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mTOR COMPLEXES PROMOTE PERIPHERAL NERVE DAMAGE AND SCHWANN CELL INJURY IN DIABETES

by

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AN ABSTRACT OF THE THESIS OF

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Background: Diabetic Neuropathy (DN) is one of the microvascular complications associated with diabetes. The clinical manifestations of DN include sensorimotor loss, paresthesia and altered pain and thermal perception. Hyperglycemia has been reported to be one of the triggers that induce peripheral nerve injury, characterized by myelin abnormalities, axonal atrophy and blunted regenerative potential. Nevertheless, the pathways that lead to the pathophysiology of diabetes-induced peripheral nerve injury has yet to be elucidated. Our group has identified reactive oxygen species (ROS) as a final key mediator in a number of diabetic complications, but their role in the etiology of DN has not been previously described. One of the major sources of ROS is the NADPH Oxidases (NOX) family, which we and others have shown to be involved in the pathophysiology of diabetic complications. More importantly, the crosstalk of NADPH oxidases with downstream or upstream signaling molecules is at the heart of the deleterious effect seen in diabetic complications.

Aim: The following study aims to investigate the role of NOX1 enzyme as sources of oxidative stress in myelinating Schwann cells (SCs) and sciatic nerves in the diabetic milieu. Furthermore, the crosstalk of the NADPH oxidases with the mTOR signaling pathway and the role of this signaling axe in SCs and sciatic nerve injury will be identified.

Methods: Mouse Schwann Cell (MSC80) cell line are used for *in vitro* studies and the C57/BL6, NOD/FVB, and Swiss Webster mice are used for the *in vivo* studies. Western blot and PCR analyses are used to assess expression of myelin proteins, NOXs, mTOR complexes associated proteins in addition to their signaling effectors. *In vitro* silencing of NOX and mTOR is also performed by transfection to further elucidate the role of the NOX/mTOR axe in diabetic peripheral injury. NADPH oxidase activity assay is used to assess NADPH-induced intracellular ROS production alongside DHE staining, and HPLC in SCs and sciatic nerves. Cellular apoptotic death is assessed using the Tunnel assays. Molecular assessment of peripheral myelin protein integrity is used to assess the PMP22-Aggregation in sciatic nerves. All of the cellular, biochemical and molecular tests are correlated with the sensory and motor changes observed by behavioral assessments in the type 1 diabetic animal models. The behavioral tests included are: Raised Beam-walking test and Hind-paw Withdrawal test.

Results: Hyperglycemia induced Nox1 upregulation and increased enzymatic activities which lead to an increase in ROS production. Oxidative stress triggered changes in the physiological integrity of SCs and function of sciatic nerves compared to normoglycemia. Hypergylcemia-induced SC and sciatic nerve injury was seen through an alteration in myelin protein expression as well as increased apoptosis concomitant with the synergistic alteration of mTOR. These alterations were correlated with sensorimotor deficits seen at the behavioral level, and these were ameliorated upon treatment with GKT, Rapamycin and PP242. The inhibition of NOX and mTOR were shown to be associated with the normalization of myelin protein levels at the molecular level in addition to the attenuation cellular apoptosis.

Conclusion: Our study shows that hyperglycemia induced oxidative stress and peripheral nerve injury through the upregulation of NOX1. This led to the activation of the mTOR pathway and dysmyelination, influencing SCs and peripheral nerve function.

