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DIABETES AND DEPRESSION: UNVEILING A POTENTIAL BIOLOGICAL LINK

by
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"Cultivate the habit of being grateful for every good thing that comes to you, and to give thanks continuously. And because all things have contributed to your advancement, you should include all things in your gratitude." - Ralph Waldo Emerson

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AN ABSTRACT OF THE THESIS OF

Rasha Khalil Barakat for Master of Science
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Title: Diabetes and Depression: Unveiling A Potential Biological Link.

Background: Depression and diabetes are prevalent diseases whose incidence rates are increasing worldwide. Epidemiological studies provide strong evidence that subjects with diabetes are at significantly higher risk of developing depression. Shared biological vulnerabilities may be involved in the comorbidity of depression and diabetes. However, the common functional and molecular mechanisms between the two disorders remain unknown. Reactive oxygen species (ROS) have been shown to be increased in both disorders. However, the sources and the mechanisms by which ROS lead to peripheral and central nervous system injury need to be elucidated.

Aim: The aim of this study is to determine the role of NADPH-induced ROS in the onset of depression and diabetes. More importantly this project will evaluate the effect of depression, on the alteration of the NADPH oxidases pathway and its effect on the onset and development of diabetic complications, specifically neuropathy using functional, behavioral, structural, and molecular testing.

Methods: A chronic stress procedure was used to induce depression in the control or non-obese type 2 diabetic mice. Sucrose test, tail suspension test, and forced swim test were performed to assess depression in mice. Raised beam walking test was used to assess behavioral malfunction in diabetic and depressed animals. RT-PCR allowed the measurement of mRNA levels of Nox1, PLP, and MBP. Western blots were used to assess the protein expression levels of Nox1 and myelin proteins. NADPH oxidase activity was used to measure the activation of the Nox enzymes and as a measurement of superoxide anion production.

Results: Behavioral assessment of the animals shows a depressed like behavior in the diabetic animals resembling that of the experimentally induced depression in the control animals. Interestingly, the experimentally depressed diabetic mice showed an increase in the severity of depression. These observations were paralleled by an increase in the NADPH-dependent superoxide production concomitant with a dysregulation in the myelination process of the central and peripheral nervous system. Results show that in the diabetic, depressed, as well as the diabetic rendered experimentally depressed the myelin proteins PLP and MBP levels were altered as well as the PMP22, one of the major myelin proteins of the peripheral nervous system. Treatment with GKT, a specific Nox1 and Nox4 inhibitor, decreased ROS production, restored the expression of myelin protein to approximately baseline levels in the brain and sciatic nerve and reversed significantly, the depressive-like behavior as well as sensorimotor dysfunction.

Conclusion: This study indicates that NADPH oxidases-induced ROS production might be a major player in the central and peripheral myelin injury inducing a depressive-like behavior in diabetes and causing an alteration in the sensorimotor function.

CONTENTS

FIGURES

TABLES

ABBREVIATIONS

CNP:	2’,3’-cyclic nucleotide-3’-phosphodiesterase
CNS:	Central Nervous System
DPN:	Diabetic Peripheral Neuropathy
DSM:	Diagnostic and Statistical Manual of Mental Disorders
FST:	Forced swim test
HPA:	Hypothalamus-Adrenal-Pituitary
MAG:	Myelin-Associated Glycoprotein
MBP:	Myelin Basic Protein
MDD:	Major Depressive Disorder
MOBP:	Myelin-associated Oligodendrocytic Basic Protein
MOG:	Myelin Oligodendrocyte Glycoprotein
MPZ or P0:	Myelin Protein Zero
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
Nox family:	NADPH Oxidase Family
P2:	Myelin Protein 2
PLP:	Proteolipid Protein
PMP22:	Peripheral Myelin Protein 22
PNS:	Peripheral Nervous system
ROS:	Reactive Oxygen Species
TST:	Tail Suspension Test

CHAPTER I

INTRODUCTION

Diabetes Mellitus is a chronic disease affecting 9.3% of the population worldwide. It is projected to be the 7th leading cause of death by 2030. This disease is classified into two types: type I and type II. Type I diabetes is characterized by the destruction of pancreatic β-cells. It affects 5-10% of the diabetic population. Type II diabetes is the most common form of the disease with 90-95% prevalence with insulin resistance being its main feature. Obesity and poor lifestyle are considered as the two major factors contributing to the onset of type II diabetes. Like any other disorder, diabetes is accompanied by micro- and macro-vascular complications. Among the the microvascular complications, neuropathy is the most common affecting 50% of diabetic patients (American Diabetes Association, 2010; Shakeel et al, 2015).

Increased clinical data reported that depression among other mood disorders can be more commonly seen in diabetic patients compared to patients suffering from other chronic disorders. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), depression is characterized by irritability or depressed mood, loss of interest or pleasure, sleep disturbance, loss of energy and fatigue, significant weight gain or loss, and change in activity and suicidal thoughts. A growing body of research suggests that the presence of depression is significantly higher in diabetic patients. Emerging evidence describes a potential association between diabetes and depression (Rustad et al, 2011; Campayo et al, 2011; Bissels et al, 1994; Korczak et al, 2011; Moulton et al, 2015; Holt et al, 2014). In the same spirit, epidemiological studies show that the prevalence of depression is two times higher in type II patients with 19.1% versus 10.7% in

the control group. The prevalence rate is three times higher in type I patients with a 12% incidence versus 3.2% for the non diabetic group (Roy et al, 2012).

The lack of basic tools and clinical tools is a limiting factor when it comes to correlating diabetes and depression. The chronic aspect of diabetic complications and their psychological burden may be the source of major stress for the patient. They include sharp pain, abnormal thermal sensations, undergoing dialysis, and a poor lifestyle among many others. However, this explanation is not enough to demonstrate the relationship between these two conditions. Although depression is considered as a symptom of diabetes, we believe that there is a biological link underlying both disorders and that their comorbidity puts the diabetic depressed patient at a higher risk of developing complications. In this study, we try to shed the light on the biological crosstalk underlying the mechanisms by which diabetes induces depression and causes more severe complications.

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

1. Diabetes and Depression: Shared Mechanisms

A growing body of evidence supports that comorbid diabetes and depression share biological vulnerabilities. Several immunological and inflammatory mediators have been involved in the pathogenesis of both diabetes and depression (Korczak et al, 2011; Moulton et al, 2015). Studies have shown an increased production of pro-inflammatory cytokines such as IL-4, IL-6, and TNF-α in diabetes and in depression (Foss-Freitas et al, 2008; Maes et al, 1999; Cyranowski et al, 2007). Elevated markers of oxidative stress such as lipid peroxidation, nitrite/nitrate overproduction, and reduced antioxidative mechanisms were present in both disorders (Martin-Gallan et al, 2007; Mylona-Karayanni et al, 2006; Khanzode et al, 2003).

Other evidence suggests that a dysregulation of the Hypothalamus-Pituitary-Adrenal cortex (HPA) axis is associated with both diabetes and depression (Moulton et al, 2015b). Elevated levels of cortisol, the hallmark of HPA axis dysregulation, are present in diabetes and depression (Pariante, 2008; Lebinger et al, 1983). Moreover, functional and structural abnormalities in the brain of diabetic and depressed patients are similar. They include a decrease in the volume of the hippocampus and cerebral atrophies (McIntyre et al, 2007, Sheline et al, 1996; Lunetta et al, 1994, Lloyd et al, 2004, Jongen et al, 2007). These shared mechanisms cause injruy to the brain of depressed and diabetic patients.

2. Myelination in the CNS

The CNS is composed of two types of cells: neurons and glia. In the brain, the ratio of neurons to glia is approximately 4:1 but in the cerebellum the neurons outnumber the glial cells. The latter group of cells is divided into three subtypes: oligodenrocytes, astrocytes, and microglia. The oligodrendrocytes are responsible for myelination in the CNS. The myelin sheath creates an insulating layer covering the axons in order to allow a fast propagation of the action potential in specific regions called “Nodes of Ranvier”. This fast propagation or conduction is known as “saltatory conduction” and depends largely on the development of compact, well-organized, and resistant myelin sheaths around the axons. This sheath is rich in lipids (70%) and proteins (30%) (Edgard & Sibille, 2012). The myelinating oligodendrocytes express proteins such as the myelin basic protein (MBP) and proteolipid protein (PLP), myelin-associated glycoprotein (MAG), 2’,3’-cyclic nucleotide-3’-phosphodiesterase (CNP), myelin olidendrocyte glycoprotein (MOG), myelin-associated oligodendrocytic basic protein (MOBP), and myelin

protein 2 (P2). These proteins work in synergy to guarantee the compaction and the maintenance of the myelin sheath. The study of the level of expression of myelin proteins present in the oligodendrocytes is one of the major tools that assess myelin injury in the CNS.

MBP is one of the most abundant proteins of the CNS with a high positive charge and several isoforms (Han et al, 2013; Muller et al, 2013; Edgar & Sibille, 2012). It is referred to as the "executive myelin protein" (Boggs et al, 2006) because mice and rats that lack a functional isoform have shivering symptoms, hypomyelination in the CNS, and die prematurely (Kwiecien et al, 1998; Readhead and Hood, 1990). MBP arises from the Golli (genes of oligodendrocyte lineage) gene complex. MBP is one the main proteins related to the etiology of multiple sclerosis (Tzakos et al, 2005). Different splicing will result in different MBP isoforms: 14, 17.22, 17.24, 18.5, 20.2, and 21.5 kDa with 18.5 being the major adult isoform. MBP plays a role in proliferation and process extension, signaling pathways (Fyn-SH3), myelin compaction and sieving, cytoskeleton assembly and turnover, phosphorylation and Ca-calmodulin binding.

PLP is found in two isoforms in the brain: DM20 the short isoform lacking 35 residues, and PLP/DM20 which represents 17-45% of the total protein amount present in the CNS myelin. PLP is also found in a smaller proportion in the PNS. The role of PLP has not yet been fully elucidated. It is known that PLP is mandatory for the physical stability of the myelin sheath, proper assembly of the myelin, conduction of nerve impulses in the brain and spinal cord, oligodendrocytic membrane adhesion, and formation of the myelin intraperiod line. Mutations in PLP can cause mental and physical retardation such as spastic paraplegia type 2 and the Pelizaeus-Merzbacher disease (Han et al, 2013, Edgar & Sibille, 2012).

3. Myelin Injury in the CNS

Several studies have reported demyelination and myelin injury mediated by depression and diabetes in the brain. However, the etiology of myelin injury in both disorders is still unknown. It has been reported that type 2 diabetic patients have larger white matter lesion and lateral ventricle volumes, and smaller grey matter volumes than non-diabetic patients (Jongen et al, 2007). Since the volume of the white matter is not affected, this suggests that there are cortical and sub-cortical atrophies in the brains of the patients. Jongen et al also mentioned a sex linked difference in the significance of the results; a separate analysis of the results demonstrated larger cerebrospinal fluid and larger lateral ventrical volumes, and smaller total brain and grey matter volumes in female diabetic patients compared to male diabetic patients. Diffusion tensor imaging studies have revealed a pathology in the white matter of depressed patients (Rajkowska et al, 2015; Liu et al, 2016; Tham et al, 2011). A reduction in the expression of oligodendrocyte and myelin genes is present in the ventral and lateral prefrontal cortex, and in the pregenual anterior cingulate. Oligodendrocyte density was also reduced in the amygdala of patients with major depressive disorder (MDD) (Bowley et al, 2002; Hamidi et al, 2004). Rajkowska & Miguel-Hidalgo suggest that a decreased gliogenesis in the hippocampus is associated with depression (2007).

