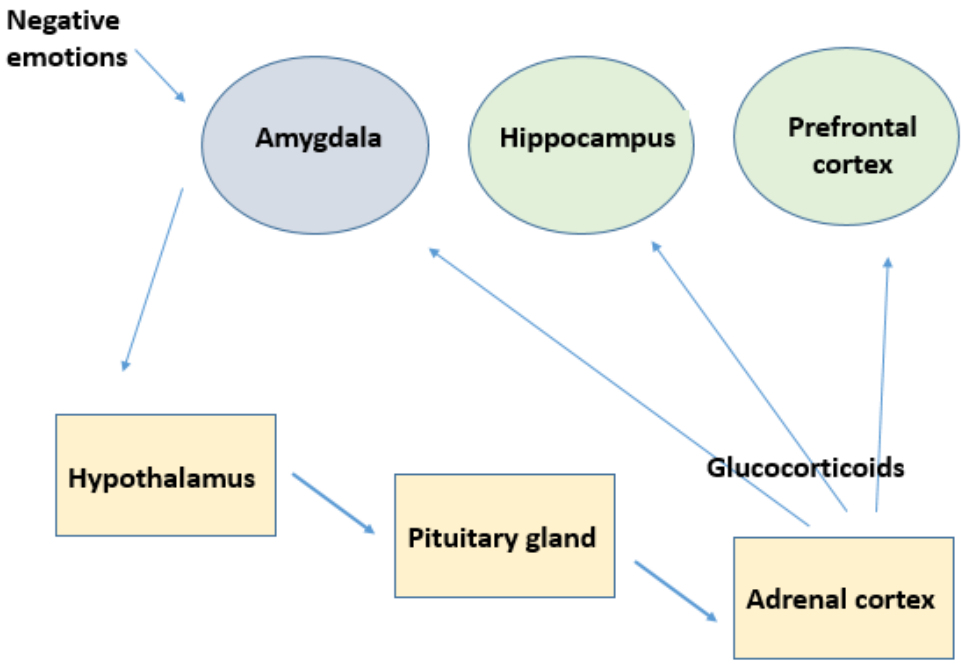

Figure 1: The amygdala-HPA axis interaction

The diagnostic tools for depression are varied. For clinical studies, a structured or semi-structured interview method is standard. Genomic, proteomic and metabolic profiling are further being investigated to identify possible biomarkers for MDD diagnosis (Smith, Renshaw & Bilello, 2013).

Over the decades, a wide range of therapeutic means have been proposed to combat depression including pharmacotherapy, psychotherapy and somatic therapy.

Most of the available antidepressants work by increasing overall synaptic monoamine concentration (serotonin, norepinephrine and dopamine). This is achieved either by preventing the monoamine reuptake into the presynaptic neuron or via reversible or irreversible inhibition of the monoamine degrading enzyme. Moreover, somatic therapy including electroconvulsive therapy, transcranial magnetic stimulation, and vagus nerve stimulation, has a widespread clinical use for the treatment of mental disorders where transient electric or magnetic currents are induced into anatomically deep brain structures. Finally, herbal medicine has been a reasonable alternative or add-on therapy for the management of mental disorders such as anxiety, depression and dementia. St. John's wort, also known as hypericum perforatum, is the only approved herbal antidepressant for the clinical management of mild to moderate depression (Fekadu, Shibeshi & Engidawork, 2017).

To investigate both the etiology and possible therapeutic agents of depression, the use of animal models becomes fundamental. However, when it comes to depression, ideal animal models remain lacking. An 'ideal' animal model offers an opportunity to understand the interplay among the molecular, genetic, environmental and epigenetic factors that may lead to depression. Some established criteria for the validation of an animal model are presented in table 1.

Table 1: Criteria for the validity of an animal model of depression

Criteria	**Manifestations**
Face validity	Behavioral observations similar to symptoms seen in depressed subjects
Construct validity	Pathophysiological alterations that occur in patients with depression (overstimulation of the HPA axis, hippocampal atrophy…)
Predictive validity	Reversal of observed behavioral alterations upon treatment (antidepressants, electroconvulsive therapy…)

Based on these aforementioned criteria, many animal models of depression have been developed. Some rely on genetic engineering, whereas others on environmental manipulations. Learned helplessness is one of widely validated animal models, in which uncontrollable and unpredictable electrical foot-shock stress potentially induces a depressive-like state in the experimental animals. Exposure to inescapable electric shock places the animal in a state of "helplessness" even when presented with an easy escape route. Another model of depression is maternal deprivation where the animals are separated from their maternal figures after birth. This mimics early unfavorable life experiences, which represent one of the key risk factors for the onset of mental disorders including MDD. Also, bilateral olfactory bulbectomy, another animal model of depression, results in immune, endocrine, behavioral, and neurotransmitter-related alterations which reflect many of the symptoms observed in patients with MDD.

Anhedonia, a core symptom of depression in both humans and animal models, reflects reduced interest or pleasure in naturally rewarding activities. In this study, the chronic mild stress (CMS) protocol was used. Katz et al pioneered the first CMS paradigm where different stressors were administered for a period of 3 weeks including electric shocks, immersion in cold water, reversal of light/dark cycle, and a variety of other stressors (1981). Rodents subjected to these different stressors over long periods (3 weeks to 3 months) demonstrated behavioral deficits, such as changes in sleep and anhedonic behavior, satisfying the face validity criterion. With respect to construct validity, increased adrenal gland weight and elevated corticosterone levels were recorded, indicating over-activation of the HPA axis. Furthermore, changes in lipids and proteins, in addition to decreased antioxidant levels and increased pro-inflammatory

markers were detected in animals subjected to CMS. In addition, various classes of clinically effective antidepressants successfully reversed these behavioral changes strengthening this model's predictive validity. However, carrying out CMS experiments are quite challenging since they are space-demanding, and time-consuming (Abelaira, Reus & Quevedo, 2013).

A. Diabetes and Depression in the Central Nervous System (CNS)

1. Diabetes and Depression: Known Shared Mechanisms

Comorbid diabetes and depression present major clinical challenge where the outcomes of each condition are exacerbated by the other (Doyle, Halaris and Rao, 2014). Both disorders share biological mechanisms, including dysfunctions in the HPA axis resulting in hypercortisolemia, as well as overproduction of pro-inflammatory mediators such as interleukin-6 (Il-6), Il-4 and tumor necrosis factor-α (TNF-α) (Moulton, Pickup and Ismail, 2015; Lopresti, Hood and Drummond, 2013; Kemp et al., 2014). Alongside chronic inflammation, obesity, a major contributor to both conditions, leads to insulin resistance, which in turn reinforces depressive symptoms (Penninx et al., 2013).

Diabetes and depression also induce structural and functional changes in various glucocorticoid receptor–rich brain regions, including the hippocampus, amygdala, and prefrontal cortex (Hoogendoorn, Roy and Gonzalez, 2017). Moreover, reduced hippocampal volume, decreased neurogenesis, cerebral atrophies and lacunar infarcts are recorded in both depressed and diabetic subjects (Ho, Sommers and Lucki, 2013; Semenkovich et al., 2015).

2. Myelin Proteins and Myelination

Within the vertebrate nervous system, motor, sensory and cognitive functions demand rapid impulse propagation, which is enhanced by the insulation of axons with myelin (Nickel and Gu, 2018). Myelin provides the structural basis for saltatory conduction, accelerating nerve communication 20–100-fold in comparison to unmyelinated axons of the same diameter (Nave and Werner, 2014). In addition, myelin ensures trophic support, which is vital for axonal survival (Yin et al., 2006).

In the CNS, sub-ventricular zone derived oligodendrocytes are the myelinating glial cells ensheathing multiple axons simultaneously (Fields, 2014; Edgar and Sibille, 2012). Myelin, this tightly compacted multi-lamellar membrane, consists of lipids (70%) and a variety of proteins (30%) (Edgar and Sibille, 2012; Aggarwal, Yurlova and Simons, 2011). The major structural myelin proteins of the CNS include proteolipid protein (PLP), myelin basic protein (MBP), although other proteins are also expressed such as 2', 3'-cyclic nucleotide-3'-phosphodiesterase (CNP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated oligodendrocytic basic protein and the myelin associated glycoprotein (MAG) (Deber and Reynolds, 1991; Snipes, 1992; Fulton, Paez and Campagnoni, 2010).

MBP, a water soluble extrinsic membrane protein, accounts for 25-30% of all myelin proteins. Being the most abundant myelin protein in the CNS, its predominance secures stable myelin sheath compaction. The human MBP occurs in three forms: 21.5, 17.2 and 18.5 kDa; the latter being the most prominent. In addition to regulating the expression of other myelin proteins, MBPs have been suggested to play a role in axonal cytoskeletal organization. With their extensive coupling to cytoskeletal structures, MBPs are capable of regulating a wide range of cellular processes, from process

extension, migration, to cell proliferation and survival (Snipes, 1992; Fulton, Paez and Campagnoni, 2010).

Interacting with MBP, PLP, an intrinsic membrane protein, is essential for adequate formation and compaction of the multilayered mature myelin sheath. Within the CNS, PLP exists in two isoforms: DM20, which is highest during embryonic development, and PLP, during postnatal development. Furthermore, PLPs have been reported to be expressed in non-myelinating cells, and have been linked to the regulation of several cellular activities including ion exchange, cell migration and apoptosis. Additional studies also report PLP to participate in signal transductions between the extracellular matrix and cell interior (Snipes, 1992; Fulton, Paez and Campagnoni, 2010).

3. Myelin Injury in the CNS

Even though the interactions of these myelin proteins with respect to one another and to the phospholipid bilayer are not yet fully understood, their precise arrangements have functional importance. A slight conformational change in any of these components perturbs the entire structure, leading to dysmylination (D'Urso, Ehrhardt and Müller, 1999). Mutations in the human PLP gene cause mental and physical retardation such as spastic paraplegia type 2 and the Pelizaeus-Merzbacher disease (Han et al, 2013; Edgar & Sibille, 2012).

Ajilore et al reported decreased cortical gray matter thickness in the medial prefrontal cortices of both diabetic and depressed subjects (Ajilore et al., 2010). In addition, type 2 diabetic patients with microvascular complications exhibited reduced global brain volumes as opposed to both healthy volunteers and those without

complications (Fang et al., 2018). Postmortem studies have documented reductions in glial density in the dorsolateral prefrontal cortex, orbitofrontal cortex, anterior cingulate cortex and amygdala of MDD subjects. In contrast, several studies proclaimed no changes within the aforementioned brain regions of MDD patients. This leaves a muddled consensus on total glial alterations in MDD (Edgar and Sibille, 2012).

On the one hand, demyelination, whether in the hippocampus or prefrontal cortex, has been observed in patients with Alzheimer's disease, temporal lobe epilepsy, MDD, and other psychotic disorders. On the other hand, veterans with PTSD demonstrated increased hippocampal myelin compared to trauma-exposed controls. Taken together, these findings indicate the importance of myelin regulation: myelin must be at an optimal level, not too little or too much, to achieve normal cognitive functioning (Nickel and Gu, 2018).

B. Diabetes in the PNS

Myelin proteins are essential for the healthy functioning of neurons. Chronic hyperglycemia tends to damage peripheral glial cells. This in turn decreases the expression of myelin proteins, eventually leading to loss of nerve conduction (Rachana, Manu and Advirao, 2016).

1. Myelin Proteins and Myelination

Neural crest-derived Schwann cells are the myelinating glia of the PNS, with a ratio of one Schwann cell to one axon (Mizisin, 2014). Similar to the CNS myelin proteins, those of the PNS are fundamental for appropriate myelin assembly and function. Even though MAG, MBP and PLP are expressed in the PNS to lesser extents,

myelin protein zero (MPZ or P0), and peripheral myelin protein 22 (PMP22) are exclusive to it (Snipes and Suter 1995; Adlkofer et al., 1995).

PMP22 is a 22 kDa glycosylated myelin protein, comprising an approximate 5% of PNS myelin proteins in both rodents and humans PMP22 has been suggested to partake in peripheral nerve myelination and cell proliferation. It is required for axonal maintenance, myelin thickness determination and stability (Adlkofer et al., 1995). Various mutations in the PMP22 gene have been coupled with hereditary demyelinating peripheral neuropathies, such as Charcot-Marie-Tooth disease, Dejerine-Sottas syndrome, as well as hereditary neuropathy with liability to pressure palsies (D'Urso, D., Ehrhardt, P. and Müller, H, 1999).

Similarly, P0, a 28kDa myelin protein, is crucial for myelin sheath tight compaction. Supporting the aforementioned, Yin et al showed that mice with one copy of the P0 gene displayed motor deficits and hypo-myelinated sciatic nerves. Taking this finding a step further, PNS myelination halted in P0-null mice, ultimately inducing severe neurological disabilities and early death (2014).

2. Myelin Injury in the PNS

Chronic hyperglycemia contributes to nerve damage by triggering a range of metabolic abnormalities. Some of these dysfunctional biochemical mechanisms include the production of advanced glycation end (AGE) products, and increased activities of both protein kinase C and polyol pathways. Inflammation, dyslipidemia, mitochondrial reactive oxygen species, and endoplasmic reticulum stress strongly bring about DPN progression in both humans and experimental models of diabetes (O'Brien, Sakowski and Feldman, 2014; Sima and Zhang, 2014).

In addition, obesity and oxidative stress, risk factors common to both DM and depression, promote apoptosis of neurons and Schwann cells via activation of programmed cell death caspase pathways (Dogiparthi et al., 2017; Pop-Busui et al., 2016; Zychowska et al., 2013).