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LIST OF ABBREVIATIONS

DN/ DPN:	Diabetic Neuropathy/ Peripheral Neuropathy
ROS:	Reactive Oxygen Species
HPLC:	High Performance Liquid Chromatography
NCV:	Nerve Conduction Velocity
MSC80:	Mouse Schwann Cells 80
SC:	Schwann cell
NG/ HG:	Normal/High Glucose
P0 or MPZ	Myelin Protein Zero
PMP22:	Peripheral Myelin Protein 22
DHE	Dihydroethidium
mTOR:	Mammalian Target of Rapamycin
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
NOD:	Non-Obese Daibetic
FVB:	Friend Virus B-type

CHAPTER I

INTRODUCTION

A. Diabetes Mellitus: General Overview

Diabetes Mellitus (DM) is a chronic, systemic malfunction recognized as a group of metabolic diseases, characterized by a failure in glucose metabolism with a disruption in carbohydrate, fat and protein metabolism. DM can be classified into two major types. The hallmark of Type I DM is the autoimmune destruction of the β-cells of the pancreas that leads to a deficiency in insulin. This is associated with risk factors that are mainly of a genetic component. However, environmental factors may play a role although they are still poorly defined. As for Type II DM, the more prevalent form, the pathology extends from primarily insulin resistance with relative reduction in insulin secretion to an insulin secretory defect with insulin resistance (International Diabetes Federation, 2015; Harcourt et al., 2013; Mayfield, J. 1998). Patients may be undiagnosed for years since the onset is gradual and the symptoms are not severe (Harris, M.I. 1993). Nevertheless, the risk of incidence increases with age, obesity and a sedentary lifestyle, which emphasizes environmental factors, besides the genetic component, as significant contributors to the disease (Alberti, K.G., & Zimmet, P.F. 1998).

Persistent hyperglycemia is associated with long-term damage and failure of many organs (American Diabetes Association, 2010) as a result of macrovascular and microvascular complications. Microvascular complications manifest as nephropathy that may lead to renal failure, retinopathy with potential blindness, and neuropathy with increased risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction (DCCT, 1993; Ali et al., 2013). By contrast, increased risk for cardiovascular disease, myocardial infarction,

cerebrovascular disorders, dyslipidemia, hypertension in addition to skin ulcers and infections arise from damage to the macrovasculature. This work will be focused on the most common and debilitating complication associated with diabetes: Diabetic Neuropathy.

B. Diabetic Neuropathy

Diabetic Neuropathy (DN) is estimated to affect approximately 50-70% of diabetic patients and tends to develop throughout disease progression, concurrent with a risk that rises with prolonged duration (World Health Organization, 2016). Defined as nerve injury that is associated with diabetes, DN may affect different types of nerves, including large-fiber sensory, small-fiber sensory, motor, and autonomic. Consequently, there are different forms of diabetes-induced neuropathies that have been described. DN can be classified as autonomic, proximal, focal, and peripheral (CallaghanBC. et al, 2012).

Diabetic autonomic neuropathy (DAN) can impair the cardiovascular, gastrointestinal, genitourinary, and sudomotor systems and can cause orthostatic hypotension, cardiac autonomic instability, and a range of symptoms that include constipation, nausea, and erectile dysfunction (Tracy and Dyck, 2008). Autonomic neuropathy may also appear in the form of treatment-induced neuropathy, also known as insulin neuritis (Gibbons and Freeman, 2015) and this is a form of painful neuropathy described in patients shortly after undergoing rapid glycemic control. Another form of autonomic neuropathy, known as diabetic radiculoplexus neuropathy (DRPN), presents sub-acutely with pain in the lower back, hip, and proximal leg followed by weakness and with a monophasic course over several months (Callaghan et al., 2015). The final form known as Diabetic mononeuropathy, can affect the median, ulnar, radial and common peroneal nerves and leads to extreme pain which may resolve spontaneously after several weeks.

As for Diabetic Sensorimotor Polyneuropathy (DPN) which is the typical type and the most frequently reported neuropathy among diabetic patients, it is characterized by a loss of sensation and motor function, paresthesia and pain that are often asymptomatic until complications are severe at late stages of diabetes (Ziegler et al., 2014; Zhang et al., 2014). Consequently, early recognition of DPN is critical. DPN affects small and large myelinated and unmyelinated fibers in a length-dependent, symmetric manner and progresses from the extremities proximally. Additionally, DPN increases the risk of ulcers, infections and calluses with diminished nociception and finally leads to gangrene and limb amputation which is why DPN is considered a disabling complication.