B. Diabetes in the PNS

One of the major forms of injury affecting the PNS is due to myelin dysregulation.

1. Myelination in the PNS

Schwann cells are the glial cells of the PNS. They are responsible for the myelination of the axons and for the rapid saltatory conduction. Myelination of the axons is needed for a proper functioning of the PNS. Glycoproteins account for 60% of the proteins in the PNS. The remaining 40% include basic proteins (20-30%) which are incorporated in the membranes of the Schwann cells, and diverse proteins each of which contributing to less than 1% of the total protein count (Garbay et al., 2000). The PNS expresses some of the myelin proteins present in the CNS such as P2, MAG, MBP, and PLP. However, some proteins such as myelin protein zero (MPZ or P0) and peripheral myelin protein 22 (PMP22) are exclusively found in the PNS.

PMP22 is a 22 kDa glycosylated myelin protein. It accounts for 2-5% of myelin proteins in the PNS (Kitamura et al, 1976; Pareek et al, 1993). PMP22 is involved in the initiation of myelination, the determination of the myelin thickness and the stabilization of the myelin sheath (Adlkofer et al., 1995). It interacts with MPZ to ensure the maintenance and compaction of the myelin sheath. PMP22 is also involved in the development and proliferation of Schwann cells. In mice lacking PMP22, there is a delay in myelination resulting in immature Schwann cells that do not wrap the axons (Han et al, 2013, Garbay et al, 2000). Thus, an aberrant change in PMP22 expression levels is expected to have effects on cellular proliferation, myelination, and apoptosis. Over 50% of the individuals with Charcot-Marie-Tooth disease have a mutation in the PMP22 gene (Li et al, 2013). However, diabetes induced myelin injury is not yet fully described.

2. Myelin Injury in the PNS

Axonal atrophy and demyelination are suggested to be two of the major pathological manifestations of Diabetic Peripheral Neuropathy (DPN) (Yagihashi et al., 1990; King et al., 1989). However, the etiology of demyelination in DPN is still unclear. Emerging experimental literature points to a key role of Schwann cells in the pathogenesis of DPN. These myelinating cells are considered a primary target of hyperglycemia (Yu et al, 2014a; Taiana et al, 2014; Cinci et al, 2015), and when exposed chronically to high blood glucose levels, they undergo oxidative, metabolic and apoptotic changes. These in vitro studies corroborated with in vivo findings describing a reduced nerve conduction velocity, axonal atrophy, and impaired axonal regeneration in experimental animal models of diabetes (Dyck & Giannini, 1996). Yet, the mechanisms by which hyperglycemia results in Schwann cell injury remain unclear.

ROS generation, hypoxia, and hyperglycemia play a role in Schwann cell dysfunction (Eckersley, 2002). Diabetes also affects the Schwann cells by enhancing the degeneration of myelinated axons. Morphological changes such as basal lamina thickening, enlarged mitochondria, and accumulation of lipid droplets were observed in nerve biopsies of diabetic patients (Bischoff, 1979).

Peripheral nerve injury and neuropathic pain have been implicated in the onset of depression (Gui et al, 2016; Murad et al, 2015; D'Amato et al, 2016). However, depression mediated PNS injury has not been described yet. More importantly, the mechanisms underlying the biological relationship between diabetes and depression in inducing PNS injury are still unknown.

C. Diabetes, Depression, and Oxidative Stress

Hyperglycemia and depression are associated with increased cellular and systemic oxidative stress. ROS is considered to be a common pathway of injury leading to diabetes and depression.

Antioxidant drugs such as N-acetyl-L-cysteine, deferoxamine, and vitamin E seem to reduce oxidative stress along with depressive behavior and hyperglycemia in animals (DeMorais et al, 2014; Arent et al, 2012). Antioxidant treatment also prevents or slows the development of DPN in diabetic animals (Cameron et al, 1994; Coppey et al, 2003; Sayyed et al, 2006). This suggests a major pathogenic role of ROS in the pathology of depression and diabetic complications such as DPN.

Several mechanistic pathways are known to be involved in the contribution of ROS production in the CNS and PNS (Edwards et al, 2008; Korczak et al, 2011). Different sources of ROS are reported to be altered in diabetes. Recent studies indicate a major role for the NADPH oxidase (Nox) family as a source of ROS in the pathology of diabetic nephropathy, retinopathy, and cardiomyopathy (Eid et al., 2009; Kowluru & Chan, 2007; Zhao et al., 2015). However NADPH oxidase involvement in depression and DPN remains unknown.

The NADPH oxidases is a family of enzymes whose main role is to produce ROS. The NADPH oxidase family includes Nox1, Nox2, Nox3, Nox4 and Nox5, DUOX1, and DUOX2. These isoforms contain an FAD binding region near the COOH-terminus, a NADPH binding domain at the COOH-terminus of the oxidases, six transmembrane domains and four highly conserved heme-binding histidines located on the third and fifth transmembrane domains (Bedard & Krause, 2007). The expression of these molecules differs across tissues. In the

nervous system, Nox2 is the main isoform present. It is made of two membrane-bound subunits, gp91^{phox} (also known as Nox2) and p22^{phox}. The interaction between p67^{phox}, p47^{phox}, p40^{phox} and Rac leads to the production of ROS and oxidative stress (Brandes et al, 2014; Bedard and Krause, 2007; Touyz et al, 2011).

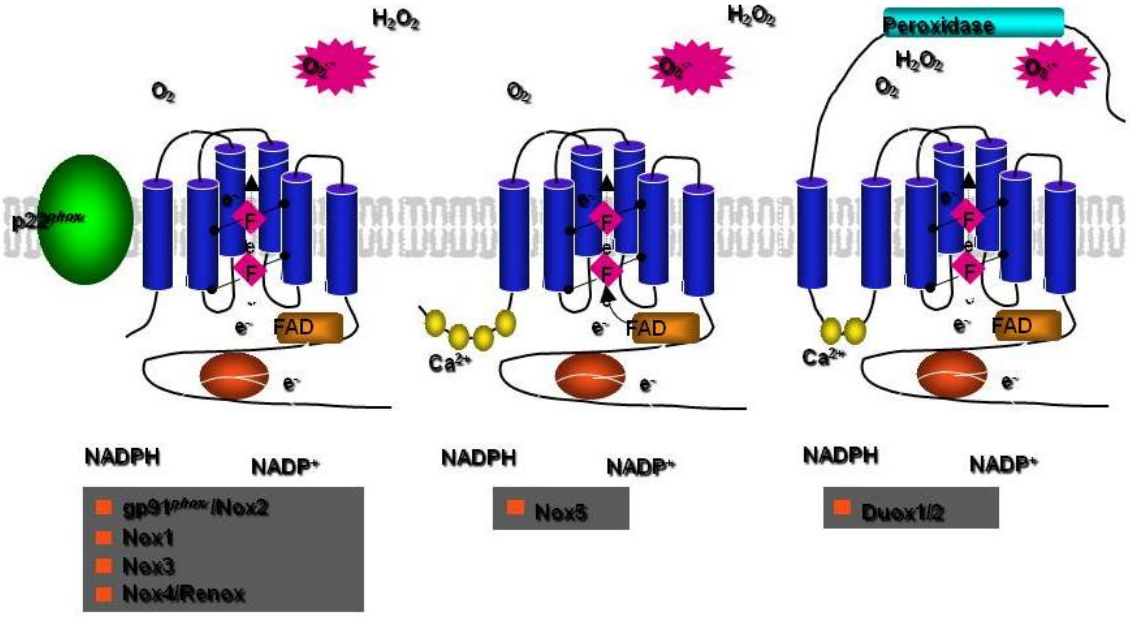


Figure 1. NADPH oxidase family of enzymes (Nassif et al, 2015)

D. NADPH and CNS Injury

Oxidative damage is thought to cause cognitive deficits such as memory, learning, and social interactions (Sato et al, 2010; Walton et al, 2013). Overproduction of ROS also causes neural structural damages (Uchihara et al, 2016). In depressed patients, higher levels of ROS markers were observed compared to the non-depressed population in areas such as the hippocampus (Che et al, 2010, Gupta et al, 2015). Animal models of diabetes show a blunted

oxidant defenses and increased activity of ROS producing enzymes in different areas of the brain. In addition, hyperglyceamia in the brain increases the levels of cytokines causing an increased generation of ROS (Pfeffer et al, 1994). In major depression and diabetes, antioxidant levels such as zinc, vitamin E, and coenzyme Q10 are lowered (Maes et al, 2011). This impairment is accompanied by increased production levels of ROS which cause DNA damage and increased lipid peroxidation. Results have shown that once oxidative stress is reduced, by blocking of NAPDH by apocyanin, or through increasing antioxidant levels such as N-acetyl-L-cysteine, the animals show reduced depressive symptoms and score better on behavioral tests (Seo et al, 2010; Arent et al, 2012). However, the role of specific Nox inhibitors and their involvement in myelin dysregulation are not yet studied.

E. NADPH and PNS Injury

NADPH-dependent ROS generation is upregulated in the dorsal root ganglion neurons in diabetic animals (Vincent et al, 2009). Increased lipid peroxidation, ROS production, and protein nitrosylation, combined with diminished levels of reduced glutathione and ascorbate (antioxidants) were all observed in dorsal root ganglia and peripheral nerves in animal models of type 1, type 2 and pre-diabetes. Antioxidant treatment of type 1diabetic animals with γ-linolenic acid, α-lipoic acid, and aldose reductase inhibitors blocks many of the indices of neuropathy (Fernyhough et al., 2010). Pharmacological inhibition of NADPH oxidase by diphenylene iodonium and apocyanin decreases ROS production and prevents the development of diabetic complication in vitro and in vivo (Cotter & Cameron, 2003; Sonta et al., 2004; Matsushima et al., 2009). Depression mediated PNS injury is not mentioned in the literature however depression

caused by neuropathic pain has been reported (Gui el al, 2016; Murad et al, 2015; D'Amato et al, 2016). Nox 1 and Nox 4 which are expressed in the CNS and PNS, were reported to cause injury in diseased states (Radermacher et al, 2013; Choi et al, 2014; Kallenborn-Gerhardt et al, 2012; Bedard & Krause, 2007). The role and function of the NADPH oxidase family in depression and DPN will be investigated in this study.

F. Hypothesis and Aim of the Study

Increased prevalence of comorbid diabetes and depression is supported by a growing body of research. Evidence supporting that diabetes and depression share biological mechanisms is growing. These origins stem from a dysregulation of the HPA axis, an overactivation of innate immunity and inflammation, and oxidative stress. They cause insulin resistance, atrophy of the hippocampus, β-cell apoptosis, circadian rhythm disturbance, endothelial dysfunction, and myelin injury. However, the mechanisms causing myelin injury in the nervous system in diabetes and depression are still uncharacterized.

Understanding the mechanisms by which diabetes and depression cause myelin injury in the body will improve the outcomes and provide a potential treatment of diabetes and depression simultaneously.

Our hypothesis states that both disorders, through oxidative stress produced by NADPH oxidase family of enzymes, contribute to the onset of myelin injury and that their comorbidity worsens and speeds up the progression of demyelination. This project will allow the study of the effect of GKT, a Nox1/Nox4 inhibitor, on the myelin proteins of the brain and sciatic nerve. From a fundamental perspective, it will allow the identification of novel molecular mechanisms

involved in depression and diabetes. From a clinical perspective, this study will pave the way for the examination of the potential of GKT in the treatment of diabetes and depression.