Even though insulin is vital for neuronal growth and survival, insulin signaling pathways are disrupted in diabetic patients either due to insulin deficiencies or insulin resistance. This disruption impairs nerve regeneration, contributing to the pathogenesis of diabetic neuropathy (Callaghan et al., 2012; Dogiparthi et al., 2017).

C. Reactive Oxygen Species in Diabetes and Depression

Oxidative stress occurs either due to elevated reactive oxygen species (ROS) levels or reduced antioxidant capacities of cells. As a result, it is implicated in carcinogenesis, neurodegeneration, atherosclerosis, aging, depression, and diabetes, causing direct or indirect ROS-mediated damage of nucleic acids, proteins and lipids (de Morais, H., et al, 2014; Ray, Huang and Tsuji, 2012).

The brain is quite sensitive to oxidative stress, partly due its high metabolic rate and the abundance of iron and copper. These interact with hydrogen peroxide, generating reactive hydroxyl radicals which may ultimately cause damage to proteins, lipids, and DNA. Moreover, neuronal membranes are packed with phospholipids containing polyunsaturated fatty acid esters. Their exposure to ROS causes a chain reaction, consequently generating lipid radicals and extensive membrane damage.

ROS control many central functions via a direct mechanistic action on the brain. Neurons are capable of transmitting and converting ROS into adequate intracellular responses, including synaptic plasticity. Furthermore, ROS play a role in

MBP modification and can induce synaptic long-term potentiation, a form of activityreliant synaptic plasticity and memory consolidation (Drougard, Fournel, Valet & Knauf, 2015).

Multiple mechanistic pathways are shown to be involved in ROS production in the CNS and PNS (Dewanjee et al., 2018; Korczak et al, 2011). The nicotinamide adenine dinucleotide (NADPH) oxidase (Nox) family is a major source of ROS that has been shown to mediate diabetic nephropathy, retinopathy, and cardiomyopathy (Eid et al., 2009; Habibur Rahman, Kumar Jha and Suk, 2016; Zhao et al., 2015). Actually, ROS overproduction has been reported to be among the many factors leading to insulin resistance (IR). This was observed in murine adipocyte cell line 3T3-L1 supplemented with ROS in the form of H_2O_2 triggered the onset of IR (Lin et al., 2004). Similarly, in vivo studies reported IR in ob/ob mice as a result of elevated ROS levels (Houstis, Rosen and Lander, 2006). However, the involvement of Noxs in depression and DPN remains unclear.

NADPH oxidases are considered “professional” ROS-producing enzymes as their primary, defined function is the generation of superoxide and/or hydrogen peroxide (H_2O_2). Catalytic Nox subunits include an N-terminal domain composed of six transmembrane helices. Four histidine residues in helices III and V incorporate two heme atoms. A cytosolic C-terminal dehydrogenase domain entails a flavin adenine dinucleotide (FAD) cofactor and an NADPH-binding site. Upon activation, electrons are transferred from NADPH to molecular oxygen, producing superoxide anions (Bedard and Krause, 2007). The latter can be dismutated into H_2O_2. Importantly, the Nox family is comprised of seven members; namely Nox1 through 5 and DUOX1 & 2. Interestingly, these isoforms differ in their tissue distribution, level of expression, nature

of ROS produced, and control by distinct signaling modulators (Cifuentes-Pagano, Meijles and Pagano, 2014).

p22phox, a membrane-bound component present in Nox1-, Nox2, Nox3 and Nox4 complexes, serves as a docking site for other regulatory subunits depending on the particular Nox system. Other regulatory subunits act either as organizers, targeting other subunits to the membrane, or as activators, directly modulating catalytic activity (Cifuentes-Pagano, Meijles and Pagano, 2015).

Nox2, which is the first to be discovered, is the most thoroughly characterized isoform. It comprises the gp91phox subunit, also known as Nox2, and p22phox. Unlike other Nox subunits, Nox4 is unusual in that it is constitutively active. Upstream activators are required for the normal functioning of all the other Noxs. Furthermore, the activity of all Nox enzymes except Nox4, necessitates the interaction among the cytosolic p47phox (organizer), p67phox (activator), p40phox and the small Rho-family GTP-binding protein, which leads to the production of ROS (Sorce et al, 2012). Nox4 on the other hand, can also bind to polymerase (DNA-directed) delta-interacting protein 2 (Polidip2) for maximal activity (Lyle et al., 2009). Finally, Nox4 appears to produce mainly H_2O_2 in contrast to Nox1, Nox2, Nox4 and Nox5 which release mainly superoxide (Takac et al., 2011; Nisimoto et al., 2014)

Interestingly, Noxs are potent superoxide anion generators in both neurons and glial cells. Immuno-histochemical approaches in rats and mice have successfully localized Noxs in different regions of the central nervous system including the hippocampus, cortex, amygdala, striatum, thalamus and hypothalamus (Drougard, Fournel, Valet & Knauf, 2015). Even though the brain is protected by the blood brain

barrier, inflammatory markers circulating in the brain can generate ROS (Radak et al 2013; Surjyadipta Bjattacharje 2013; Popa-Wagner et al 2013).

D. NADPH and CNS Injury

Elevated ROS levels were remarked in depressed patients compared with control subjects (Jiménez-Fernández et al., 2015). Additionally, decreased plasma concentrations of essential antioxidants such as vitamin C, vitamin E, and coenzyme Q1 were detected in MDD patients (Maes et al, 2011; Lopresti, Hood and Drummond, 2013).

ROS overproduction induces neural structural damages associated with cognitive deficits such as impaired memory, learning skills and social transactions (Uchihara et al, 2016; Walton et al, 2013). In depressive etiologies, ROS overproduction was remarked in depressed patients compared with control subjects (Jimenez-Fernandez et al, 2015). Elevated ROS levels were also observed in the hippocampi of depressed patients compared to healthy subjects (Black et al., 2015; Gupta et al, 2015, de Morais et al., 2014). This state of oxidative injury was shown to occur through NADPH-dependent mechanisms whereby blockage of NADPH via apocyanin, or increasing antioxidant supplementation such as N-acetyl-L-cysteine, reduced depressive symptoms in both humans and animals, and also improved cognitive and behavioral performance, respectively (Seo et al, 2010; Arent et al, 2012; Wayhs et al., 2013; Bhatti et al., 2018). However, the role of specific Nox inhibitors and their involvement in myelin dysregulation are poorly understood.

E. NADPH and PNS Injury

Numerous data denote peripheral nerve injuries induced by glucose-mediated oxidative stress, ultimately leading to neuronal apoptosis and loss of Schwann cells (Feldman et al., 2017; Feldman, et al., 1997; Russell, et al., 2001). By the same token, hyperglycemia mediates dorsal root ganglion (DRG) neuron injury either via direct NADPH overstimulation or hyperglycemia-induced AGE exposure (Vincent et al., 2009, Vincent et al, 2005a; Shakeel, 2015). Additionally, antioxidant administration such as glutathione, vitamin E, vitamin C, and/or lipoic acid ameliorates DN progression and counteracts oxidative stress (Papanas and Ziegler, 2016).

F. Hypothesis and Aim of the Study

Our hypothesis suggests that NADPH-induced oxidative stress, which constitutes the final outcome of both disorders; diabetes and depression, promotes myelin alterations within the hippocampus and prefrontal lobe, alongside peripheral myelin alterations possibly leading to peripheral nerve injury. To validate the suggested hypothesis, three specific aims were addressed. The first aim assesses the role of NADPH-induced ROS production in depression and diabetes-induced depressive-like behaviors in animal models. The second aim examines whether depression can cause myelin alterations at the level of the PNS. And the final aim tends to investigate whether the effect of comorbid diabetes and depression may further contribute to peripheral myelin alterations possibly exacerbating peripheral injury. Interestingly, from a clinical perspective, studying the effect of an antidepressant selective serotonin reuptake inhibitor (SSRI) (escitalopram) or a specific Nox4 inhibitor (GKT) on central

and peripheral myelin alterations may unravel novel therapeutic approaches in treatment of diabetes, depression and DPN.

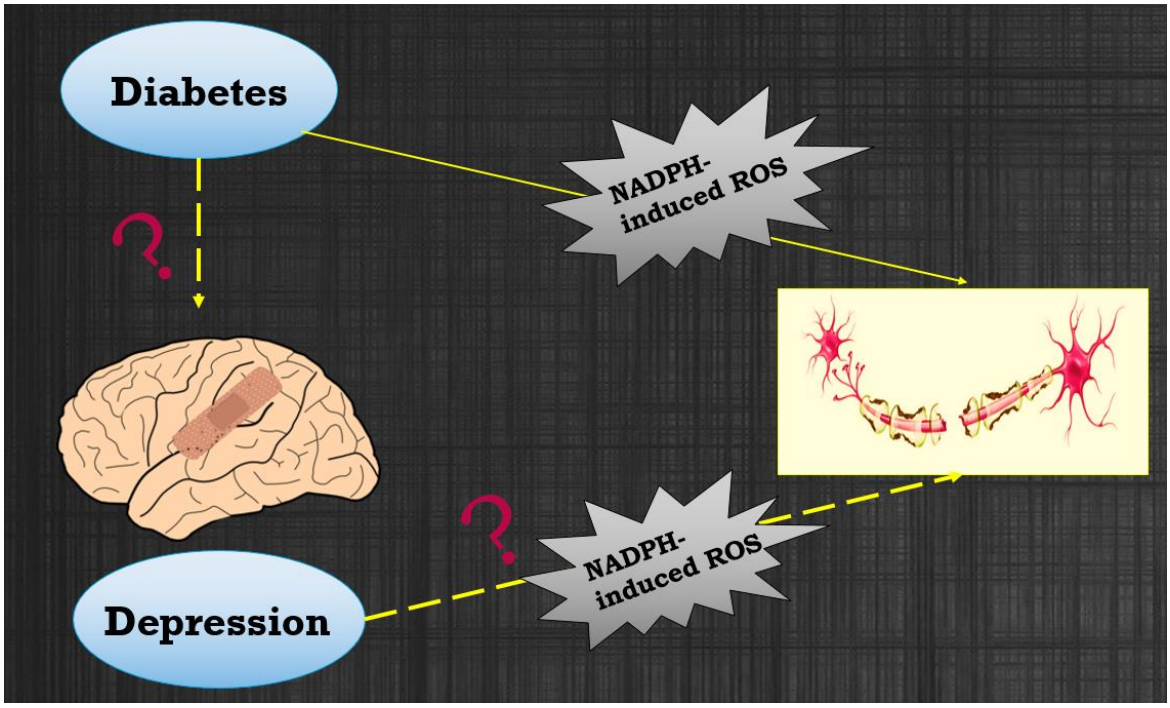

Figure 2: Causative Reagents of Central and Peripheral myelin alterations

CHAPTER II

MATERIALS AND METHODS

A. Experimental Design

In this study, two types of mice were utilized: the FVB/NJ control wild-type mice and the MKR transgenic non-obese type 2 diabetic mice. Both types were bred at the American University of Beirut. At eight weeks old 8, the MKR mice developed type 2 diabetes. At week 9, mice were randomly divided into eight groups, and were exposed to a 28-day chronic mild stress (CMS) protocol. At week 13, these mice were subjected to behavioral testing including the beam walking (BW), forced swim (FST) and tail suspension tests (TST) to assess sensorimotor dysfunction and depressive-like symptoms prior to any treatment. These rodents were then treated for 10 weeks, with an SSRI (escitalopram) or GKT (a specific Nox4 inhibitor), and reassessed for behavioral changes alongside the sucrose preference test (SPT). At week 25, all mice were sacrificed for molecular testing. Details pertaining to each section are provided below.

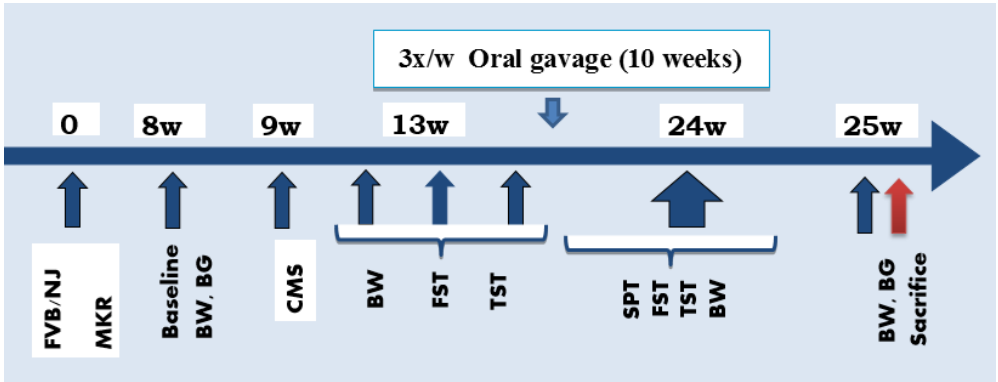

Figure 3: The Experimental Design of this Study

B. Animal Models

In this study, a total of 36 male mice were used. A non-obese type 2 diabetic mouse model was utilized. A non-obese type 2 diabetic mouse model was utilized. Within this transgenic mouse model, type 2 DM develops approximately after eight weeks due to functionally inactivating the insulin-like growth factor-1 receptor (IGF-1R) in the skeletal muscle. Marked with insulin resistance, hyperinsulinemia, and hyperglycemia, these mice are representative of non-obese human type 2 DM (Mallipattu et al., 2014). FVB/NJ mice belonging to the same age group were used as control. These eight week-old mice were divided into eight groups as illustrated in table 2.