Physiological studies have described DPN to be associated with a reduced nerve conduction velocity, reduced intraepidermal nerve fiber density, hyperalgesia, allodynia, axonal degeneration, and abnormal neurotropism. However, although the pathophysiology of DPN is poorly understood, it is now widely accepted that the degree of peripheral damage is correlated with the significant pathological interactions between neurons, Schwann cells, and the microvascular endothelium (Vincent et al., 2009; Vincent et al., 2013). Treatments that address DPN involve glycemic management and symptomatic relief (Genuth, S. 2006). Despite that, optimal management has been reported to be of limited preventative benefit with regards to the micro-vascular complications especially neuropathy (DCCT 1995; UKPDS 1998). Hence, further investigations are pivotal for a better understanding of the cellular mechanisms involved in the pathogenesis of diabetic neuropathy.

C. Schwann Cells: Physiology and Pathophysiology in Diabetes

1. *Myelin Proteins and Myelination in the Peripheral Nervous System*

Schwann cells (SCs) are the myelin-producing cells and the chief regulators in nerve - axon interactions in the peripheral nervous system (PNS). The functional organization during development, myelination, growth and regeneration of nerve fibers after injury are dependent on SC integrity and functionality. During development, proliferation of SCs is terminated in response to various signals such as the axonal growth factor and neuregulin 1, and this signaling cascade triggers the differentiation of SCs that will either myelinate individual axons or bundle multiple unmyelinated axons (Schulz et al., 2014). Through the myelin sheath, a specialized, multi-layered and insulating extension of SC membranes, SCs engulf a nerve axon to produce a myelinated fiber (Sherman, D. L., & Brophy, P. J. 2005). Two SCs meet at an unmyelinated segment, known as the Node of Ranvier where the conductance of electric potentials along nerve fibers is facilitated via saltatory conduction (Garbay et al., 2000).

Furthermore, the myelin sheath is comprised of lipids (71%) and proteins (29%) and its integrity is critical for SC and nerve function. The peripheral myelin proteins are relatively small and characterize compact myelin membranes. Glycoproteins, including myelin protein zero (P0), peripheral myelin protein (PMP22), myelin associated glycoprotein (MAG) and periaxin, account for 60% of total myelin proteins. The remaining 40% of proteins include basic proteins (20-30%), incorporated in the Schwann cell's membranes, and diverse proteins. These proteins are critical for the physiology of SCs and their myelin sheaths (Bilbao, J. M., & Schmidt, R. E. 2015).

- ***Myelin Protein Zero (P0)***

P0 is the predominant protein (60%) of the PNS that functions as a cell adhesion molecule. Initial studies showed that P0 mediates homophilic plasma membrane cell adhesion following its transfection into cultured non-adherent cells (D'Urso et al., 1990). Any alteration in P0 expression levels will have deleterious effects on the development and the preservation of nerve fibers as well as their myelin sheath. Defects in P0 expression have been involved in disorders due to loss of compaction such as in dysmyelinating neuropathies (Shy, ME., 2006), while the overexpression of P0 results in arrest of myelination and failed axon sorting (Yin et al., 2000). Nevertheless, P0 works alongside with other myelin proteins to attain its function as altered P0 expression alone does not lead to total dysmyelination (Giese et al., 1992).

- ***Peripheral Myelin Protein (PMP22)***

PMP22 accounts for about 2-5% of peripheral myelin proteins and its production is exclusive to SCs (Jetten, A. M., & Suter, U. 2000). Its expression is essential for SC growth and function. In one study, motor nerves obtained from the PMP22 deficient mice revealed immature SCs with failed myelin formation (Adlkofer et al., 1995), emphasizing the role of PMP22 in initiating the myelination process. Furthermore, mutations in the PMP22 gene during development was shown to affect SC differentiation, myelination, and led to SC apoptosis (Sahenk et al., 2003; Jun et al., 2013). These mutations have been associated with genetic disorders and are considered the most common cause for inherited neuropathies or Charcot-Marie-Tooth (CMT) disease (Katona et al., 2009).