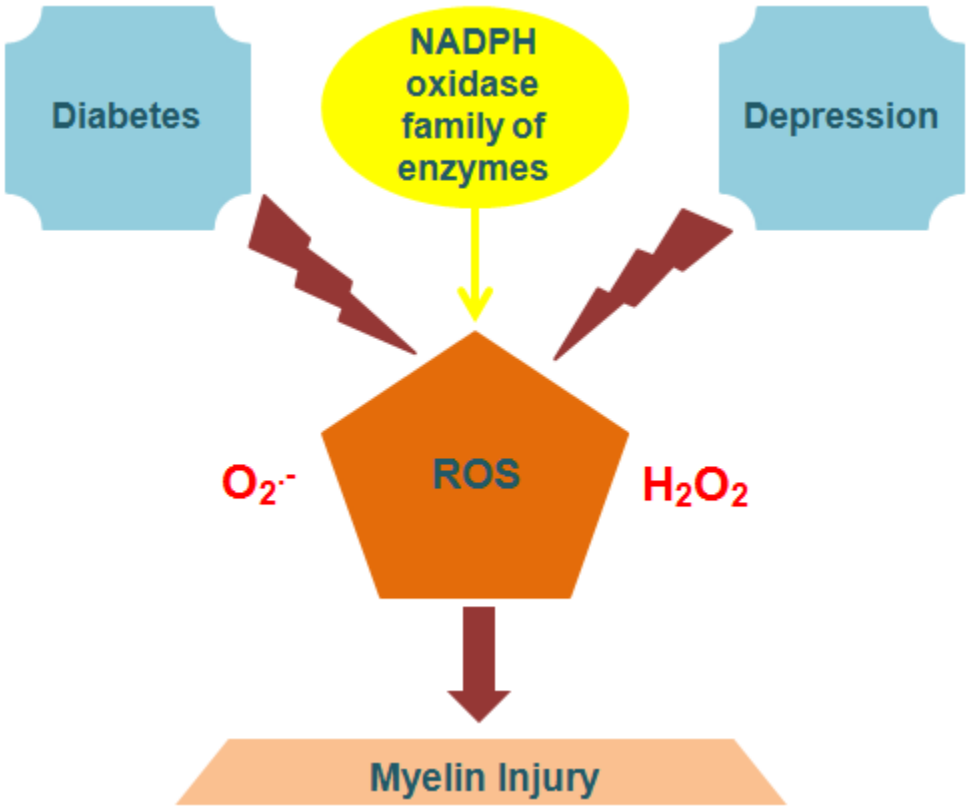


Figure 2. Proposed model of myelin injury in diabetes and depression

CHAPTER II

MATERIALS AND METHODS

A. Animal Models

Non-obese type 2 diabetic mouse model (MKR mice) was used for this study. They are transgenic mice with a dominant-negative insulin-like growth factor-I receptor (KR–IGF-IR) specifically targeted to the skeletal muscle leading to peripheral insulin resistance and impaired insulin action paralleled by an increase in blood glucose levels. Diabetes in these mice develops relatively at an early age rendering these mice an excellent model to study the molecular mechanisms underlying the development of human type 2 diabetes and its complications. FVB/NJ mice of similar age were used as a control strain for this animal model.

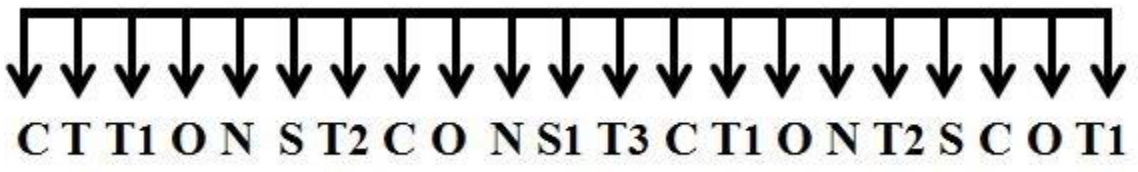
6 week-old mice were separated into 6 groups: control, diabetic, control depressed, diabetic depressed, control depressed treated with GKT #137833, and diabetic depressed treated with GKT #137833. The control depressed, diabetic depressed, control depressed with GKT, and diabetic depressed with GKT were subjected to the depression treatment for 6 weeks. GKT #137833 was dissolved in DMSO and administered 3 times a week intraperitoneally at a dose of 2.5 mg/kg.

All animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and had free food and water access. The Institutional Animal Care and Use Committee of the American University of Beirut approved all protocols. After the performance of the behavioral

studies, the animals were euthanized and the sciatic nerves and brains were extracted to study myelin injury. Biochemical analysis was done on the nerves and on two regions of the brain (prefrontal cortex and hippocampus) to assess the defects at the molecular level. These two regions of the brain were shown to display an abnormally low activity in PET scans in parallel to other areas of the brain of depressed patients (Videbech, 2000; Alves et al, 2006). Sciatic nerves were extracted to assess the molecular changes occurring in neuropathy.

B. Depression Treatment (Chronic Mild Stress Procedure)

The protocol followed in our study is the one mentioned by Kumar et al (2010) which is a modified version of the protocol mentioned by Molina et al. (1990) and Murua et al. (1991). Since the study dealt with diabetic mice, we substituted the food deprivation stress with a tail suspension stressor. The protocol was performed to induce depression and anxiety in animals. Different stressors were administered each day in order to maximize the unpredictable nature of the stress procedure. The stressors were performed between 9:00 AM and 2:00 PM for 6 weeks. The protocol included the following stressors in addition to a stress free day per week:



C-cold swim (12ºC, 5min); T-tail pinch (30s); T1-(tail suspension test, 5min), O-overnight illumination; N-no stress; S-swimming at room temperature (23±2ºC, 15min); T2-tail pinch (60s); S1 (23±2ºC, 10min); T3-tail pinch (90s)

Swimming

Mice were put in a transparent recipient filled with 15 cm of water. A water temperature of 23±2°C should be maintained for the 10 or 15 min swim test. For the 5 min cold swim test, the water temperature should be around 12 °C. The experimenter should be aware that the animal's limbs are not in contact with the bottom of the recipient.

For the assessment of depression: the forced swim test was performed in a recipient filled with 15 cm of water at 23±2°C. The test was filmed for 5 minutes. Immobility time was scored when the mouse would float without moving its limbs or drown.

Tail pinch

The mice were placed on the table and their tails were pinched manually at a constant pressure for 30, 60, or 90 seconds.

Tail suspension

The mice's tails were taped to a beam 50 cm high. An underpad was place beneath the mouse for safety. The duration of the test lasted 5 minutes. The experimenter should be aware that the animal's body is aligned with its tail since during the entire duration of the test.

For the assessment of depression: the tail suspension test was done in the same conditions. However, it lasts for 6 minutes and should be recorded for assessment of immobility. Immobility duration was scored when the mouse would let itself hang without moving its head or limbs.

Overnight illumination

The cages containing the mice were placed in a quiet room with constant lighting overnight. The experimenter should make sure that every cage is exposed to light.

After 6 weeks, animal depression was assessed by the sucrose preference test, the forced swim test, and the tail suspension test.

C. Sucrose Preference Test

Two bottles were placed inside every cage, one containing 1% sucrose and the other one drinking water. Every 3 or 4 days, the bottles' positions were swapped to prevent the possibility of a side preference in drinking. Sucrose consumption was assessed in all animals. Every 3-4 days, both bottles were weighed. At the end of the study, the final weight of the bottles was recorded. Subtracting the final weight from the initial weight will give us the amount of liquid consumed in grams. Sucrose preference was calculated according to this formula:

Sucrose consumption (%) = [Amount of sucrose solution consumed(g) / total amount of fluids consumed (g)] * 100

A decrease of sucrose consumption below 65% is considered a criterion for anhedonia. Mice that fit this criterion had shown features of depression in previous studies (Moreau et al, 1992; Vogel et al, 1990; Willner, 1997).

D. Peripheral Nerve Injury

The peripheral injury was assessed by the raised beam walking test. It is a behavioral test used to assess motor dysfunction in animals. The setup consists of a rod of 1.2 cm diameter and 70 cm length. At one end of the rod we set a secure platform to house the animal. First, the mouse was allowed to adapt to the set-up and to the platform. Then, the animal will be securely put at the end of the rod opposite to the safe platform. Once the mice have succeeded in reaching several times the platform with the help of the investigator, the test will be recorded in 3 trials. We will monitor the ability to reach the platform. The **time** needed to reach the end of the rod, the **speed** at which the movement occurs, the **number of stops** and the number of times the mice **slips** on the rod without falling (faults) will be counted.

E. Western Blot Analysis.

Hippocampii and sciatic nerves were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 50 mM Tris-hydrochloride, 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 polyclonal anti-Nox1 (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-PLP (1:250, abcam), mouse polyclonal anti-MBP (1:250, Millipore), rabbit

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.

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

mRNA expression in prefrontal cortices was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method and the SYBR green system. 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 RT^2qPCR Primers (Integrated DNA Technologies, Inc.,Coralville, IA, USA), for Nox 1, PLP, and MBP. 26S was used as internal reference gene.

Primers	**Sequence**	**Annealing TºC**
Nox1	**F: 5'-TCCATTTCCTTCCTGGAGTG-3'** **R: 3'-CCCAACCAGTACAGCCACTT-5'**	**60ºC**
PLP	**F: 5'-TACCCTGGCTAAAGCAGAGC-3'** **R: 3'-GAGGTGGTGTTCGAGGTGTC-5'**	**59ºC**
MBP	**F: 5'-GCCCTGACTGTTGTATGGCT-3'** **R: 3'-TCATTTGGAACATACATTCTGGCA-5'**	**57ºC**
26S	**F: 5'-AGGAGAAACAACGGTCGTGCCAAAA-3'** **R: 5'-GCGCAAGCAGGTCTGAATCGTG-3'**	**57-60ºC**

Table 1. Oligonucleotide primer sequences and conditions employed for RT-PCR

G. NADPH Oxidase Assay

NADPH oxidase activity was measured in the prefrontal lobe, hippocampus, and sciatic nerve 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.

H. Statistical Analysis

Results are expressed as mean ± SEM from multiple independent experiments. Statistical significance is assessed by ANOVA and the Student T-Test. Significance is determined as probability (p) <0.05.

CHAPTER III

RESULTS

A. Mice with type 2 diabetes develop a depressive like behavior

Diabetes has been shown to induce a depressive like behavior in human as well as in animal models of diabetes (Sharma et al, 2010; Kleinridders et al, 2015). To determine whether diabetes induces symptomatological and pathophysiological like behavior, we performed a panel of behavioral tests on 4 months old MKR mice, representing early onset of diabetes. All behavioral tests were performed in the morning to avoid differences in locomotor activity and other variables affected by circadian rhythm. The tail suspension (TST) and the forced swimming tests (FST) assess behavioral despair. The TST revealed a 66% increase in the immobility time of the diabetic animals when compared to their control littermates (Figures 3A). These results were quite comparable to the control depressed mice. Importantly, the TST reveals that induction of depression in diabetic mice significantly worsen the despair behavior seen during the onset of diabetes where a 64% increase in the immobility time was recorded when compared to diabetic mice and a 11% immobility time increase was observed when compared to control depressed mice. These alterations were also noticeable using the forced swimming test. However the results obtained by the FST were not significant (Figures 3B).