① Control (n=3)	⑤Diabetic (n=5)
② Control mice where depressive-like behavior was induced (Depressed) (n=4)	⑥ Diabetic mice where depressive-like behavior was induced (Diabetic depressed) (n=4)
③ Control mice where depressive-like behavior was induced (Control depressed treated with GKT) (n=5)	⑦ Diabetic mice where depressive-like behavior was induced (Diabetic depressed treated with GKT) (n=5)
④Control mice where depressive-like behavior was induced (Control depressed treated with escitalopram) (n=5)	⑧Diabetic mice where depressive-like behavior was induced (Diabetic depressed treated with escitalopram) (n=5)

Table 2: Grouping of 36 Male Mice into Eight Subsets

All treatments were administered three times a week via oral gavage. GKT # 137831, manufactured in our lab, is a highly potent inhibitor of Nox4 and to a lesser extent Nox1. It was dissolved in methyl cellulose and was given at a dose of 40mg/kg/day. Escitalopram, a selective serotonin reuptake inhibitor (SSRI), was purchased from the AUB pharmacy and was given at a dose of 20mg/kg/day.

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. Plasma glucose levels (PGL) were monitored weekly via tail vein punctures using a glucometer (ACCU-CHEK Performa; USA) (Eid et al, 2009).

C. Chronic Mild Stress Procedure

The protocol employed in this study is a modified version of those proposed by Kumar et al (2010), Molina et al (1990) and Murua et al (1991). Due to the presence of diabetic animals, food deprivation was substituted with other stressors. The protocol aimed at inducing depressive-like symptoms in mice. Different stressors were administered daily for 28 consecutive days with a stress free day/week (Franceschelli et al, 2014; Zhu et al, 2014; Kompagne et al, 2008; Katz, Roth and Carroll, 1981), to prevent the animals from getting accustomed to these stressors, maximizing unpredictability.

In this study, the following stressors were used. The change of mate (M) 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 (1981). Titling the cage (T) 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 anhedonic behavior. In addition, pre-exposure to 95 dB of white noise (N) for 6-8 hours has been

demonstrated to initiate a highly active behavioral profile in mice. Wetting the bedding of the cages (W) 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 (R) 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 Helena et al light/dark cycle manipulation (D'-D'') further contributes to anhedonic behavior, alongside increased corticosterone and decreased brain-derived neurotrophic factor levels in the hippocampus (2013).

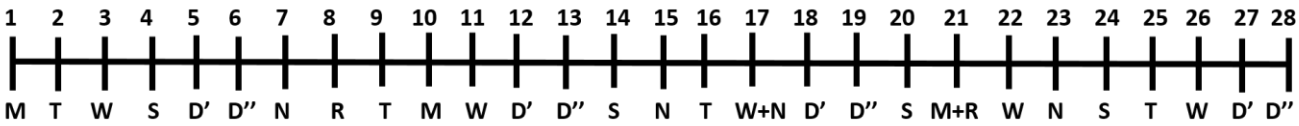

Figure 4: Different Stressors of the 28-Day Chronic Depression Protocol. M: Mate Change (2h); T: Tilted Cage at 45° (8h); W: Wet Cage (8h); S: Stress-free day; D'-D'': Light/dark cycle manipulation (48h); N: White Noise (8h); R: Response to Rat Urine (4h)

D. Behavioral Assessments

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.

1. Sucrose Preference Test

Anhedonia, defined as the inability to experience pleasure from enjoyable tasks, is a core symptom of depression. The sucrose preference test (SPT) is a reward-based test, used as an indicator of anhedonia. SPT was performed over a week period and twice throughout the study: following chronic mild stress protocol and after treatment provision. The purpose of the first SPT is to signify the presence of depressive-like symptoms attributed to chronic stress. The second serves to determine the effectiveness of the drugs delivered (Huynh et al., 2011).

In summary, the test was performed as followed: Two bottles; one of 1% sucrose solution and the other of tap water, were placed in the randomly assigned sides of each home cage, and were switched once every three days to eliminate side preference artifact. Both bottles were weighed before and after use (Gross and Pinhasov, 2016; Kompagne et al., 2008). After one week, mice's intake of water and 1% sucrose were measured. Sucrose preference was calculated as the percentage of total liquid intake ascribed to 1% sucrose solution, according to the equation:

$$\text{Sucrose Preference} = \left(\frac{Sucrose\ Intake\ (g)}{Sucrose\ Intake\ (g)+Water\ Intake\ (g)}\right) *100$$

Rodents are born with an innate preference for sweet tastes. Sucrose consumption below 65% is indicative of anhedonia (Strekalova et al., 2011). In previous studies, mice meeting this criterion, exhibited depressive-like symptoms (Vogel et al, 1990; Willner, 1997).

2. Forced Swim Test

The forced swim test (FST) was first developed in 1977 by Porsolt et al as a

model for identifying the clinical efficacy of antidepressants. This test is also used to assess depressive-like behaviors in animal models of psychiatric disorders. When rats 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. Thus, each mouse was placed in a beaker with a depth of 30 cm filled with water of 24 ± 2°C, and was allowed a five-minute swim session, without having its limbs come in contact with the bottom of the container. In addition to floating, the immobility time of mice was recorded, reflecting increased depressive-like behavior.

3. Tail Suspension Test

In a manner analogous to the FST, the tail-suspension test (TST) is a behavioral test designed to screen for potential antidepressant drugs, as well as assess depressive-like behaviors. TST incorporates suspending mice above the ground by their tails. The procedure includes a suspension bar and tape. It is important to note that the tape should be strong enough to carry the weight of the mouse being tested. After taping the tails of mice to a beam of 50 cm in height, immobility time was recorded for six minutes (Can et al., 2011).

4. Raised Beam Walking Test Assay

Motor coordination and balance assessment is performed via the raised beam walking test (Luong et al., 2011). This task is useful for the detection of subtle deficits in motor skills and balance which may not be pinpointed out by other motor tests, such as the Rotarod. Animals were placed on a rod of 1.2 cm diameter, 70 cm length and around 50cm above a flat surface. At one end of the rod, a secure platform was set to house the animal. First, the mouse was allowed to adapt and then trained to stay upright and walk across the elevated narrow beam to its safe platform three times before recording its performance. The time taken to cross, the speed (calculated using the following formula: speed= distance/time), the number of stops and the number of faults/slips were recorded for analysis.

E. Tissue Removal (Hippocampus, Prefrontal lobe and Sciatic Nerve)

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 (2013), and Chiu et al (2007).

To harvest the sciatic nerves of these mice, each mouse was then pinned to a dissecting board in a supine position. 70% ethanol was used to disinfect the skin. Using a razir blade, a deep cut was made at the base of the tail along the vertebral column. Both hind limbs were held together in position using one hand and the base of the tail was held with the other. To expose the sciatic nerves, the tail was pulled away from the hind limb at the point of the cut. The part of the nerve that runs along the lumbo-sacral region was exposed and appears as a thick whitish cord. This step was performed with caution because excessive pulling force could thin or break it, or even distort the histological morphology of the myelin. (Bala, Tan, Ling & Cheah, 2014).

F. Molecular Assessments

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

1. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

mRNA expression in both hippocampi and prefrontal cortices of mice was analyzed by real-time RT-PCR using the ΔΔCt method and the SYBR green system (Eid et al, 2009, El-Merahbi et al., 2014, Daoud et al., 2016). Total RNA was extracted from the prefrontal lobe +lysate using TRIZOL reagent (Sigma Aldrich, Steinheim, Germany) and converted into cDNA using the Revert First Strand cDNA Synthesis Kit according to the protocol. cDNA was quantified using RT- PCR Biorad CFX384 with SYBR green dye and mouse RT2qPCR Primers (Integrated DNA Technologies, Inc., Coralville, IA, USA), for Nox 1, Nox 4, PLP, MBP and PMP22. 26S was used as internal reference gene (Eid et al, 2009).

Table 3: Oligonucleotide primer sequences and conditions employed for RT-PCR

Primers	Sequence	Annealing TºC
26s	F: 5'-AGGAGAAACAACGGTCGTGCCAAAA-3' R: 5'-GCGCAAGCAGGTCTGAATCGTG-3'	57-60ºC
MBP	F: 5'-TACCCTGGCTAAAGCAGAGC-3' R: 5'-GAGGTGGTGTTCGAGGTGTC-3'	57ºC
PLP	F: 5'-GCCCTGACTGTTGTATGGCT-3' R: 5'-TCATTTGGAACATACATTCTGGCA-3'	58ºC
NOX1	F: 5'-TCCATTTCCTTCCTGGAGTG-3' R: 5'-CCCAACCAGTACAGCCACTT-3'	60ºC
NOX4	F: 5'-ACTCCCTTCGCCTCTCTTC-3' R: 5'-CCTTCCCTTGTTCACTCATC-3'	60 ºC
PMP22	Sense: 5'-AATGGACACACGACTGATC-3' Anti-sense: 5'-CCTTTGGTGAGAGTGAAGAG-3'	52 ºC

2. NADPH Oxidase Activity Assay

NADPH oxidase activity was measured in sciatic nerves, prefrontal lobes and hippocampi of mice. Proteins were extracted from sciatic nerves using cooled mortar and pestle by smashing the frozen nerve and suspending the remnants in the lysis buffer (20 mM KH2PO4 (pH 7.0), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). 25 μg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin (behaving as the electron acceptor), and 100 μM NADPH (acting as the substrate for the NADPH oxidase). Photon emission expressed as relative light units (RLU) was measured every 30 s for **4** mins in a luminometer. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent (Eid et al 2009)

3. Western Blot

Mouse sciatic nerves 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 18 polyclonal anti-PMP22 antibody (1:1000, 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)

G. Statistical Analysis

Results are expressed as mean ± SD from multiple independent experiments. For both behavioral and molecular assessments, normality tests were done to ensure that the sample distributions of both the treated and non-treated 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

A. Metabolic Parameters of All Eight Groups

Table 2 summarizes the body weights (BW), plasma glucose levels (PGL) and the hemoglobin A1C levels (HbA1C) at the 25th week prior to sacrifice. PGL were significantly different in diabetic animals relative to control littermates. Moreover, the treatments did not affect the glucose levels of these animals.

Group	BW (g)	PGL (mg/dL)	HbA1C (mmol/mL)
Control (n=3)	29.13 ± 2.42	121.75 ± 3.78	4.275 ± 0.31
Control/depressed (n=4)	31.1 2 ± 1.15	130 ± 10.42	4.675 ± 0.33
Control/depressed/GKT (n=5)	29.02 ± 1.64	118.6 ± 8.67	4.25 ± 0.25
Control/depressed/Escitalopram (n=5)	27.95 ± 0.92	126.25 ± 1.7	5.05 ± 0.50
Diabetic (n=5)	34.38 ± 1.18	449.4 ± 33.05*	9.525 ± 0.51*
Diabetic/depressed (n=4)	32.42 ± 3.18	174.6 ± 59.1*	10.05 ± 0.77*
Diabetic/depressed/GKT (n=5)	30.56 ± 1.49	245.8 ± 145.8	10.05 ± 0.77
Diabetic/depressed/Escitalopram (n=5)	30.4 ± 1.55	323 ± 111.29	9.425 ± 0.48

Table 4: The body weights (BW), plasma glucose levels (PGL) and hemoglobin A1C levels (HbA1C) of all 36 male mice at week 25, prior to sacrifice. *p<0.05, comparison with respect to C.

B. Mice with Type 2 Diabetes Develop Depressive-like Symptoms

Using the FST (Figure 5A), the immobility time of the control/depressed (58.67 ± 8.4), diabetic (61.2 ± 10.0), and diabetic/depressed (73.2 ± 8.4) mice is

significantly greater than that of their control littermates (20.5 ± 8.405). Furthermore, the immobility time of the diabetic (61.2 ± 7.7) and the diabetic/depressed (73.2 ± 5.4) animals is significantly greater than that of the control/depressed group (58.67 ± 7.7). In the TST (Figure 5B), the immobility time of the control/depressed (78.28 ± 10.25), diabetic (120.6 ± 12.3), and the diabetic/depressed mice (106.4 ± 10.24) is notably greater than that of the control group (40.71 ± 10.25).