Anhedonia is another major criterion for the diagnosis of depression. A depressed individual or animal would not engage in pleasurable activities. We have used the sucrose preference test or the sucrose consumption test to assess anhedonia. Consumption above 65% reflects that the

mouse is feeling “pleasure”. The control group has a sucrose preference above 65% meaning that the animals are feeling “pleasure”. Our results show that the diabetic animals as well as the control-depressed animals have a sucrose preference below 65% when compared to the control FVB mice. Induction of depression in the diabetic animals revealed a more significant decrease in sucrose consumption of when compared to diabetic animals (20% decrease) or control-depressed animals (9% decrease).

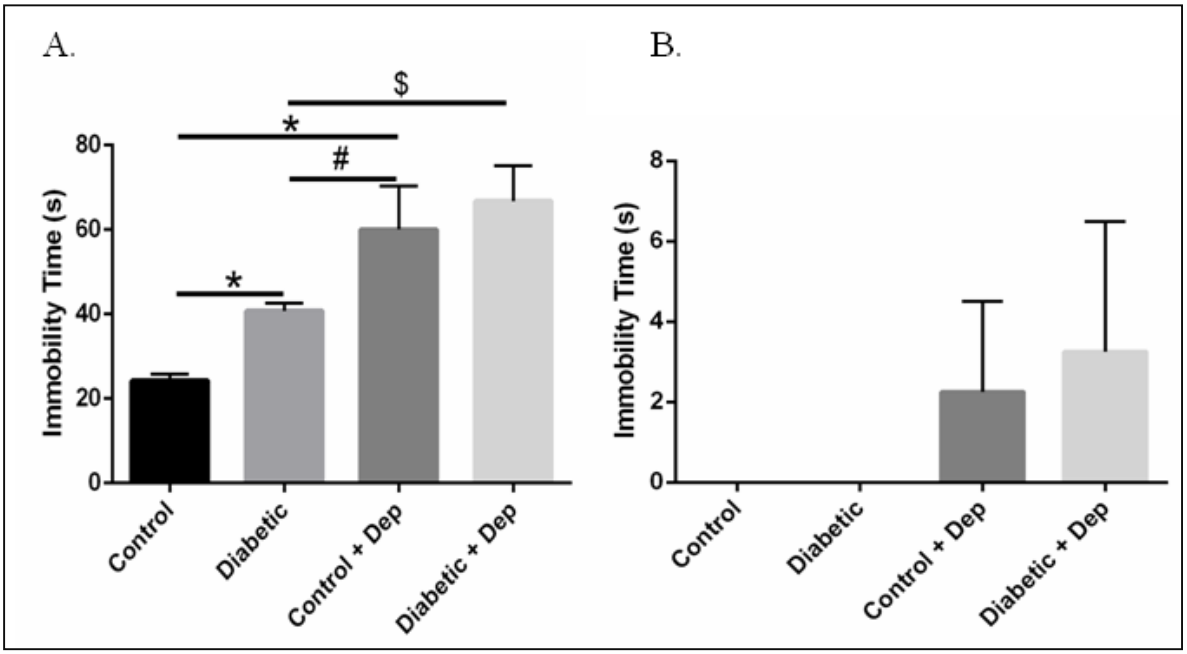


Figure 3. Assessment of depressive-like states in control, diabetic, control/depressed, and diabetic/depressed animals.
Barograms representing the tail suspension (A), and the forced swimming (B) tests. Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic vs. control/depressed. $P<0.05, diabetic/depressed vs. diabetic (n=4).

B. Diabetes and depression cause alterations in the levels of the central myelin proteins: MBP and PLP.

Although depression involves an overall reduction in brain activity, some parts of the brain are more affected than others. In brain-imaging studies using PET scans, depressed people display

abnormally low activity in the prefrontal cortex, and the hippocampus in parallel to other parts of the brain (Videbech, 2000; Alves et al, 2006). Myelin alteration has recently emerged to play a role in mental illness (Chambers et al, 2004; Haroutunian et al, 2014; Fields, 2008), however its role in diabetes-induced depression is not yet defined. For that, we studied the expression of PLP and MBP, two myelin genes, responsible for the myelination process in the CNS. Our data show that MBP mRNA levels were reduced in diabetic, control/depressed, and diabetic/depressed mice when compared to their control littermates (Fig 4A). mRNA levels of PLP were significantly reduced in the control depressed animals when compared to the control group. Surprisingly, in the diabetic and diabetic/depressed groups PLP mRNA levels were increased when compared to the control group (Fig 4B).

Moreover, we showed that in the hippocampus of diabetic, depressed, and diabetic depressed animals, protein expression of MBP is increased compared to the control group (Fig 4C, E). Similarly, PLP protein expression was also upregulated in control depressed and diabetic depressed animals however, it was downregulated in diabetic mice (Fig 4D, F). Comorbid diabetes and depression caused a greater dysregulation in the myelin proteins.

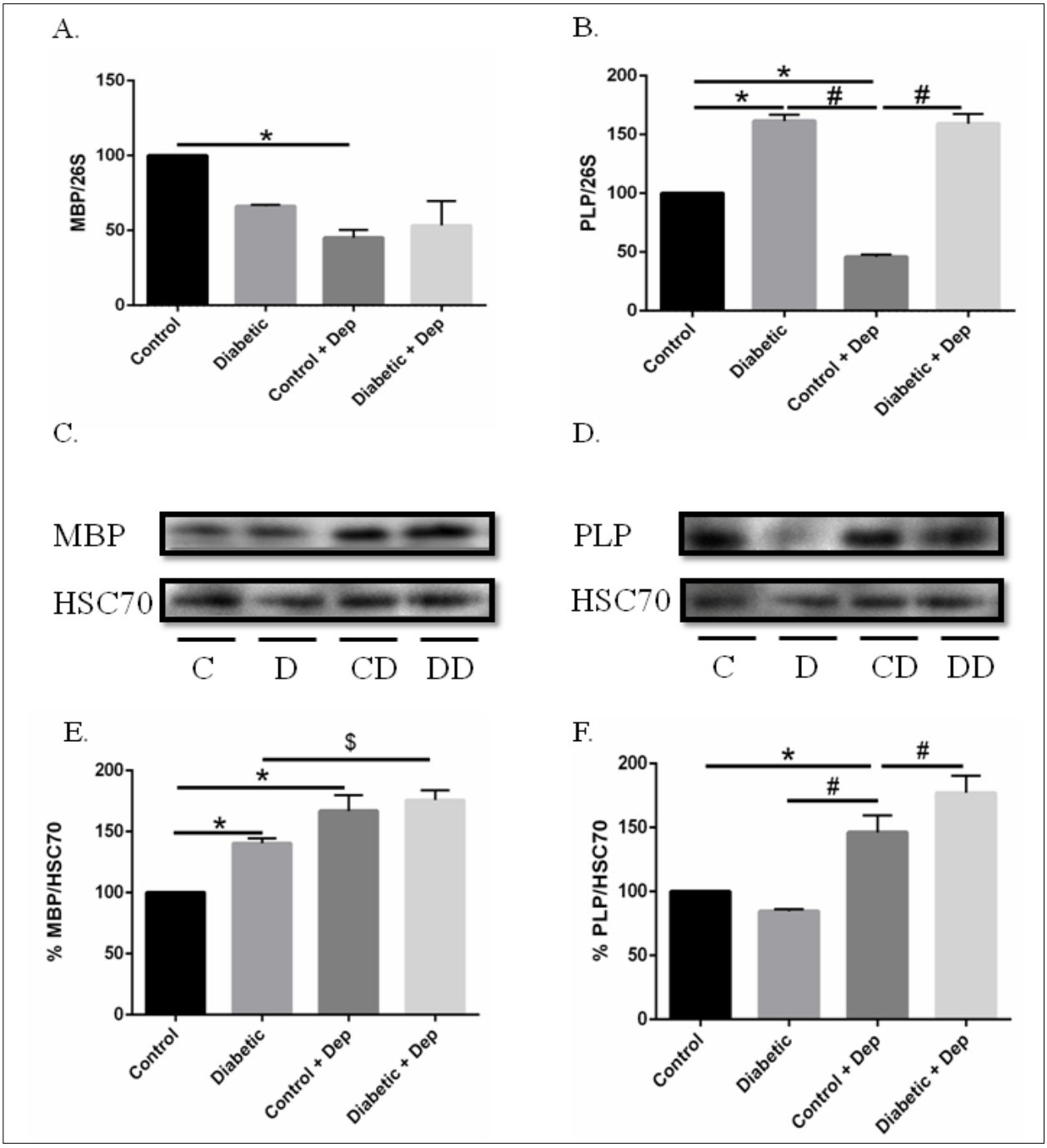


Figure 4. Diabetes and depression cause myelin injury in the prefrontal lobe and hippocampus.
Expression of PLP and MBP in the prefrontal lobe and hippocampus of control (C), diabetic (D), control/depressed (CD), and diabetic depressed (DD) groups. A and B. mRNA levels of MBP (A) and PLP (B) in the prefrontal lobe are measured by RT-PCR (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic and diabetic/depressed vs. control/depressed. C and D. Representative MBP (C) and PLP (D) western blot images in the hippocampus. HSC70 was used as the loading control. E and F. Quantitative results of MBP (E) and PLP (F) levels in the hippocampus. Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic and diabetic/depressed vs. control/depressed. $P<0.05, diabetic/depressed vs. diabetic.

C. NADPH oxidase Nox1 expression is up-regulated with diabetes, depression, and with both disorders in the hippocampus and in the prefrontal lobe.

ROS production through the NADPH oxidase system has been described to play a role in diabetic complications specifically in diabetes induced renal and heart injuries (Eid et al, 2009; Zhao et al, 2015). However nothing is known about the role of NADPH oxidases in depression or in the depressive-like behavior seen in diabetes. Nox1 and Nox4 were assessed in this study, however we focused on the Nox1 isoform. We assessed Nox1 mRNA levels in the prefrontal lobe and the protein expression of this NADPH oxidase subunit in the hippocampus. Our results show that mRNA levels of Nox1 are increased in the prefrontal lobe of the diabetic, control/depressed, and diabetic/depressed mice when compared to the control FVB animals (Fig 5A). In parallel our results show an alteration in ROS production in the hippocampus as assessed by the NADPH oxidase activity that indirectly measures NADPH dependent superoxide production (Fig 5B). Interestingly, the hippocampus did not express Nox4 subunits, while Nox1 protein expression in the hippocampus was increased in the diabetic, control/depressed, and diabetic/depressed mice when compared to their control littermates (Fig 5C, D). Similarly, an increase in NADPH oxidase activity in the hippocampus was detectable in diabetic, depressed, and diabetic/depressed animals (Fig 5E). 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.