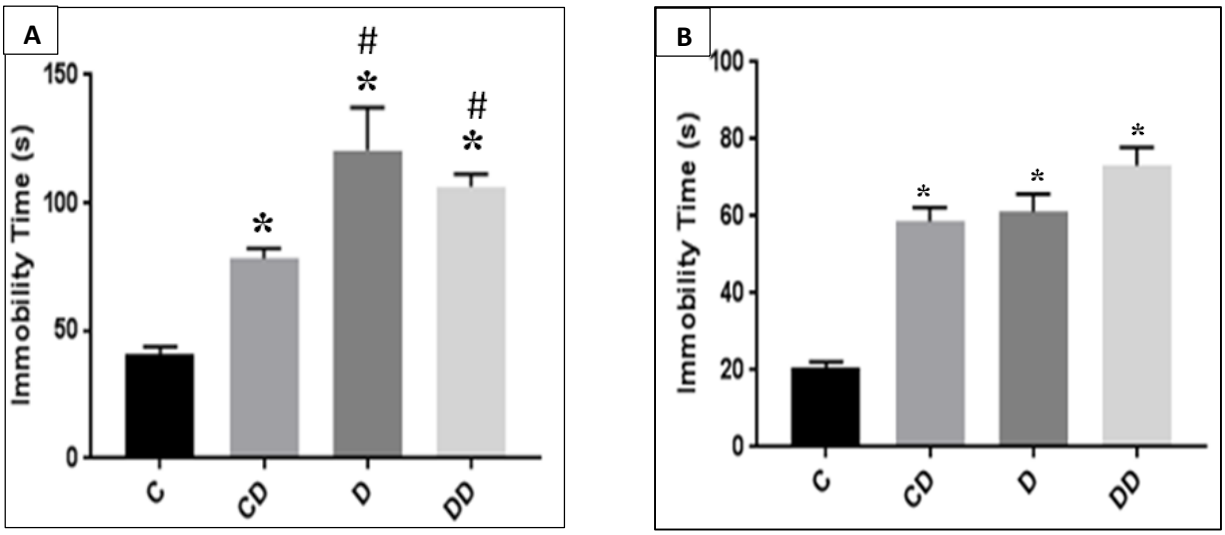

Figure 5: ***Assessment of Depressive-like Behaviors in Control (C) (n=3), Diabetic (D) (n=5), Control/Depressed (CD) (n=4), and Diabetic/Depressed (DD) (n=4) animals.*** *Barograms represent the tail suspension (A), and the forced swim (B) tests. Values are the mean ± SD. * p<0.05, comparison with respect to C. # p<0.05, comparison with respect to CD.*

C. Diabetes, And Diabetes Paired with Depression, Upregulate MBP and PLP

Within the hippocampus, the MBP mRNA levels of the diabetic (336.1 ± 73.52) and diabetic/depressed animals (618.6 ± 73.52) are significantly greater than those of the control (100 ± 73.52). In addition, MBP is significantly upregulated in the diabetic/depressed animals when compared to the diabetic group (Figure 6A). The values of the control groups in figures A, B, C and D have been standardized to 100. As

illustrated in Figure 6B, the PLP mRNA levels within the hippocampi of the control/depressed (342 ± 92.69), diabetic (531.2 ± 92.69), and diabetic/depressed mice (782 ± 92.69) are significantly greater in comparison to the control FVB/NJ mice (100 ± 92.69). Moreover, when compared to the control/depressed mice, PLP is significantly upregulated in the diabetic, and diabetic/depressed batches. As seen in the MBP mRNA levels, the PLP mRNA levels of the diabetic/depressed are significantly greater than those of the diabetic.

Within the prefrontal lobe, the MBP mRNA levels of the diabetic (463 ± 55.58), and diabetic/depressed mice (806.3 ± 55.58) are notably greater than those of the control (100 ± 55.58). In addition, the MBP mRNA levels of both diabetic, and diabetic/depressed animals are significantly greater in comparison to the control/depressed group (199.5 ± 55.58). Moreover, MBP is significantly upregulated in the diabetic/depressed when compared to the diabetic (Figure 6C). As shown in figure 4D, the PLP mRNA levels of the control/depressed (371.5 ± 75.21), diabetic (455.2 ± 75.21) and diabetic/depressed (686 ± 75.21) are significantly greater than those of the control (100 ± 75.21). Furthermore, PLP is significantly upregulated in the diabetic, and diabetic/depressed animals in comparison to the control/depressed mice (371.5 ± 69.64).

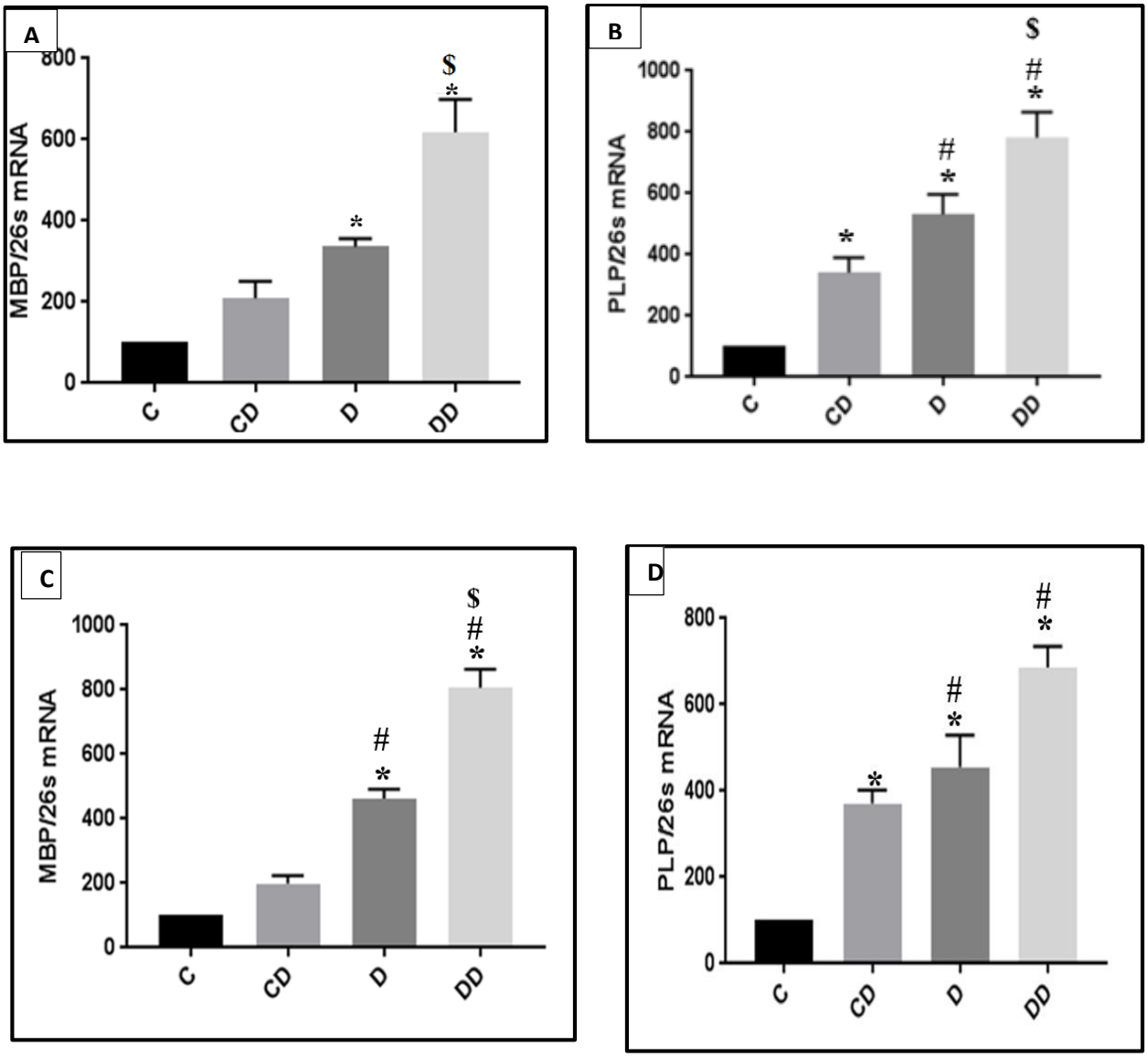

Figure 6: Diabetes alone, and diabetes coupled with depression, upregulate MBP and PLP. MBP and PLP mRNA levels were measured by RT-PCR in the hippocampi (A&B) and prefrontal lobes (C&D) of control (C) (n=3), diabetic (D) (n=5), control/depressed (CD) (n=4), diabetic/depressed (DD) mice (n=4). Values are the mean ± SD. * $p<0.05$, comparison with respect to C. # $p<0.05$, comparison with respect to CD. $ $p<0.05$, comparison with respect to D.

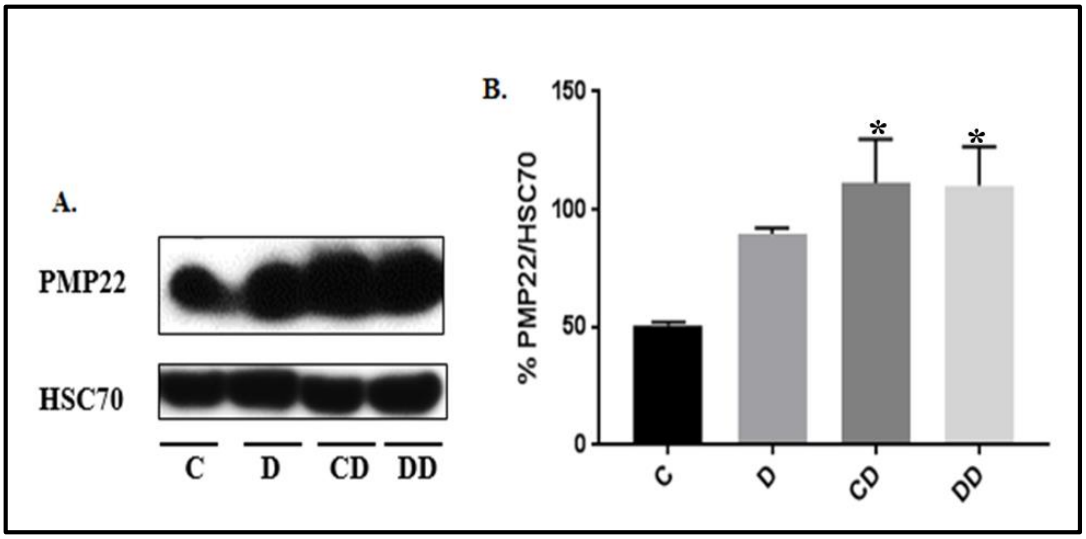

Figure 7: The presence of myelin alterations in the sciatic nerves of control/depressed (CD) (n=4) and diabetic/depressed (DD) (n=4) animals compared to the control (C) (n=3) and diabetic (D) (n=5) groups. A. Representative PMP22 western blot image. HSC70 was used as the loading control. B. Quantitative results of PMP22 in the sciatic nerves of C, D, CD, and DD mice. Values are the mean ± SD.*P<0.05, comparison to the C.

D. Characterizing Myelin Injury in the PNS

PMP22 protein expression was measured in all groups to assess whether diabetes, depression, and/or diabetes coupled with depression induce PNS injury. Our results indicate that PMP22 protein levels are significantly overexpressed in both control/depressed (111.2 ± 17.56) and experimentally depressed/diabetic mice (110.2 ± 17.56) in comparison to the control FVB mice (50.94 ± 17.56) (Figures 7A&B).

E. Type 2 Diabetes and Depression Affect Fine Motor Coordination

Examining whether diabetes and/or depression alter motor coordination 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 control/depressed mice. The beam walking test helps us shed light on a probable

correlation between depression and peripheral nerve injury. Our results showed that the average speed of the control/depressed (5.9 ± 0.77), diabetic (4.06 ± 0.99), and diabetic/depressed (4.71 ± 0.78) was significantly greater than that of the control (7.75 ± 0.77) (Figure 8A). Additionally, the diabetic (3.4 ± 0.72) and diabetic/depressed mice (3.05 ± 0.59) showed a greater number of foot slips in comparison to both the control (0.75 ± 0.59) and control/depressed mice (1.63 ± 0.53) (Figure 8B). With respect to the number of foot stops, the control/depressed (1.7 ± 0.4), the diabetic (2.4 ± 0.4), and the diabetic/depressed animals (1.7 ± 0.36) is significantly greater than that of the control (1.25 ± 0.4). Interestingly, diabetic mice showed a greater number of foot faults when compared to the control/depressed group (Figure 8C).

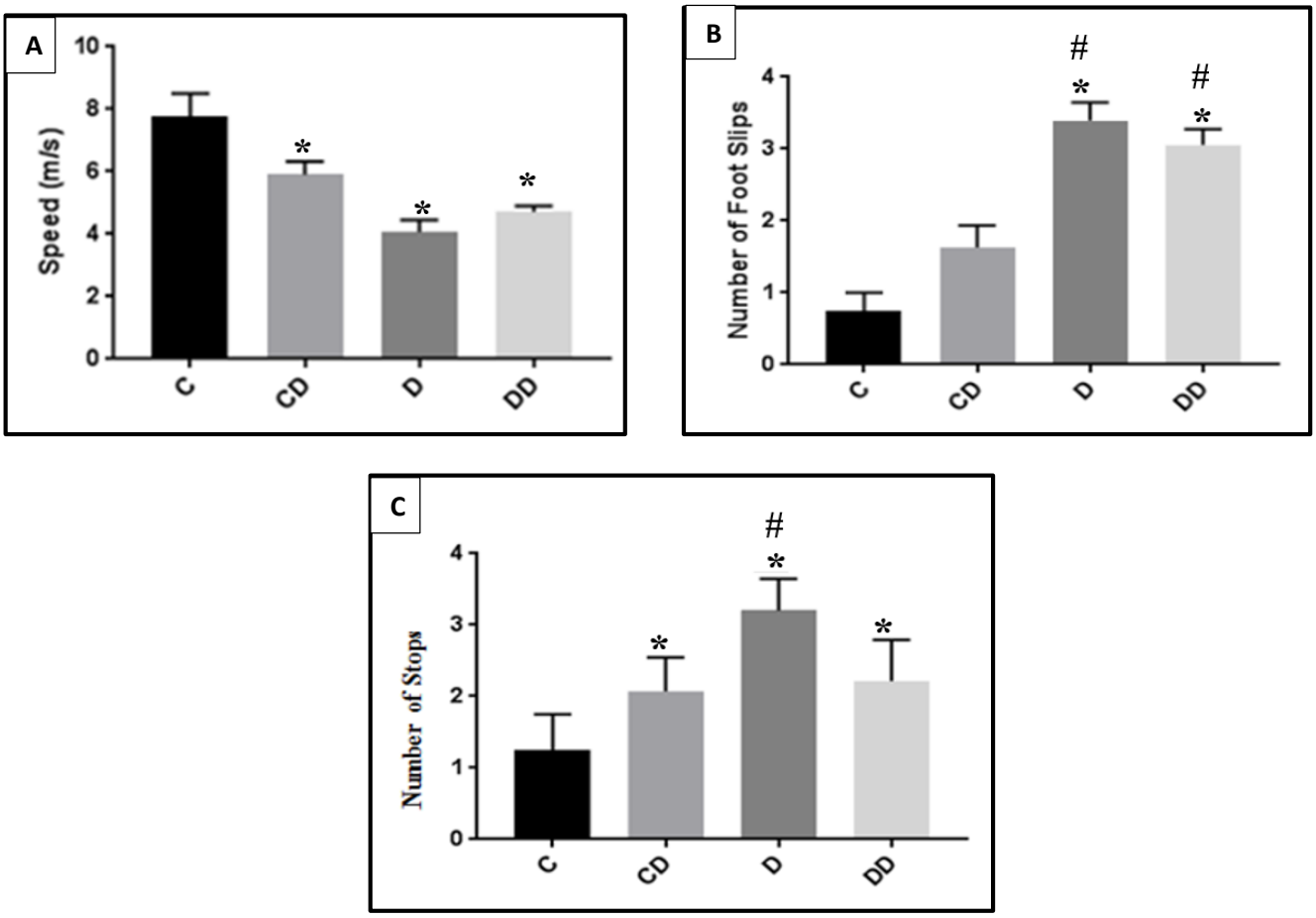

Figure 8: ***The Effect of Hyperglycemia, Depression, and Hyperglycemia Combined with Depression on Motor Coordination.*** *Barograms represent the average speed (A), number of foot slips (B), and number of stop (C) of control (C) (n=3), diabetic (D) (n=5), control/depressed (CD) (n=4) and diabetic depressed (DD) (n=4) mice assessed by the raised beam walking test. Values are the mean ± SD. * p<0.05, comparison with respect to C. # p<0.05, comparison with respect to CD.*