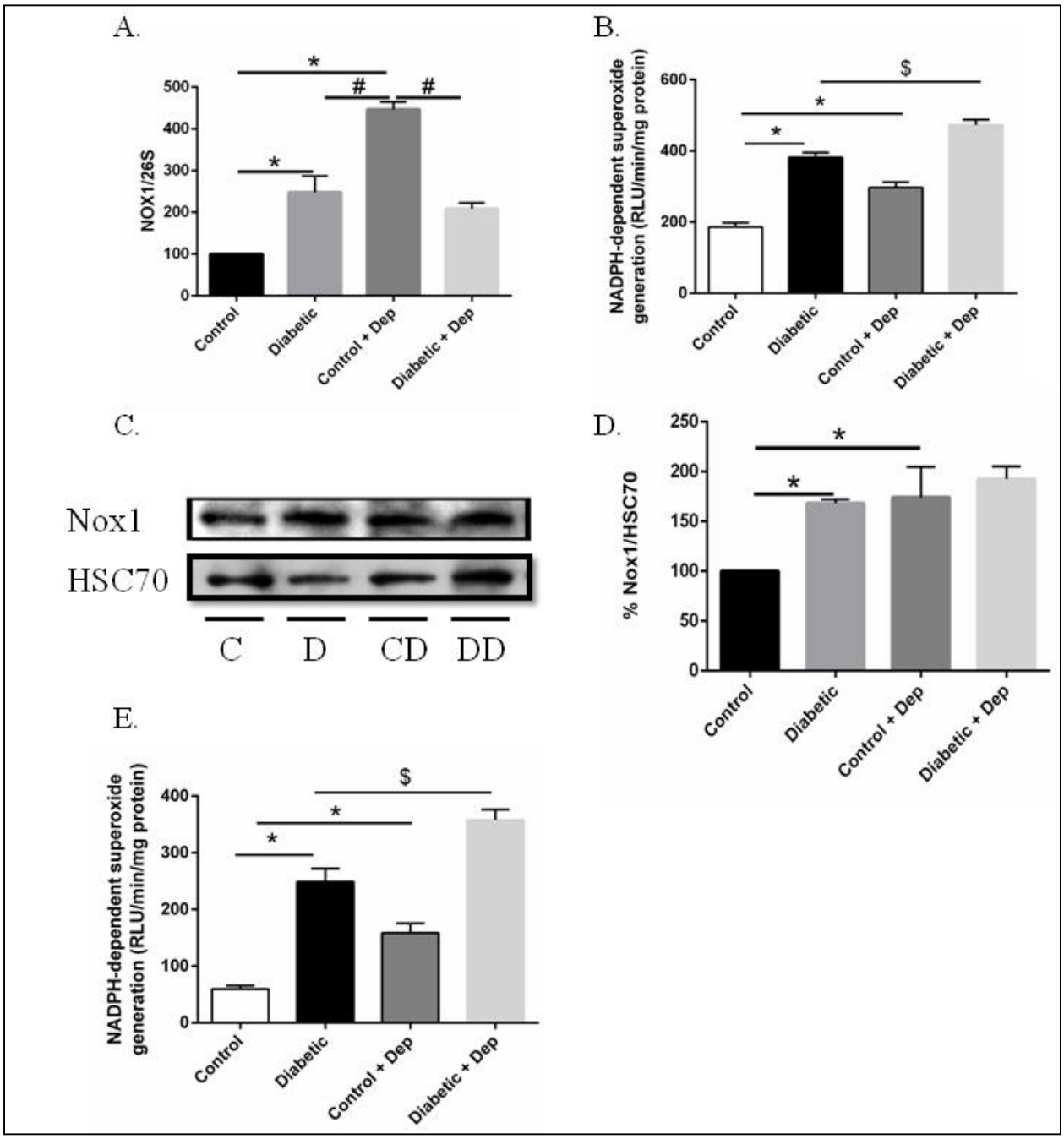


Figure 5. ROS generation in the CNS of diabetic, depressed, and diabetic depressed animals.
A. Nox1 mRNA levels were measured by RT-PCR in the prefrontal lobe of control, diabetic, control/depressed, diabetic/depressed mice (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic and diabetic/depressed vs. control/depressed. B. The superoxide anion generation assessed by evaluating the NADPH oxidase activity was measured in the prefrontal lobe of control, diabetic, control/depressed, and diabetic/depressed animals (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. $P<0.05, diabetic/depressed vs. diabetic. C. Representative Nox1 western blot image in the hippocampus. HSC70 was used as the loading control. D. Histogram showing the quantification of the western blot analysis.

Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. E. The superoxide anion generation was assessed in the hippocampus of control, diabetic, control/depressed, and diabetic/depressed animals (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. $P<0.05, diabetic/depressed vs. diabetic.

D. Type 2 diabetes and depression affect fine motor coordination.

We next examined if diabetes and/or depression alter motor coordination. We also, studied if induction of depression in diabetic mice exacerbates motor injury when compared to diabetic or control depressed mice. These experiments will allow us to highlight, if any, the correlation of depression with peripheral nerve injury. Deficit in fine motor coordination was assessed using the balance beam test or beam-walking test (Luong et al., 2011). This test examines the ability of the animal to walk on a narrow elevated beam (12 mm width beam and 50 cm above the table top) and to remain upright. Depressed mice showed a slower pattern of movement, more foot faults and made more stops then the control group (Fig 6B, C, D). Diabetic mice had the same pattern of decrease in locomotor coordination as the depressed mice. Interestingly, the number of foot slips was more pronounced in diabetic/depressed animals when compared to control/depressed or diabetic animals (Fig 6C).

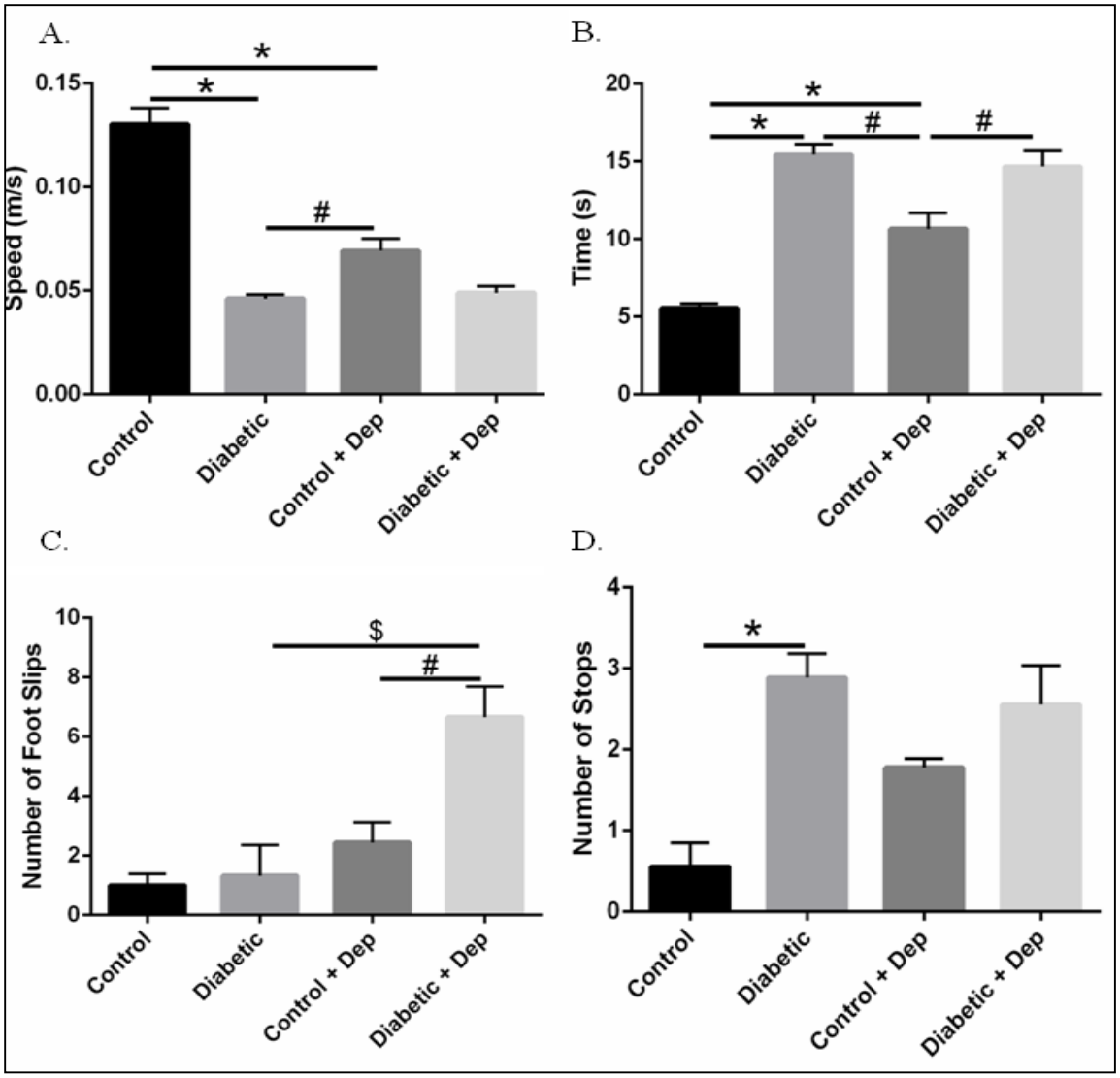


Figure 6. The effect of hyperglycemia, depression, and hyperglycemia combined with depression on motor coordination.
Barograms representing the average speed (A), time (B), foot slips (C) and stops (D) of 12week-old control animals, diabetic animals, control depressed animals, and diabetic depressed animals assessed by the raised beam-walking test (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic and diabetic/depressed vs. control/depressed. $P<0.05, diabetic/depressed vs. diabetic.

E. Myelin injury is present in the peripheral nervous system.

Next, we assessed the myelin pattern in the sciatic nerve of these groups of mice. In the central nervous system, myelin injury was detected through an alteration in the levels of PLP and MBP.

In the PNS, Schwann cells carry out the process of myelination. PMP22, one the glycoproteins of the PNS, is involved in the initiation of the determination of the myelin thickness, myelination, and the stabilization of the myelin sheath (Adlkofer et al., 1995, Garbay et al, 2000). Any alteration, up-regulation or down-regulation, in the expression of this myelin protein would have lead to the formation of non-functional myelin (Niemann et al, 2006). In order to assess whether diabetes, depression, and diabetes/depression induce PNS injury, PMP22 protein expression was measured in these groups of mice. Our results show that diabetes whether occurring alone or co-occurring with depression alters the protein expression of PMP22 in the sciatic nerves of mice compared to the control animals. This alteration was also observed in the control/depressed animals (Fig 7).

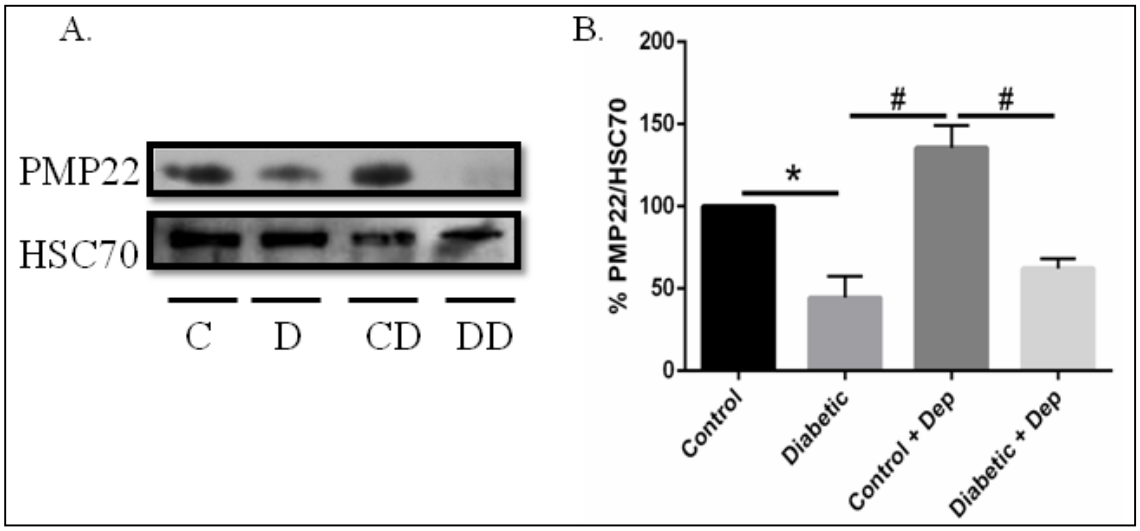


Figure 7. Myelin injury is present in the sciatic nerve of diabetic and depressed animals. A. Representative PMP22 western blot image. HSC70 was used as the loading control. B. Quantitative results of PMP22 in the sciatic nerves of control, diabetic, depressed, and diabetic/depressed mice. Values are the mean ± SEM. *P<0.05, diabetic vs. control. #P<0.05, diabetic and diabetic/depressed vs. control/depressed.

F. NADPH-dependent superoxide production in the sciatic nerves of diabetic and depressed animals.

Next we investigated if ROS production through an NADPH oxidases dependent pathway was altered in the sciatic nerves of the control, diabetic, depressed, and diabetic depressed groups (Fig 8). Our data showed an increase in the NADPH oxidases activity in the sciatic nerve of the diabetic mice similar to that observed in the control depressed group. Induction of depression in the diabetic mice increased further the NADPH dependent superoxide generation when compared to the values obtained from the control/depressed and the diabetic animals. These finding suggest that depression occurring during the onset of diabetes might lead to an increase in ROS production that in turn can exacerbate peripheral neuropathy complication.

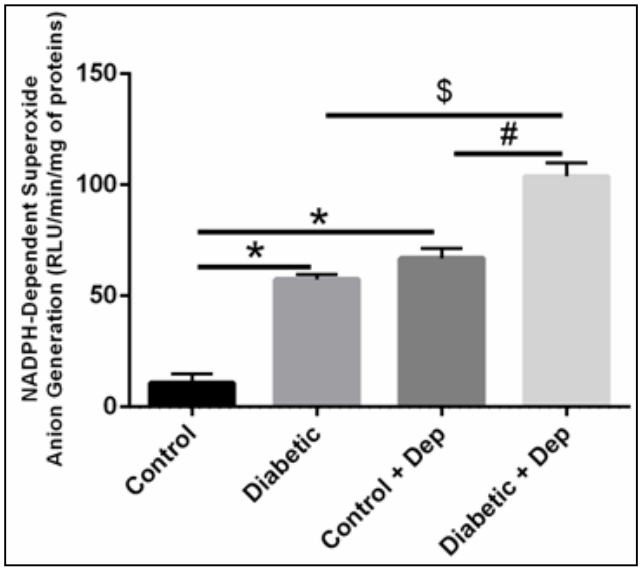


Figure 8. Diabetes and depression induce NADPH-dependent superoxide production in the PNS.
The superoxide anion generation assessed by evaluating the NADPH oxidase activity was measured in the sciatic nerve of control, diabetic, control/depressed, and diabetic/depressed animals (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic/depressed vs. control/depressed. $P<0.05, diabetic/depressed vs. diabetic.

We next examined if this alteration in the NADPH oxidase-induced ROS production especially the alteration of the Nox1 subunit had a key role in the development of the depressed like behavior seen in the diabetic, depressed and diabetic/depressed animals. For that, we used a Nox1 and Nox4 inhibitor (GKT) that was developed by Genkyotex (GKT #137833).

G. Nox1/Nox4 inhibition reduces ROS generation in the CNS and PNS

GKT treatment blocked NADPH-induced ROS production in the prefrontal lobe (Fig 9A), Hippocampus (Fig 9B) and in the sciatic nerves (Fig 9C) of the diabetic, control/depressed and diabetic/depressed animals.

Interestingly our results show a reversal in the depressive like behavior in the diabetic animals and the depressed diabetic animals, associated with amelioration of the mice motor coordination. More importantly, our results show that myelin alteration whether seen in the brain or the peripheral nervous system was corrected when ROS production was inhibited with the NADPH oxidases inhibition.

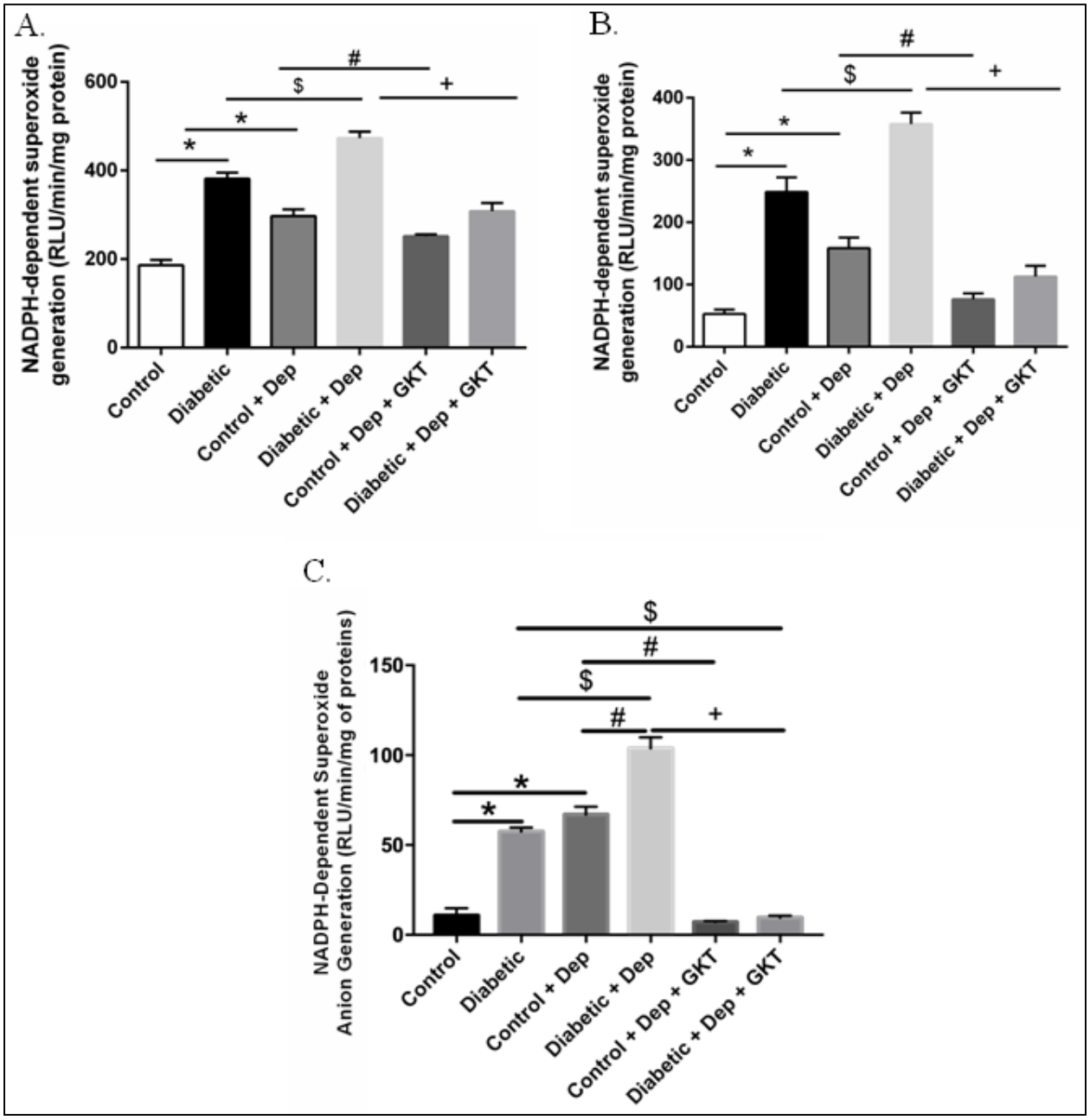


Figure 9. GKT reduces oxidative stress in the prefrontal lobe, hippocampus, and sciatic nerve
The superoxide anion generation was assessed in the prefrontal lobe (A), hippocampus (B), and sciatic nerve (C) of control, diabetic, control depressed, diabetic depressed, control depressed treated with GKT (CDG), and diabetic depressed treated with GKT (DDG) animals (n=3). Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic/depressed and control /depressed + GKT vs. control/depressed. $P<0.05, diabetic/depressed and diabetic/depressed + GKT vs. diabetic. +P<0.05, diabetic/depressed + GKT vs. diabetic/depressed.

H. Inhibition of Nox1 or Nox4 reverses depressive-like behavior

To examine the effect of GKT in the reversal of depressive-like behavior, the TST was performed. However, we did not perform the FST because the animals were used to the stress of swimming which was 3 times per week for 6 weeks. Thus, using the FST as a tool to assess depressive-like behavior in treated animals would not give significant results. The TST demonstrated that the control depressed treated mice had a 88 % decrease in immobility time when compared to control depressed mice. However, the diabetic depressed treated mice had a 40% decreased immobility time when compared to diabetic depressed mice (Fig 10). Our results also show that anhedonia assessment using the sucrose preference test was decreased below 65% in depressed, diabetic and diabetic/depressed animals. This effect was reversed by GKT treatment in the depressed as well as in the diabetic/depressed group, where sucrose consumption was beyond 65%.

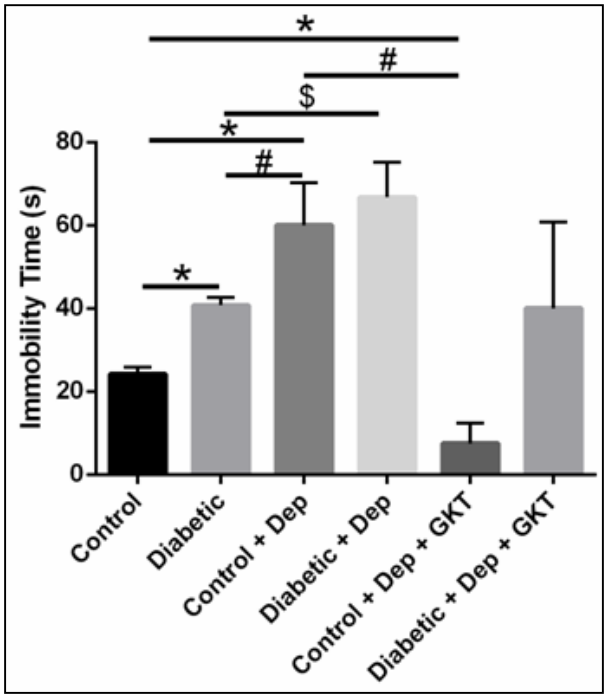


Figure 10. GKT reduces hopelessness and anhedonia.
Barograms representing the TST. Values are the mean ± SEM of 4 mice in each group. *P<0.05, diabetic, control/depressed and control/depressed+GKT vs. control. #P<0.05, diabetic, and control/depressed+GKT vs. control/depressed. $P<0.05, diabetic/depressed vs. diabetic.

I. Nox1 and Nox4 inhibition reduces myelin injury in the CNS

GKT treatment of the control/depressed mice and the diabetic mice where depression was induced (diabetic/depressed) reversed the myelin dysregulation that was observed in the depressed animals as well as the diabetic/depressed animals whether at the prefrontal lobe levels or the hippocampus levels (Fig 11A, B, C, D, E, F). Our results show that the MBP and PLP mRNA levels of prefrontal lobe of the control/depressed mice as well as the diabetic/depressed mice returned to levels similar to that of the FVB control group (Fig 11A, B). Moreover, our data shows that in the hippocampus, the dysregulation of the protein expression of MBP and PLP was reversed with GKT treatment to levels similar to the control mice (Fig 11 C, D, E, F).