F. Upregulation of NOX1 and NOX4 in diabetic/depressed mice within the CNS

Our results show that Nox1 is significantly upregulated in the hippocampi of diabetic/depressed (1519 ± 331.7) and diabetic mice (423.3 ± 317.2) compared to that of the control (100 ± 331.7) (Figure 9A). The values of the control groups in figures A, B, C and D have been standardized to 100. Moreover, Nox1 is significantly upregulated in the diabetic/depressed mice in comparison to both the control/depressed (192.7 ± 307.1)

and diabetic mice (423.3 ± 291.3) (Figures 9A). Similarly, the mRNA levels of Nox4 of both diabetic (348.4 ± 110.6) and diabetic/depressed (596 ± 115.6) groups are markedly greater than those of the control FVB mice (100 ± 115.6). Interestingly, Nox4 is significantly upregulated in the diabetic/depressed mice (596 ± 101.6) when compared to diabetic mice (348.4 ± 101.6) (Figure 9B). Within the prefrontal lobe, Nox1 mRNA levels are significantly greater in the diabetic (1110 ± 726.2) and diabetic/depressed (4600 ± 679.3) animals in comparison to the control (Figure 9C). With respect to that of the control/depressed (175.2 ± 628.9), Nox1 in the diabetic/depressed mice (4600 ± 628.9) is significantly upregulated. Additionally, when compared to the diabetic mice (1110 ± 679.3), Nox1 mRNA levels in the diabetic/depressed group (4600 ± 679.3) are significantly greater. Nox4 is significantly upregulated in the control/depressed (257.6 ± 275.5), diabetic (451.5 ± 263.4), and diabetic/depressed (1805 ± 275.5) in comparison to that of the control (Figure 9D). In contrast to the control/depressed (257.6 ± 255), Nox4 mRNA levels in the diabetic/depressed (1805 ± 255) are significantly elevated. Nox4 also seems to be upregulated in the diabetic/depressed mice (1805 ± 242) when compared to the diabetic group (451.5 ±242) (Figure 7D). Also, the NADPH-dependent superoxide anion production within the hippocampi of the diabetic (1607 ± 148.3) and diabetic/depressed (2090 ± 148.3) is significantly greater than that of the control. Moreover, when compared to the control/depressed group (764.9 ± 148.3), NADPH-dependent superoxide anion generation is significantly greater in those of the diabetic (1607 ± 148.3) and diabetic/depressed animals (2090 ± 148.3) (Figure 9E). Intriguingly, the diabetic/depressed mice (2090 ± 148.3) showed a greater NADPH-dependent superoxide anion production compared to the diabetic group (1607 ± 148.3). Similar findings were recorded in the prefrontal lobe of these animals, where control/depressed

(945.5 ± 59.56), diabetic (1451 ± 59.56), and diabetic/depressed mice (1904 ± 59.56) showed a significant increase in NADPH-dependent superoxide anion production in comparison to their control littermates (257.8 ± 59.56) (Figure 9F). When compared to the control/depressed group (945.5 ± 59.56), the diabetic and diabetic/depressed mice recorded a significant increase in the NADPH-dependent superoxide anion generation. Similar to the findings observed within the hippocampus, diabetic/depressed (1904 ± 59.56) mice showed a significant increase in the NADPH-dependent superoxide anion generation when compared to the diabetic group (1451 ± 59.56).

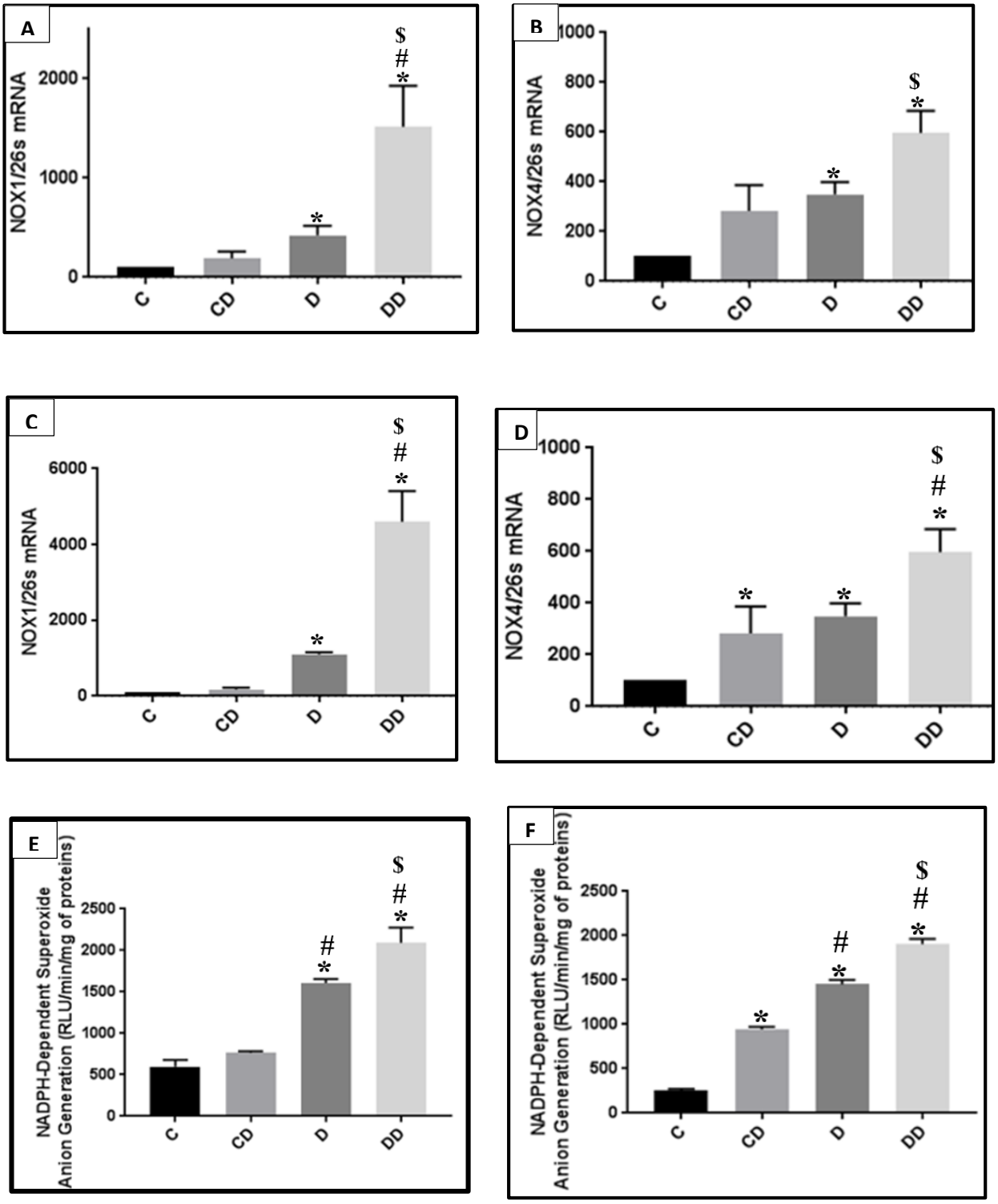

Figure 9: **Elevated NADPH-induced superoxide production in diabetes, depression and both disorders combined.** Nox1 and Nox4 mRNA levels were measured by RT-PCR in the hippocampi (A,B,E) and prefrontal lobes (C,D,F) of control (C) (n=3), diabetic (D) (n=5), control/depressed (CD) (n=4), diabetic/depressed (DD) mice (n=4). Values are the mean ± SD. * $p<0.05$, comparison with respect to C. # $p<0.05$, comparison with respect to CD. $ $p<0.05$, comparison with respect to D.

G. NADPH-dependent superoxide generation in sciatic nerves of diabetic, depressed, and diabetic/depressed mice

Examining ROS production through a NADPH-reliant pathway in the sciatic nerves of the control, diabetic, control/depressed, and diabetic/depressed groups was our next step. Our findings show a significant increase in NADPH oxidase activity within the sciatic nerves of control/depressed (1210 ± 108.4), diabetic (1783 ± 108.4) and diabetic/depressed mice (2358 ± 108.4) compared to the control (650.5 ± 108.4) (Figure 10). In addition, NADPH oxidase activity was significantly greater in the diabetic group compared to the control/depressed. In parallel, inducing depression in the diabetic group further increased NADPH-dependent superoxide generation compared to that of both the diabetic and control/depressed groups.

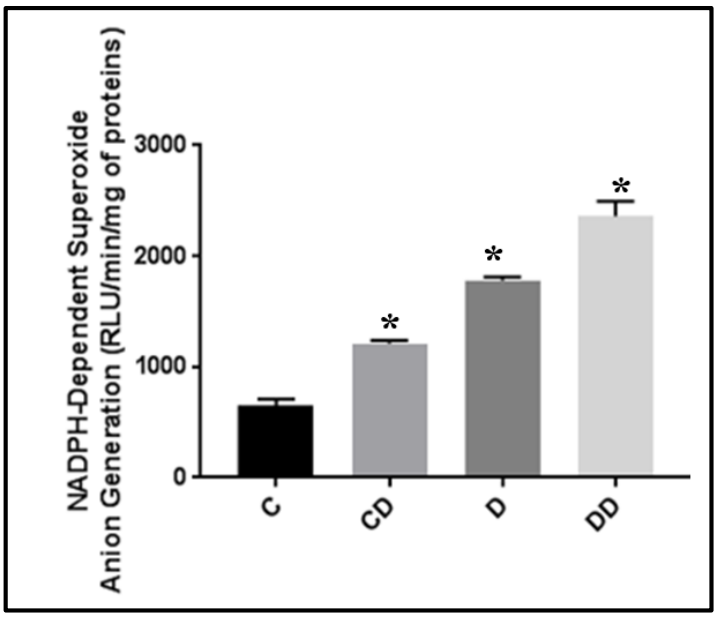

Figure 10: Diabetes and depression induce NADPH-dependent superoxide production in the periphery. The superoxide anion generation assessed by evaluating the NADPH oxidase activity was measured in the sciatic nerves of control (C) (n=3), diabetic (D) (n=5), control/depressed (CD) (n=4), and diabetic/depressed animals (DD) (n=4). Values are the mean ± SD. *P<0.05, comparison with respect to C.

H. GKT or Escitalopram display anti-depressive properties

As mentioned in section B of the results, using the FST and TST (Figure 11A&B), the immobility time of the control/depressed, diabetic, and diabetic/depressed mice is significantly greater than that of the control. In the FST (Figure 11A), our results showed a significant reduction in the immobility time of diabetic/depressed animals treated with either escitalopram (27.4 ± 4.9) or GKT (41 ± 4.9) in comparison to that of the diabetic/depressed (93.8 ± 4.9). Similarly, the immobility time of control/depressed animals treated with either escitalopram (22.25 ± 5.52) or GKT (49.4 ± 5.23) significantly decreased in comparison to that of the control/depressed mice (42.75 ± 5.23). Resorting to the TST (Figure 11B), a significant reduction in the immobility time of diabetic/depressed animals treated with either escitalopram (31.6 ± 4.9) or GKT (55.4 ± 4.9) in comparison to that of the diabetic/depressed (93.8 ± 4.9). With respect to the control/depressed mice (58± 5.25), GKT (37.25 ± 5.25) significantly reduced the immobility time.