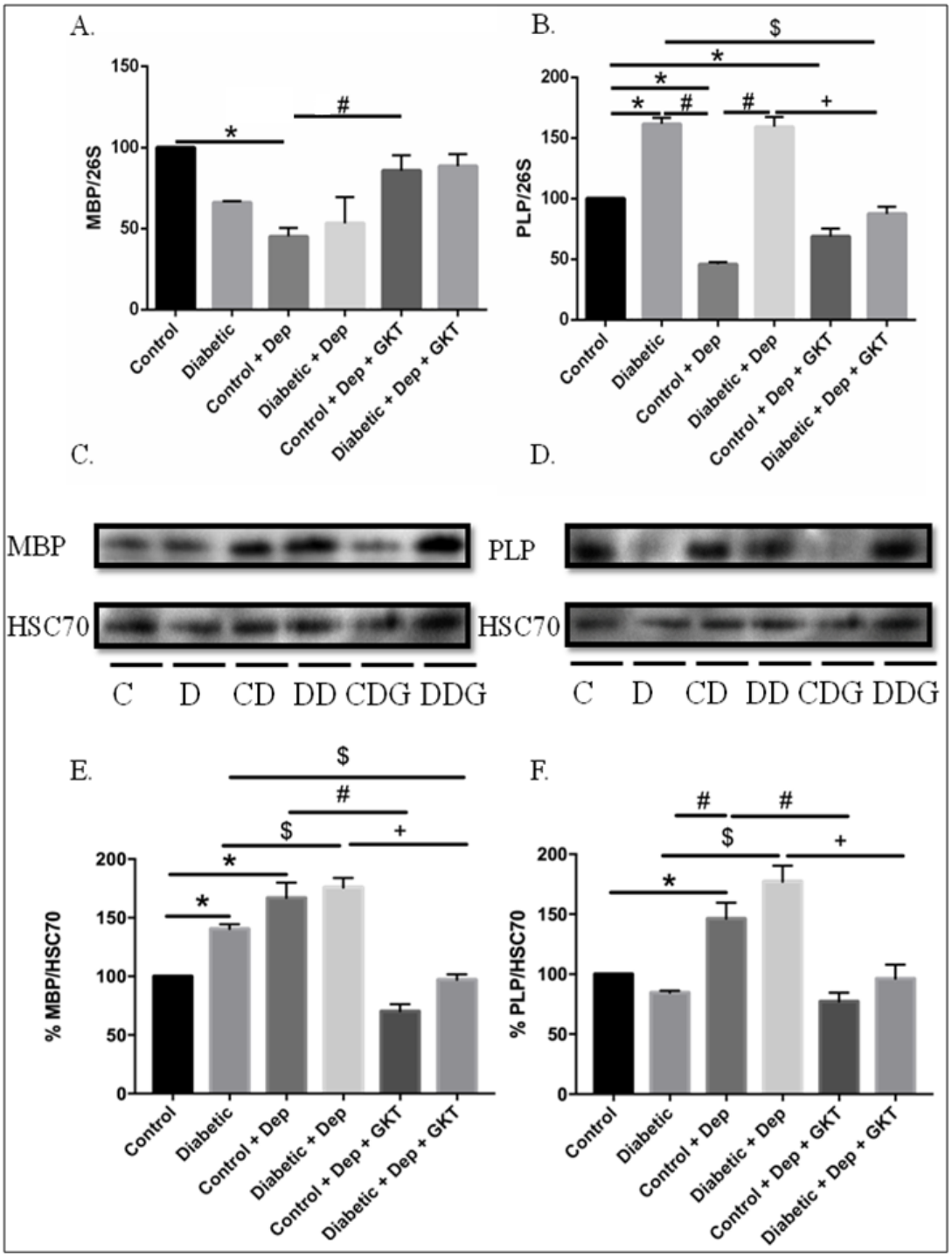


Figure 11. GKT treatment decreases myelin injury in the prefrontal lobe and in the hippocampus.
A and B. mRNA levels of MBP (A) and PLP (B) in the prefrontal lobe are measured by RT-PCR (n=3). Values are the mean ± SEM. *P<0.05, diabetic, control/depressed, and control/depressed+GKT vs. control. #P<0.05, diabetic, diabetic/depressed, and control/depressed + GKT vs. control/depressed. $P<0.05, diabetic/depressed +GKT vs. diabetic. +P<0.05, diabetic/depressed + GKT vs. diabetic/depressed.C and D. Representative MBP (C) and PLP (D) western blot images in the hippocampus. HSC70 was used as the loading control. E and F. Densitometric quantification of MBP (E) and PLP (F) are represented by barograms. Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic and control/depressed + GKT vs. control/depressed.

$P<0.05, diabetic/depressed and diabetic/depressed +GKT vs diabetic. +P<0.05, diabetic/depressed + GKT vs. diabetic/depressed.

J. Inhibition of Nox1 or Nox4 ameliorates motor coordination

To further investigate the efficiency of GKT treatment on the PNS injury, the beam walking test was used for the assessment of fine motor coordination. GKT treatment ameliorated fine motor skills in control/depressed and diabetic/depressed mice (Fig 12). The mice were faster, stopped less, and had few foot slips when compared to the diabetic animals, control/depressed animals, and diabetic/depressed groups (Fig 12).

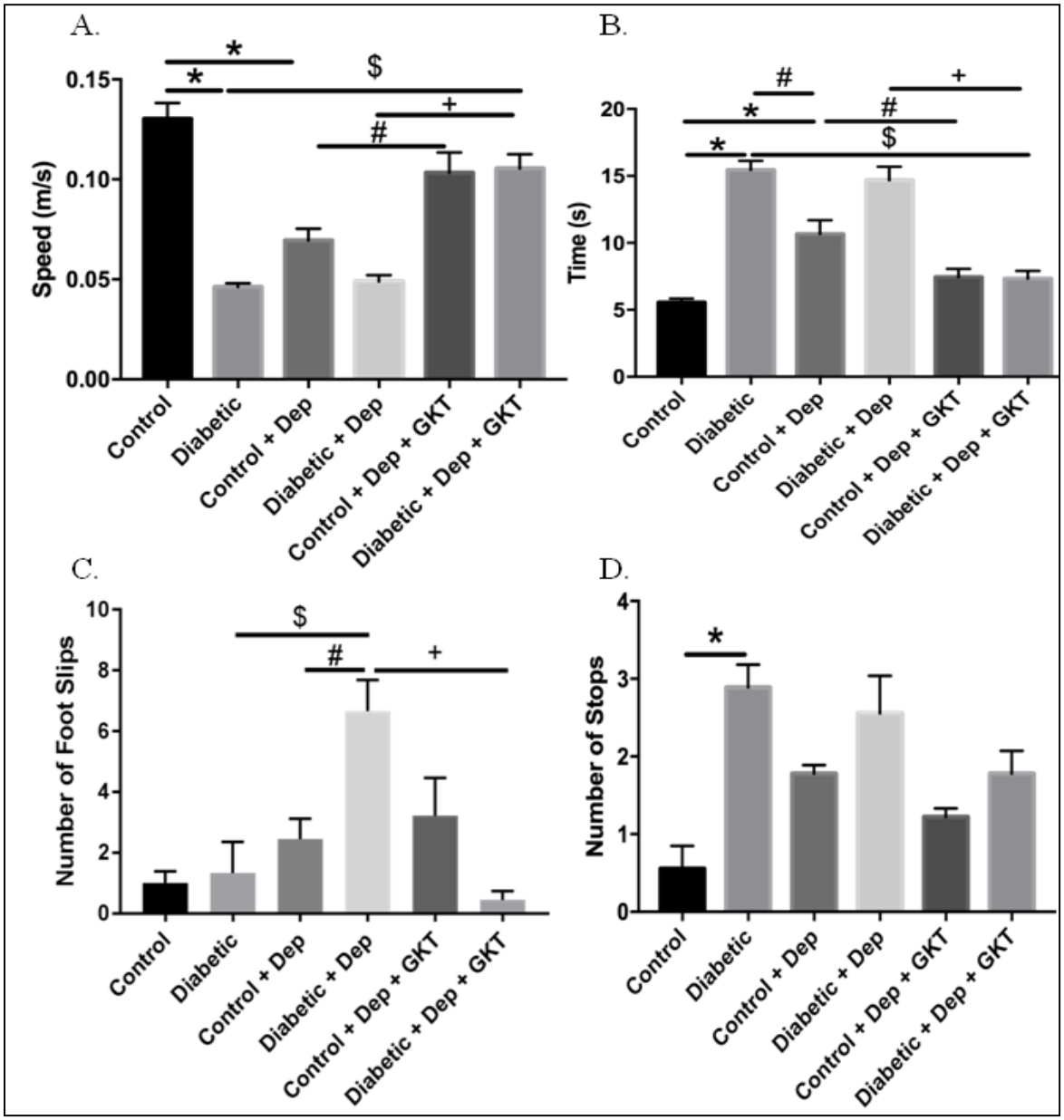


Figure 12. Improvement of motor coordination with GKT administration
Barograms representing the average speed (A), time (B), foot slips (C) and stops (D) of 12 week-old control, diabetic, control depressed, diabetic depressed, control depressed + GKT, and diabetic depressed + GKT (n=3). Motor coordination was assessed by the raised beam-walking test. Values are the mean ± SEM. *P<0.05, diabetic and control/depressed vs. control. #P<0.05, diabetic, diabetic/depressed, and control/depressed + GKT vs. control/depressed. $P<0.05, diabetic/depressed and diabetic/depressed + GKT vs. diabetic. +P<0.05, diabetic/depressed + GKT vs. diabetic/depressed.

K. Effect of Nox1/Nox4 inhibition on myelin injury in the sciatic nerve

To confirm the improvement seen in the beam walking test in the treated mice, we studied the effect of GKT at the molecular level. Our results showed that PMP22 protein expression was regulated by GKT; PMP22 was downregulated in the control depressed treated group compared to the control depressed group whereas this protein was upregulated in the diabetic depressed treated group compared to the diabetic depressed group (Fig 13). Even though, the protein expression of PMP22 was altered by GKT, the drug did not succeed in reversing myelin injury.

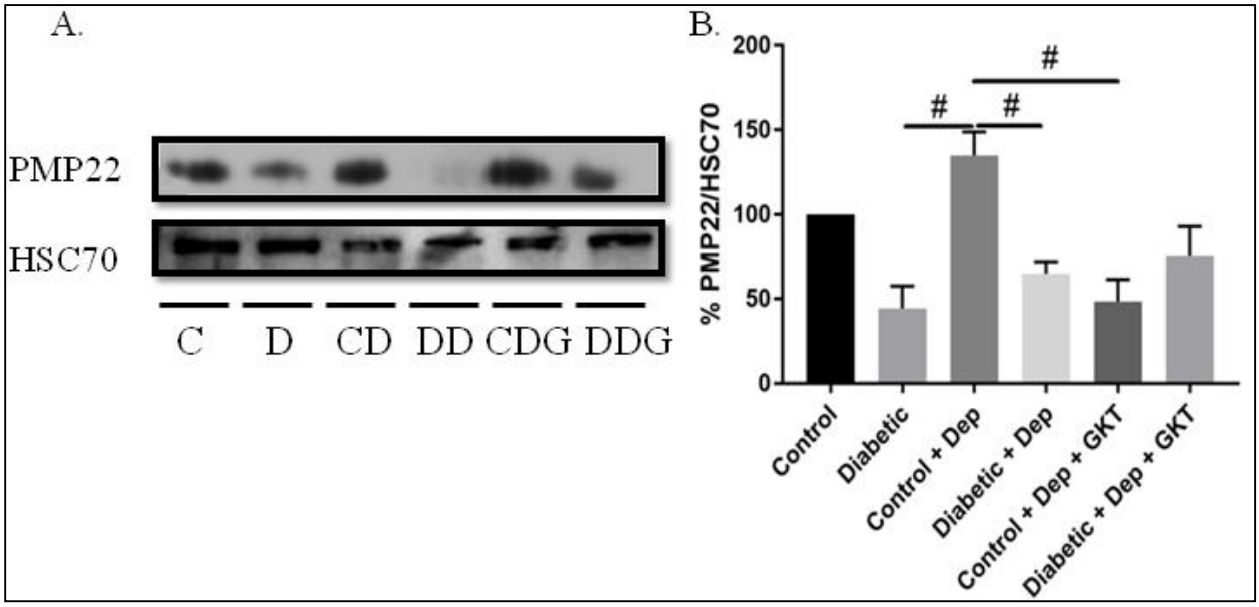


Figure 13. Effect of GKT on myelin injury in the sciatic nerve.
A. Representative PMP22 western blot images. HSC70 was used as the loading control. B. Quantitative results of PMP22 in the sciatic nerves of control, diabetic, depressed, and diabetic depressed, depressed treated, and diabetic depressed treated mice. Values are the mean ± SEM. #P<0.05, diabetic/depressed, diabetic, and control/depressed + GKT vs. control/depressed.