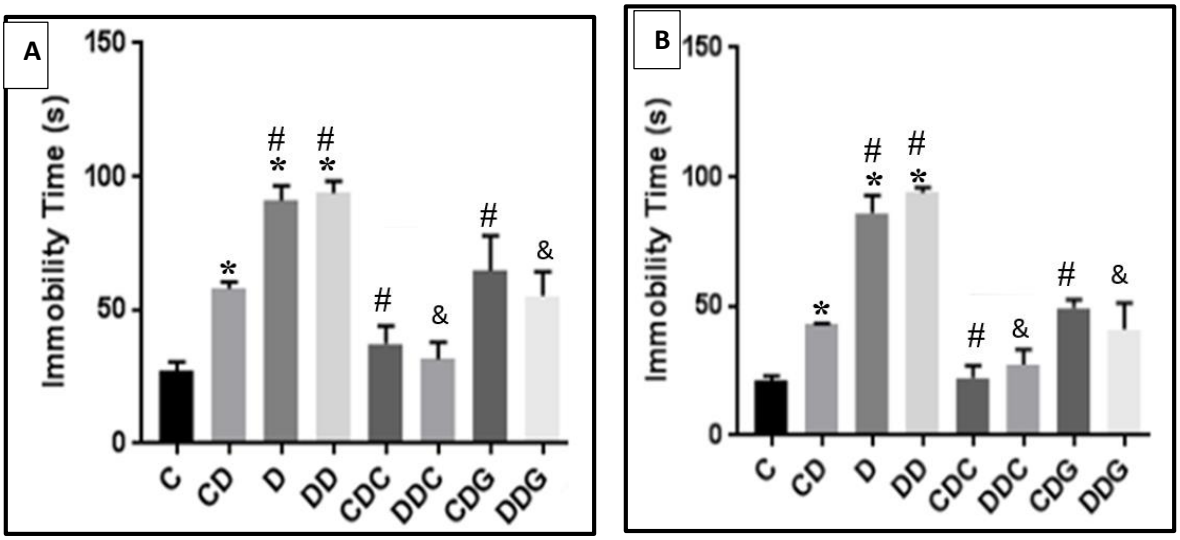

Figure 11: A selective serotonin reuptake inhibitor displays anti-depressive properties. Barograms represent the tail suspension (A), and the forced swim (B) tests. Values are the mean ± SD. The number of animals in each group are divided accordingly: control (C) (n=3), control/depressed (CD) (n=4), diabetic (D) (n=5), diabetic/depressed (DD) (n=4), control/depressed/escitalopram (CDC) (n=5), diabetic/depressed/escitalopram (DDC) (n=5), control/depressed/GKT (CDG) (n=5), diabetic/depressed/GKT (DDG) (n=5). * $p<0.05$, comparison with respect to C. # $p<0.05$, comparison with respect to CD. & $p<0.05$, comparison with respect to DD

To evaluate the anhedonic behaviors of all 'depressed' mice and confirm the effectiveness of escitalopram and GKT, SPT was performed. Both control/depressed (56.16 ± 0.49) and diabetic/depressed mice (61.25 ± 0.49) showed a significant reduction in sucrose consumption compared to the control (90.22 ± 0.54) and diabetic (77.8 ± 0.54) groups, respectively. In addition, both control/depressed treated with either escitalopram (82.9 ± 0.52) or GKT (74 ± 0.49), and diabetic/depressed groups treated with either escitalopram (87.4 ± 0.46) or GKT (78.4 ± 0.46) showed a significant increase in sucrose consumption as opposed to the control/depressed (56.16 ± 0.49) and diabetic/depressed (61.25 ± 0.46) mice, respectively (Figure 12).

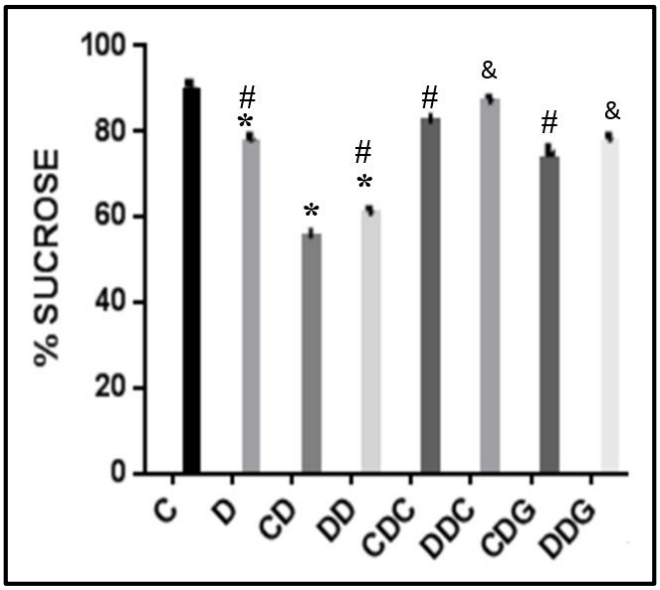

Figure 12: Chronic antidepressant treatment reverses anhedonia in 'depressed' mice. Barograms represent the percentage of sucrose consumption preference. Values are the mean ± SD. The number of animals in each group is divided accordingly: control (C) (n=3), control/depressed (CD) (n=4), diabetic (D) (n=5), diabetic/depressed (DD) (n=4), control/depressed/escitalopram (CDC) (n=5), diabetic/depressed/escitalopram (DDC) (n=5), control/depressed/GKT (CDG) (n=5), diabetic/depressed/GKT (DDG) (n=5). *$p<0.05$, comparison with respect to C.

I. GKT or escitalopram downregulate MBP expression in comorbid diabetes and depression

Our results show that treating diabetic/depressed mice with either escitalopram (90.84 ± 59.13) or GKT (334.1 ± 63.86) causes a significant reduction in the MBP mRNA levels within their hippocampi in comparison to the diabetic/depressed group (Figure 13A). Similarly, treating diabetic/depressed mice with either escitalopram (219.4 ± 84.97) or GKT (538.8 ± 84.97) causes a significant reduction in the PLP mRNA levels within their hippocampi in comparison to the diabetic/depressed group (Figure 13B). With respect to the prefrontal lobe, diabetic/depressed animals treated with either escitalopram (204.3 ± 44.48) or GKT (347 ± 48.04) showed a significant reduction in MBP mRNA levels when compared to the diabetic/depressed mice (Figure 13C). Furthermore, diabetic/depressed mice treated with either escitalopram (289.4 ± 86.03) or GKT (458.8 ± 86.03) showed a significant reduction in the PLP mRNA levels compared to the diabetic/depressed untreated group (Figure 13D).

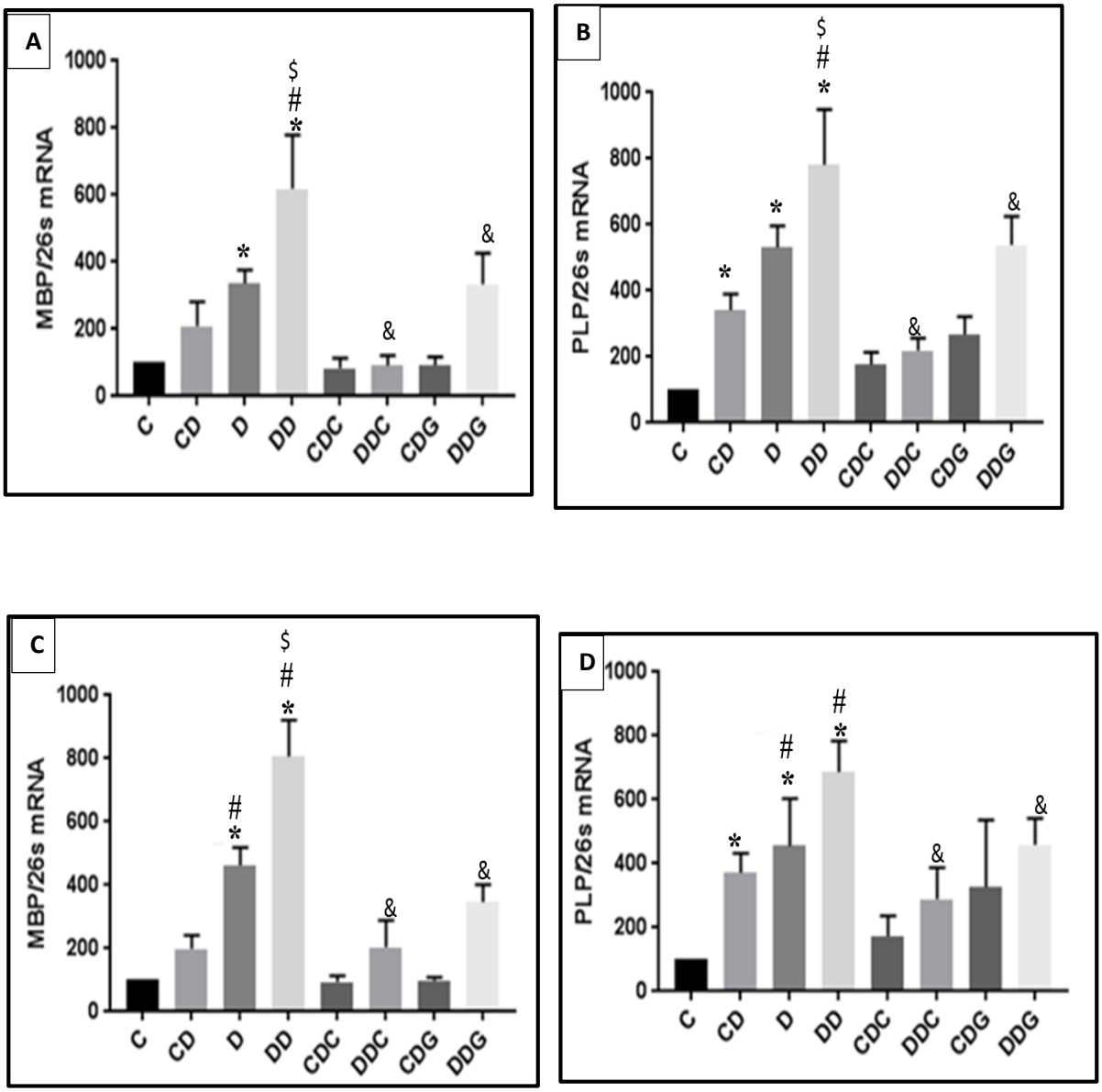

Figure 13: **Escitalopram or GKT restore MBP mRNA levels in comorbid diabetes and depression.** MBP and PLP mRNA levels were measured by RT-PCR in the hippocampi (A&B) and prefrontal lobes (C&D) of all eight groups. Values are the mean ± SD. The number of animals in each group is divided accordingly: control (C) (n=3), control/depressed (CD) (n=4), diabetic (D) (n=5), diabetic/depressed (DD) (n=4), control/depressed/escitalopram (CDC) (n=5), diabetic/depressed/escitalopram (DDC) (n=5), control/depressed/GKT (CDG) (n=5), diabetic/depressed/GKT (DDG) (n=5). *p<0.05, comparison with respect to C. # p<0.05, comparison with respect to CD. $ p<0.05, comparison with respect to D. & p<0.05, comparison with respect to DD

J. GKT, or Escitalopram successfully reduce NADPH-induced ROS production centrally and peripherally in diabetes paired with depression

As outlined in segments E and F of the results section, both diabetic and diabetic/depressed mice exhibited a significant decrease in NADPH-induced ROS generation within their hippocampi and prefrontal lobes in comparison the control. Intriguingly, either specific Nox4 inhibition (664.3 ± 114.2) or selective serotonin reuptake inhibition (1035 ± 114.2) reduced NADPH-dependent ROS production in the hippocampi of diabetic/depressed mice (2090 ± 114.2). Control/depressed mice treated with either escitalopram (767.3 ± 114.2) or GKT (664.3 ± 114.2) showed a general tendency for decrease without any significance in reduction when compared to the control/depressed mice (764.9 ± 114.2) (Figure 14A).

Also, within the prefrontal lobes of diabetic/depressed mice (1904 ± 92.64), either specific Nox4 inhibition (376.2 ± 92.64) or selective serotonin reuptake inhibition (527.8 ± 92.64) reduced NADPH-dependent ROS production significantly. Similar to the findings observed in the hippocampus, control/depressed mice treated with either escitalopram (869.1 ± 92.64) or GKT (582.7 ± 92.64) showed a general tendency for decrease with no significant reduction in comparison to the control/depressed (945.5 ± 92.64) (Figure 14B).

As for the PNS, either escitalopram (694.8 ± 185.3) or GKT (929.9 ± 185.3) reduced NADPH-dependent ROS levels of diabetic/depressed mice (2358 ± 185.3) (Figure 14C). Similarly, either escitalopram (581.7 ± 185.3) or GKT (529.1 ± 185.3) caused a significant reduction in ROS levels utilizing the specific NADPH oxidase pathway in control/depressed mice.