In summary, our findings suggest that diabetes induces a depressive like behavior in animals. This behavior is mediated by an increase in ROS production through an NADPH oxidase pathway. Importantly, our results suggest that diabetes-induced depression exacerbates diabetic complications, especially peripheral nerve complications lead by a deregulation in Schwann cells function. Blockade of the NADPH oxidases-induced ROS production reverses to a certain extent the pattern of injury observed in diabetic and in non-diabetic animals where the chronic mild stress procedure was applied.

CHAPTER IV

DISCUSSION

Diabetes has been associated with a wide range of brain alterations including depression. It is reported that depression affects patients with diabetes even more than those with chronic diseases (Moulton et al, 2015; Korczak et al, 2011). However, the relation between these two conditions is not well characterized. The first logical explanation that comes to mind is that patients with diabetes become depressed as a result of being diagnosed with diabetes and, because of the influence that it will have on their life either physically, socially or economically. Yet, the biological link(s) between diabetes and depression is still under investigation. Using non-obese type 2 diabetic animals, we show that depressive like behavior can be a direct consequence of diabetes. Thus, diabetic mice showed signs of "hopelessness" assessed by the tail suspension test and experienced "anhedonia" as measured by the sucrose preference test. These results correlated with already published data by other groups showing that type 2 diabetic mice present with signs of depression and anxiety. Yu et al found that type II diabetic patients have a decreased sucrose preference (2014), furthermore, Kleinridders et al show that mice with a brain specific knockout of insulin receptor have a long period of immobility using the tail suspension test and the force swimming test (2015), and Sharma et al reported that db/db mice exhibit a long duration of immobility as assessed by the force swimming test (2010).

Impaired myelination has been recently associated with diabetes and depression. The myelin sheath guarantees the rapid saltatory conduction of the nerve impulse. Lipids form 70%

of the myelin whereas the proteins represent 30% (Edgard & Sibille, 2012). In the central nervous system (CNS), PLP and MBP are two of the most abundant myelin proteins. Their expression levels should be tightly regulated to provide myelin stability and guarantee proper nerve conduction. In this study, type 2 diabetic mice, as well as diabetic mice where depression was experimentally induced (diabetes/depression) show an alteration in the MBP and PLP mRNA levels and protein expression in the prefrontal lobe and the hippocampus mimicking the alteration seen in the experimentally induced depressed mice. Thus diabetes and depression play a role in myelin injury. In line with our observations, several studies reported that CNS myelin proteins are altered in diabetes and depression. Kawashima et al (2007) and Mitro et al. (2012) reported a dysregulation in MBP and PLP levels in the spinal cord and in the brain of diabetic animals as well as in the brain of diabetic patients (Honer et al, 1999). Moreover, Fuchsova et al (2015) and Rajkowska et al (2015) showed a decrease in the mRNA levels of proteins belonging to the PLP family in the hippocampus and in the ventral prefrontal cortex of depressed humans. Fernandez et al show that myelin proteins of the hippocampus are altered in chronically stressed rodents (2010). Interestingly, we observed that in non-obese type 2 diabetic MKR mice where depression was experimentally induced, MBP and PLP myelin protein were significantly altered when compared to the proteins levels observed in depressed control or diabetic MKR mice.

Diabetes has been shown to induce peripheral nerve injury that in turn prompts the depressive behavior in human and rodents (Gui et al, 2016; Murad et al, 2015; D'Amato et al, 2016). But, does depression per se induce peripheral nerve injury? Is there any biological pathway that can correlate depression to peripheral nerve injury?

To our knowledge this is the first preliminary report describing alteration of the major myelin proteins in mice with depressive-like behavior. Our results show that depression

upregulates PMP22, one of the major myelin proteins in the peripheral nervous system (PNS) paralleled by an increase in motor behavior injury as assessed by the beam walking test. More importantly, experimentally induced-depression in type 2 diabetic mice induced more foot slips, a major component reflecting more deficits in fine motor coordination, with the knowledge that type 1 or type 2 diabetes are described by our group and others 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).

Is there a biological link between diabetes and depression? Does this biological link explain the aggravation of injury seen in the CNS and the PNS of the diabetic mice rendered experimentally depressed?

Reactive oxygen species (ROS) production has been portrayed to play a major role in the development of depression and of diabetic complications including DPN (Baynes et al, 1991; Maes et, 2011; Korczak et al, 2011). However the mechanisms leading to ROS production in the central nervous system or the peripheral nervous system in diseases specifically in depression and diabetes are poorly described. The NADPH oxidase family of enzymes is one of the main producers of ROS. NADPH oxidases are largely implicated in the complications of diabetes and in depression (Eid et al, 2009; Zhao et al, 2015; Kowluru & Chan, 2007; Seo et al, 2010; Walton et al, 2013). Our results show that NADPH dependent superoxide generation as well as Nox1, mRNA and protein expression, are upregulated in the prefrontal lobe and in the hippocampus of the type 2 diabetic MKR mice, as well as in the depressed control animals. The levels of NADPH oxidases and the protein expression of Nox1 were further induced in the diabetic mice rendered experimentally depressed. These finding are supported by the reported data of Réus et al showing an increase in oxidative damage in the prefrontal cortex, hippocampus, amygdala,

and striatum of diabetic rats (2015). Similarly Che et al describes an increase in oxidative stress in the hippocampii of depressed patients (2010). Furthermore, Seo et al describe that repeated stress in mice promotes depressive behavior through an increase in NADPH oxidase activity (2012). Our finding highlighting the role of ROS in depression and diabetes-induced CNS injury were paralleled by similar findings in the sciatic nerves of depressed, diabetic and diabetic/depressed animals. Thus, corroborating with previous studies by Vincent et al (2009) describing the presence of oxidative damage in type 1, type 2, and pre-diabetic animals. Taken together, our results suggest that diabetes and depression are inducing an increase in ROS production through the NADPH oxidase family of enzymes which is leading to myelin injury in the CNS and in the PNS.

Several experimental studies reported the blockade of ROS, using different types of antioxidants, in the treatment of depression and peripheral injury. The combination of N-acetyl-L-cysteine and deferoxamine increased sweet food intake in depressed rats and decreased oxidative stress (Arent et al, 2012). Vitamin E and N-acetyl-L-cysteine reduced depressive-like behavior in animal models of type 1 diabetes (DeMorais et al, 2014; Réus et al, 2015). Likewise, N-acetyl-L-cysteine reduced structural deficits seen in the sciatic nerves of diabetic rats. Li et al showed that luteolin administration in diabetic rats improved nerve blood flow and nerve conduction velocities, and alleviated abnormal sensation (2015). Other publications also reported that the use of antioxidants such as α-lipoic acid helped in the treatment of pain in type 2 diabetic patients (Garcia-Alcala et al, 2015), while in type 1 diabetes patients, Pop-Busui et al found that the use of antioxidants had no beneficial effect on DPN (2013). To our knowledge the role of NADPH in CNS and PNS injury whether due to depression or diabetes is poorly characterized.

In this study we used GKT, a specific Nox 1 and Nox 4 inhibitor, developed by Genkyotex S.A. GKT was administered 3 times per week at a dose of 2.5mg/kg, intraperitoneally. GKT treatment decreased diabetes, depression, and diabetes/depression-induced NADPH dependent superoxide production in the hippocampii and the prefrontal lobes of these mice. In parallel, GKT was also effective in reducing ROS production in the sciatic nerves of the described animals. Importantly, the use of GKT to reverse NADPH oxidase activity shows a restored feeling of "pleasure" and a decrease in "helplessness" in the treated groups of mice. These findings were complemented by a significant improvement in the sensory motor behavior of the different treated groups of mice. Interestingly, our results indicate that GKT treatment reversed the dysregulation seen in the myelin protein expression of the CNS (MBP and PLP) and PNS (PMP22) and restore it to levels quite identical to that of the control group of mice. GKT effect was studied, so far, in the context of diabetic nephropathy and chronic obstructive pulmonary disease (COPD). GKT showed promising results in ameliorated the pulmonary injury accompanying COPD, thus suggesting its potential use as a target for COPD treatment (Hollins et al, 2016). In diabetic nephropathy, GKT intake resulted in reduced albumin excretion in the urine, mesangial matrix expansion, podocyte loss, and glomerular hypertrophy (Gorin et al, 2015). The drug currently passed phase I clinical trials and is being used to treat patients suffering from diabetic nephropathy.

In summary, and to conclude, our results suggest that NADPH-dependent ROS production is at the center of the structural changes. They were described in our study as alteration in the myelin protein, observed in the hippocampus, prefrontal lobe and sciatic nerve. Also, increased NADPH oxidase activity paralleled by an increase in Nox1 protein expression is at the center of the depressive like behavior seen in diabetes, depression and especially in

diabetic mice rendered experimentally depressed. This myelin injury is not only present in the CNS; depression and diabetes are contributing to the onset of myelin injury in the PNS as well and their comordity is exacerbating the injury in both the PNS and CNS. Remarkably, our obtained results suggest that GKT treatment can be used in depression, and in diabetic-induce PNS complications. Hence, the use of GKT may be therapeutically useful in the treatment of DPN and/or depression.

In our future studies, we will try to find the most suitable dose and method of GKT administration since GKT is a specific blocker of Nox1 and Nox4. A dose that is too high could result in the death of animals. The bardoxolone trial (BEACON) which involved total blocking of ROS production resulted in a high mortality rate among participants (De Zeeuw et al, 2013). This is why it is important to find the appropriate dose and treatment duration that will result in the reversal of myelin injury in diabetic and depressed patients.

Pitfalls and Future Directions

The present study was subject to pitfalls. First, the small amount of tissue was a limiting factor for running more experiments such as DHE stain, and RT-PCR analysis. In our future studies, we will increase the number of animals to tackle the issue of the limited amount of tissue. A larger number of animals will also provide more significance to our results. In this study we focused on Nox1 to study ROS generation and PMP22 to assess myelin injury in the sciatic nerve. In the future, we will focus on Nox4 which is also involved in ROS production, as well as MPZ to evaluate sciatic nerve injury. Even though we managed to establish a depression model and depressive-like behavior assessment tests, the open field test, the elevated plus maze, and the dark/light box will be performed to provide an assessment for anxiety in the animals since it is a main symptom of depression. In addition, a control group treated with an anti-depressant was not included in the study. The use

of such a drug requires a special permission from the Ministry of Health which was not available. However, a control treated with an antidepressant group will be included in our future studies.

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