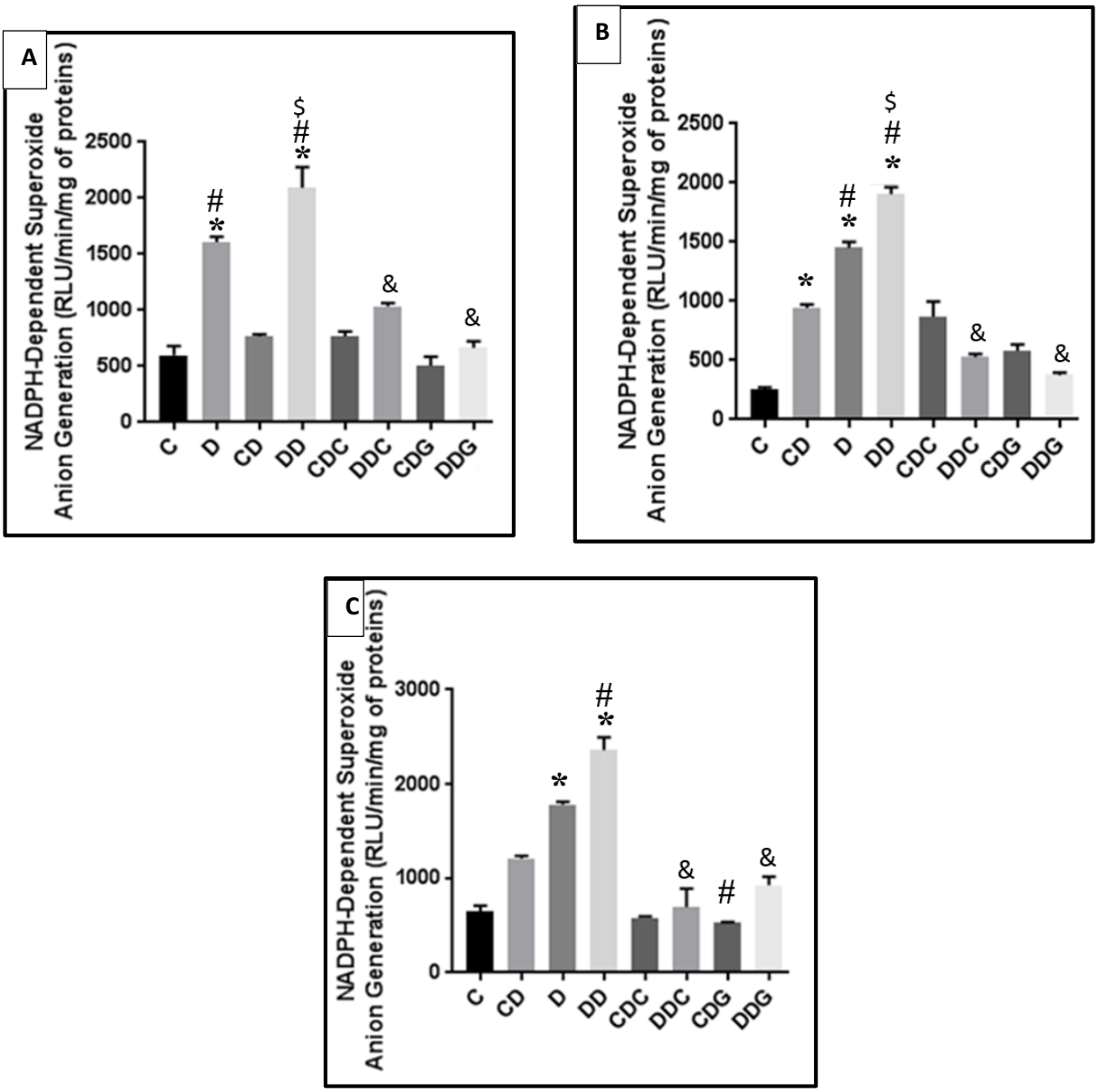

Figure 14: Escitalopram or GKT reduce oxidative stress in the hippocampi, prefrontal lobes, and sciatic nerves of diabetic/depressed animals. The superoxide anion generation was assessed in the hippocampi (A), prefrontal lobes (B), and sciatic nerves (C) of all groups. Values are the mean ± SD. The number of animals in each group is divided accordingly: control (C) (n=3), control/depressed (CD) (n=4), diabetic (D) (n=5), diabetic/depressed (DD) (n=4), control/depressed/escitalopram (CDC) (n=5), diabetic/depressed/escitalopram (DDC) (n=5), control/depressed/GKT (CDG) (n=5), diabetic/depressed/GKT (DDG) (n=5). *$p<0.05$, comparison with respect to C. # $p<0.05$, comparison with respect to CD. $ $p<0.05$, comparison with respect to D. & $p<0.05$, comparison with respect to DD

K. GKT, or Escitalopram ameliorate motor coordination in diabetes paired with depression

Using the raised beam walking test, our results showed that control/depressed mice treated with escitalopram (5.96 ± 0.38) showed a significant increase in the average speed calculated when compared to the control/depressed batch (3.51 ± 0.36) (Figure 15A). Diabetic/depressed mice treated with either escitalopram (5.62 ± 0.41) or GKT (5.43 ± 0.36) demonstrated a significant increase in their locomotion pattern in comparison the diabetic/depressed group (1.38 ± 0.34) (Figure 15B). With regard to the number of foot slips, either escitalopram or GKT caused a significant decrease in the control/depressed mice (Figure 15C). Similarly, a noteworthy reduction was also observed in the number of foot slips of diabetic/depressed mice treated with either escitalopram (0.25 ± 0.69) or GKT (1.6 ± 0.65) in comparison to the diabetic/depressed group (8.25 ± 0.65) (Figure 15D).

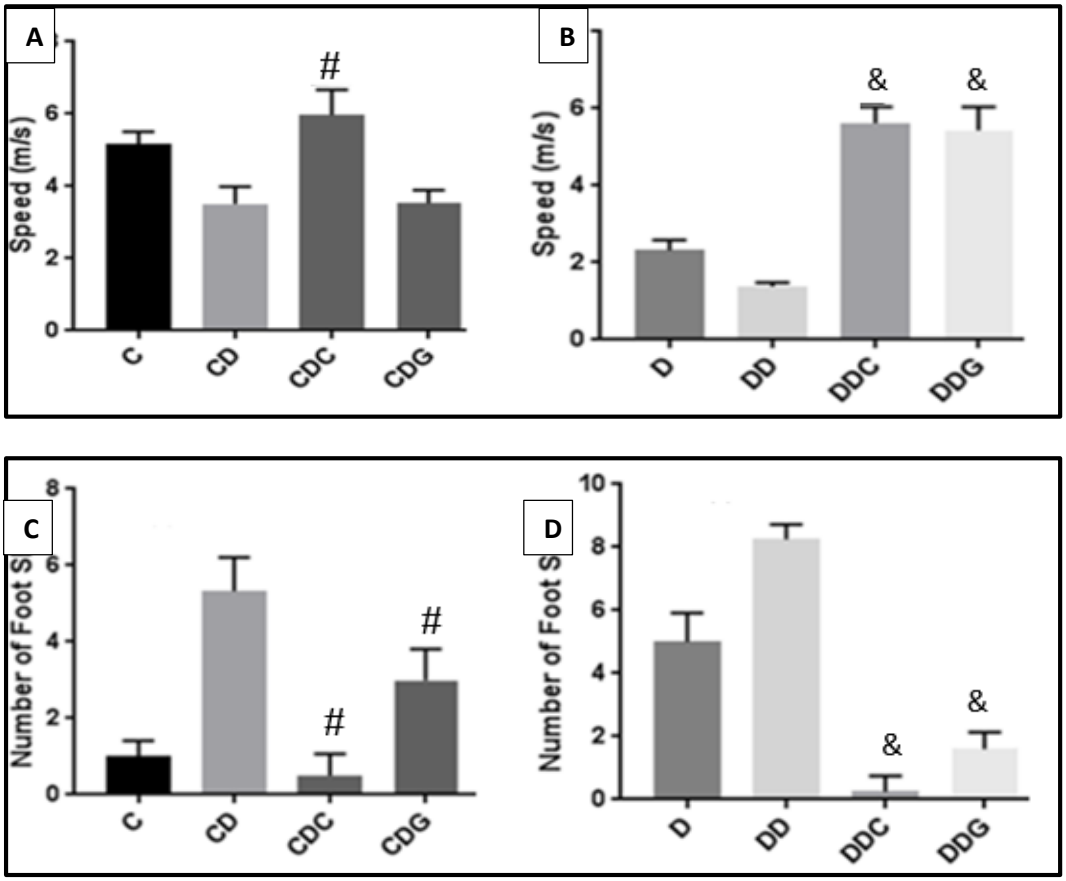

Figure 15: Improvement of motor coordination with GKT, or Escitalopram. Barograms representing the average speed (A&B), and number of foot slips (C&D) of all eight groups. Motor coordination was assessed by the raised beam-walking test. Values are the mean ± SD. Values are the mean ± SD. The number of animals in each group is divided accordingly: control (C) (n=3), control/depressed (CD) (n=4), diabetic (D) (n=5), diabetic/depressed (DD) (n=4), control/depressed/escitalopram (CDC) (n=5), diabetic/depressed/escitalopram (DDC) (n=5), control/depressed/GKT (CDG) (n=5), diabetic/depressed/GKT (DDG) (n=5). # $p<0.05$, comparison with respect to CD. & $p<0.05$, comparison with respect to DD

L. Effect of Nox4 inhibitor, and an SSRI on peripheral myelin alterations

To confirm the improvement observed in the raised beam walking test in the treated mice, we studied the effect of GKT, and escitalopram at the molecular level**.** Our results show that the PMP22 protein expression was notably downregulated in the diabetic/depressed mice treated with each of these drugs individually (Figure 16A). Quantifying these results showed a significant downregulation in the expression of

PMP22 protein within the control/depressed animals treated with either escitalopram (39.45 ± 13.47) or GKT (51.78 ± 13.47) in comparison to the control/depressed group (111.2 ± 13.47). Furthermore, PMP22 protein expression was significantly downregulated in the diabetic/depressed animals treated with either escitalopram (42.99 ± 13.47) or GKT (37.53 ± 13.47) in comparison to the diabetic/depressed group (110.2 ± 13.47) (Figure 16B).

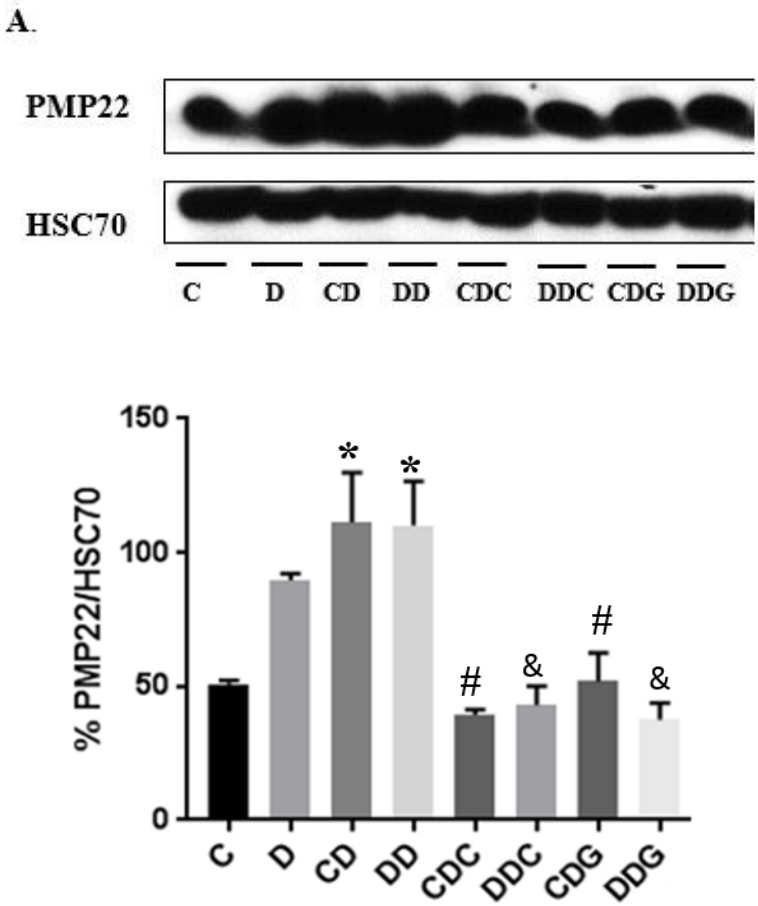

Figure 16: Effect of Nox1/Nox4 inhibitor or SSRI on peripheral myelin alterations. A. Representative PMP22 western blot images. HSC70 was used as the loading control. B. Quantitative results of PMP22 in the sciatic nerves of all 8 groups. * $p<0.05$, comparison with respect to C. # $p<0.05$, comparison with respect to CD. & $p<0.05$, comparison with respect to DD.

CHAPTER IV

DISCUSSION

Characterized by chronic hyperglycemia, diabetes has been implicated in fundamental structural and functional brain alterations. Depression, one of the chronic complications of diabetes, is greatly prevalent in diagnosed diabetic patients (Bădescu, S et al, 2016; Moulton et al, 2015). Consequently, a bidirectional relationship exists between diabetes and depression, where each disorder exacerbates the conditions of the other (Ho, N et al, 2013; Semenkovich, K et al, 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, their role in the context of diabetic peripheral neuropathy and myelin injury is still unclear.

Diabetes has been implicated in producing a depressive-like behavior in both humans and animal models of diabetes (Bădescu S. et al, 2016; Kleinridders et al, 2015). Investigating whether diabetes induces depressive-like symptoms and pathophysiology, we performed a panel of behavioral tests on the mice described in Table2. Our results from the TST show that the immobility time of control/depressed, diabetic and diabetic/depressed mice was significantly greater than that of their control littermates. Similar results were recorded using the FST. Interestingly, the TST demonstrated a significant increase in the immobility time of diabetic/depressed mice compared to that of control/depressed. Taking these data together, using non-obese type 2 diabetic mice, we demonstrated that depressive-like symptoms can be a direct result of diabetes, where diabetic mice exhibited emotional despair assessed by both tail

suspension and forced swim tests. These results are in sync with already published data by other researchers, showing signs of "hopelessness" and depressive-like behaviors in type 2 diabetic animals. According to Yu et al, type 2 diabetic patients recorded decreased sucrose preference, expressing their inability to experience pleasure from rewarding/enjoyable activities (2014). Moreover, Kleinridders et al confirmed that mice with a brain-specific knockout of insulin receptor remained immobile for longer periods of time assessed by TST and FST (2015).

Anhedonia, another major 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 greater than 65% is indicative of "pleasure" (Strekalova et al., 2011). The control group showed a sucrose preference value greater than 65%; reflecting the "pleasurable" state of mice. In contrast to the control group, control/depressed, diabetic/depressed as well as diabetic mice documented a sucrose consumption value less than 65%, presenting probable depressive-like symptoms.

The next objective was to determine the existence of any myelin alterations in diabetes-induced depression. As previously described, the myelin sheath, a stable protective membrane insulating axons, enhances rapid transmission of electrical impulses. Lipids constitute 70% of the myelin, whereas proteins account for 30% (Edgard & Sibille, 2012). The regulation of myelin protein expression should be under exquisite control to ensure myelin stability and adequate saltatory conduction. A large number of studies have validated the detrimental effects of depression on overall brain activity reduction (Cerullo et al., 2014; Dusi et al., 2015). Impaired myelination has been recently associated with diabetes and depression (de Hoz and Simons, 2014;

Haroutunian et al, 2014). However, its role in diabetes-induced depression is not yet defined. Thus, we investigated the mRNA expressions of two myelin genes: PLP and MBP, which are responsible for the myelination process in the CNS. Our data indicate that MBP mRNA levels in both the hippocampi and prefrontal lobes of diabetic and diabetic/depressed mice are significantly greater than those of control FVB mice. Furthermore, MBP mRNA levels in diabetic/depressed mice are significantly higher than those of either control/depressed or diabetic mice alone. Similarly, both diabetic and diabetic/depressed mice show a remarkable increase in PLP mRNA levels within these two brain areas in comparison to the control. Even though PLP mRNA levels of diabetic/depressed mice are much higher than those of the control/depressed within the two aforementioned brain regions, our results show that compared to diabetic mice, PLP upregulation of the diabetic/depressed group is solely confined to the prefrontal lobe. Consequently, comorbid diabetes and depression display a greater dysregulation in central myelin gene expression. In line with our observations, alterations in central myelin proteins were recorded in diabetes and depression. Kawashima et al (2007) and Pesaresi et al (2010) reported alterations in MBP mRNA levels within the spinal cords and brains of streptozotocin-treated diabetic rats, respectively. According to Rajkowska et al (2015), and Fuchsova et al (2015), decreased PLP mRNA expression was detected in the ventral prefrontal cortices and hippocampi of patients with MDD. Moreover, Fernandez et al showed altered central myelin mRNA levels in the hippocampi of chronically stressed rodents (2010).

Concerning the peripheral nervous system, diabetes has been implicated in peripheral nerve injury, which in turn elicits depressive-like behaviors in human and animal models of diabetes (Gui et al, 2016; Murad et al, 2015; D'Amato et al, 2016).

Results from our groups and others show that diabetes induces peripheral nerve injury by affecting the myelin integrity of the sciatic nerve. Yet results correlating the depressive-like state to peripheral injury are limited. Also, studies describing the effect of depression combined with diabetes on further exacerbating peripheral injury are non-existent. For that, we assessed myelin injury in the sciatic nerves of all eight groups. Our results show that depression alone, as well as depression coupled with diabetes, upregulates the expression of PMP22, one of the crucial myelin proteins of the PNS. In addition, the raised beam walking test results are indicative of motor behavior injury, further supporting the previous observation. Both diabetic and diabetic/depressed mice exhibit a significant slower pattern of movement as opposed to their control littermates. Moreover, both control/depressed and diabetic/depressed mice display a greater number of foot faults compared to the control FVB mice. Interestingly, compared to control and control/depressed mice, both diabetic and diabetic/depressed mice represent a greater number of foot slips. Moreover, there seems to be an increasing trend between the control/depressed and control mice, where the former showed a greater number of foot slips in comparison to the control. Similarly, the control/depressed, diabetic and diabetic/depressed mice record a significantly greater number of foot stops when compared to the control group. Furthermore, the diabetic group experience more foot slips and stops compared to the control/depressed. These findings reflect prominent deficits in fine motor coordination. As previously established by our group and others, whether type 1 or 2, DM has been shown to alter sensorimotor coordination and to dysregulate peripheral myelin protein (Hao et al 2015; Yu et al, 2008; Lee et al, 2009, Askwith et al, 2009).

Investigating the possible involvement of NADPH-induced superoxide generation in diabetes-induced-depressive-like behaviors has also been conducted in this study. Aberrant ROS production has been shown to participate in one of the underlying mechanisms leading to the onset of depression and of diabetic complications including DPN (Baynes et al, 1991; Maes et, 2011; Korczak et al, 2011). Addiotnally, contribution of augmented ROS production by the NADPH oxidase family of enzymes to oxidative stress and disease progression is becoming increasingly evident. NADPH oxidases are largely implicated in diabetic complications (Eid et al, 2009; Zhao et al, 2015; Seo et al, 2012; Walton et al, 2013). However, the role of NADPH oxidases in depression or depressive-like behaviors manifested in diabetes is yet to be described. For the scope of this study, Nox1 and Nox4 were assessed in the two previously mentioned fundamental brain regions: hippocampus and prefrontal lobe. Our results show that Nox1 is significantly upregulated in both the hippocampi and prefrontal lobes of diabetic/depressed and diabetic mice compared to that of the control. Moreover, Nox1 is significantly upregulated in the diabetic/depressed mice in comparison to the control, control/depressed and diabetic mice. Similarly, the mRNA levels of Nox4 of both diabetic and diabetic/depressed groups are markedly greater than those of the control FVB mice. Moreover, in both areas of the brain, Nox4 is greatly upregulated in the diabetic/depressed mice as opposed to the diabetic group. Even though Nox4 mRNA levels of the diabetic/depressed mice within the prefrontal lobe are greater than those of the control/depressed, no such significance was recorded in the hippocampus. In parallel, our results show an immense increase in ROS production in both the hippocampi and prefrontal lobes of diabetic/depressed as well as diabetic mice compared to the control. Assessed by the NADPH oxidase activity, which indirectly

measures NADPH-dependent superoxide production, ROS levels of diabetic/depressed mice within the hippocampus are profoundly greater than those produced by the control/depressed batch. Surprisingly, NADPH-induced ROS production is much higher in diabetic mice as opposed to the diabetic/depressed. These data suggest that the depressive like behavior seen in the diabetic animal can be due to an increase in NADPH-oxidases dependent ROS production. Réus et al further supported these findings, indicating increased oxidative damage in the prefrontal cortex, hippocampus, amygdala, and striatum of diabetic rats (2015). While, Che et al reported elevated oxidative stress in the hippocampi of depressed patients (2010). Furthermore, Seo et al elucidated that chronic stress in mice promotes depressive behavior via an increase in NADPH oxidase activity (2012). It is worth noting that, ROS has been implicated in neuronal and glial apoptosis, in addition to disrupting the blood-brain barrier (BBB), producing further damage to the brain (N. Han et al, 2008; M. Katsu et al, 2010).

Similarly, a marked increase in NADPH-dependent superoxide generation was recorded in the sciatic nerves of control/depressed, diabetic, and diabetic/depressed mice compared to the control batch. In addition, NADPH oxidase activity was significantly greater in the diabetic group compared to the control/depressed. In parallel, inducing depression in the diabetic group further increased NADPH-dependent superoxide generation compared to that of both the diabetic and control/depressed groups. Taken together, our results suggest that comorbid diabetes and depression induce ROS overproduction via the NADPH oxidase family of enzymes, possibly leading to central and peripheral myelin alterations.

On another note, owing to their safety profile and their efficacy, SSRIs are of the most commonly prescribed antidepressants worldwide. They have been

recommended for depressed patients with diabetes due to their hypoglycemic and antidepressant properties (Markowitz et al, 2011; Deuschle, 2013). Additionally, SSRIs are better tolerated due to their lack of anticholinergic, antihistaminic, and cardiac adverse effects (Baptista-de-Souza et al, 2014). Among a wide range of SSRIs readily available in the marketplace, (escitalopram), an antidepressant approved by the U.S. Food and Drug Administration (FDA), was employed in this study.

After ensuring the presence of depressive-like characteristics in both control/depressed and diabetic/depressed groups, all mice were subjected to a ten-week treatment process where GKT, and escitalopram were administered orally three times a week. Prior to sacrifice, the same set of behavioral tests were conducted to investigate the efficacy of these drugs in reversing depressive symptoms. After ten weeks of treatment, TST and FST revealed that both control/depressed and diabetic/depressed mice were immobile for significantly longer periods of time in comparison to control FVB mice, reflecting their continuous depressive states. Intriguingly, diabetic/depressed mice were immobile for significantly longer periods of time as opposed to the control/depressed group. Moreover, the immobility time was significantly greater in the diabetic mice as opposed to both the control and control/depressed groups. Both tests recorded a significant reduction in the immobility time of both control/depressed and diabetic/depressed mice treated with an SSRI antidepressant (escitalopram) compared to the diabetic/depressed batch. Even though GKT significantly reduced the immobility time of the treated diabetic/depressed mice in both tests, it showed no significant reduction in the control/depressed group. Interestingly, both drugs showed a significant reversal of the depressive-like states of the treated groups, with GKT to a lesser extent.

To evaluate the anhedonic behaviors of all 'depressed' mice and confirm the effectiveness of escitalopram and GKT, SPT was performed. Both control/depressed and diabetic/depressed mice showed a significant reduction in sucrose consumption compared to the control and diabetic groups, respectively. In addition, both control/depressed and diabetic/depressed groups treated with escitalopram and GKT showed a significant increase in sucrose consumption as opposed to the control/depressed and diabetic/depressed mice, respectively. Therefore, this reduction in sucrose preference can be gradually reversed by either chronic antidepressant treatment or specific Nox4 inhibition.

Even though the effect of GKT hasn't been studied in the context of DPN, it has shown promising results in both chronic obstructive pulmonary disease (COPD) and diabetic nephropathy. Not only was GKT implicated in ameliorating pulmonary injury accompanying COPD (Hollins et al, 2016), but also reduced albumin excretion in urine, mesangial matrix expansion, podocyte loss, and glomerular hypertrophy (Gorin et al, 2015). After passing phase I clinical trials, this drug is currently being used to treat patients suffering from diabetic nephropathy.

We then examined the potential effects of an SSRI antidepressant, and a specific Nox4 inhibitor on *PLP* and *MBP* mRNA levels in both the hippocampi and prefrontal lobes of the 8 groups of mice. As previously noted, control/depressed, diabetic and diabetic/depressed mice expressed higher MBP mRNA levels compared to control FVB mice. Treatment with either escitalopram or GKT significantly reduced the elevated MBP mRNA levels of the control/depressed and diabetic/depressed groups in the two designated brain regions. Furthermore, both diabetic and diabetic/depressed mice show a remarkable increase in the PLP mRNA levels within these two brain areas

in comparison to the control. However, only escitalopram caused a marked decrease in the PLP mRNA levels of diabetic/depressed mice, rendering GKT ineffective. Therefore, chronic selective serotonin reuptake inhibition, via treating depressive-like symptoms in diabetic subjects, may also reverse central myelin protein dysregulation. Similar findings were also reported by Kroeze et al. (2016).

To further investigate the efficiency of GKT, and escitalopram on PNS injury, we utilized the beam walking to assess fine motor coordination. Diabetic/depressed mice were much slower and took a significantly longer time to cross the beam in comparison to diabetic mice alone. Upon treatment with both drugs, both diabetic and diabetic/depressed mice demonstrated significant faster patterns of movement. Even though the control/depressed group took much longer to cross the beam when compared to the control, reversing depressive-like symptoms via escitalopram administration minimized total time immensely. Interestingly, unlike GKT, both escitalopram accelerated the locomotion of control/depressed animals. In addition, control/depressed alongside diabetic/depressed mice recorded a greater number of foot slips compared to both control and diabetic groups respectively. Despite the minimal efficacy of GKT in reducing the number of foot slips of control/depressed mice, escitalopram profoundly diminished the number of foot slips, ameliorating locomotor coordination (PRINSSEN et al., 2006).

To confirm the improvement observed in the raised beam walking test in the treated mice, we studied the effect of GKT, and escitalopram at the molecular level**.** Our results show that the PMP22 protein expression was notably downregulated in the diabetic/depressed mice treated with each of these drugs individually (Figure 14). Similarly, escitalopram and GKT downregulated the expression of PMP22 protein

within the control/depressed animals. Taking the behavioral and molecular analyses together, these findings could implicate an important role for SSRI and Nox4 inhibitors in reversing peripheral myelin injury in depression alone, as well as in diabetes coupled with depression.

The current findings present the possibility of an SSRI-mediated reversal of peripheral myelin alterations. These observed central and peripheral myelin improvements could be explained by a reduced NADPH-induced ROS generation. Our results showed that both diabetic and diabetic/depressed mice exhibited a significant decrease in NADPH-induced ROS generation within their hippocampi and prefrontal lobes. However, compared to the control/depressed animals, only GKT reduced central NADPH-dependent ROS production. As for the PNS, the two drugs reduced NADPH-dependent superoxide anion levels of diabetic/depressed mice. Similarly, escitalopram and GKT successfully reduced superoxide anion generation utilizing the specific NADPH oxidase pathway in control/depressed mice.

To conclude, we provide through this work evidence of a causal link between diabetes and depression with manifestations of nerve dysfunction in both the CNS and PNS. Central and peripheral myelin alterations triggered by the dual effect of hyperglycemia and depression was shown to be dependent on oxidative status disruption through an NADPH-dependent mechanism, which was shown to be restored upon the targeted blockade of Nox isoforms and SSRIs. The following study thus lends support to the significance of approaching diabetes and depression from many angles, and not from a standard one-sided therapy.

A. Limitations of the Study

This study doesn't exclude itself from limitations. Since time was a limiting factor, no histological techniques were performed in this study. This would've 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. Including other experiments, such as the nerve conduction velocity test and the hind paw withdrawal test, for the assessment of nerve damage and nociception respectively, would've further complimented our results. Screening for the combined therapeutic effect of SSRI and GKT was missing. Having limited number of mice per condition provided us with restricted amount of tissue where additional bioanalytical studies couldn't be carried out. Even though we managed to better the already-existing depression model by introducing various chronic stressors, other behavioral studies including the open field test, the elevated plus maze, and the dark/light box were lacking. Such tests further assess anxiolytic-like behaviors in these animals since anxiety is highly associated with depression.

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