Together, the heterophilic interactions of P0 and PMP22 determine the precise arrangement and function of myelin. Subsequently, any alteration in expression of either protein would modulate the function of SCs, neuronal function, myelin assembly as well as signal exchange to and from the PNS. In contrast to the central nervous system, the PNS has a strong regenerative potential enabling recovery in damaged tissue. In fact, SCs are the primary contributors in directing the regenerative response after peripheral nerve injury. While neurons begin to develop new axons, SCs are reprogrammed rapidly, de-differentiating into non-myelinating highly proliferating cells that secrete neurotrophic factors, remove myelin debris, recruit macrophages and create physical tracks that direct regrowing axons to target tissues (Zhou, Y., & Notterpek, L. 2016; Painter et al., 2014).Accordingly, the genes coding for myelin transcription factor, myelin proteins such as P0 and PMP22 as well as the enzymes of cholesterol synthesis will be downregulated (Chen et al., 2007). Thus, this effective ability to trigger the proper transcriptional repair response after axonal injury is disrupted after SCs damage.

The role of P0 and PMP22 are not well characterized in diabetic peripheral neuropathy. However our understanding of their physiological and pathophysiological function come from Charcot-Marie-Tooth CMT1A studies. One of the potential mechanisms underlying myelin injury in Charcot-Marie-Tooth CMT1A, a progressive hereditary neuropathy, is PMP22 aggregation (Tobler et al., 2002; Fortun et al., 2003; Fortun et al., 2006). In normal SCs, PMP22 is prone to misfolding and most (~80%) of the newly synthesized protein is rapidly degraded in the proteasome. However, in CMT1A, the PMP22 gene is either mutated or overexpressed, which will in turn result in an increased amount of misfolded protein targeted for degradation leading to the formation of cytosolic protein aggregates. This leads to myelination deficits after the accumulation of aggregates in the cytosol and the reduction in the amount of proteins

targeted to the plasma membrane (Fortun et al., 2003; Fortun et al., 2006; Rangaraju, S., & Notterpek, L. 2011). In one study conducted in homozygous PMP22 transgenic rats, SCs were reported to be arrested at the promyelinating stage in the sciatic nerves, suggesting a failure in SC transition from the proliferative stage to the nonproliferative, myelinating phenotype. This transition is a key step for myelination initiation (Niemann et al., 2000). Although SCs exhibited abundant myelin gene expression, namely P0 and PMP22, the myelin profiles were absent in these animals, indicating that PMP22 overexpression accumulates in a late Golgi-cell membrane compartment and blocks late steps of myelin assembly (Niemann et al., 2000).

2. *Schwann Cell and Peripheral Nerve Injury in Diabetes*

The main source of energy in the PNS is glucose, which is processed by SCs and assigned to axonal repolarization (Bradbury, M.W.B., & Crowder, J. 1976). Inefficiency in glucose uptake regulation makes the PNS highly susceptible to chronic hyperglycemia (Leinninger et al., 2006; Hinder et al., 2013). Numerous *in vitro* studies showed that hyperglycemia results in an increased apoptotic activity (Wu et al., 2012; Liu et al., 2016) and reduced proliferative potential (Gumy et al., 2008) in cultured rat primary SCs. Other investigations revealed that the underproduction of neurotrophic factors due to chronic hyperglycemia led to SC de-differentiation (Dey et al., 2013; Hao et al., 2015). Similarly, electron microscopy of sciatic nerves from diabetic rats exhibited disordered myelin sheaths and SCs with shrunken vacuoles, validating formerly described SC dysfunction at the morphological level (Liu et al., 2016). In this regard, SCs have been shown to respond to nerve and myelin injury through a series of degenerative processes, whereby they undergo high-glucose induced loss of axonal association (Dey et al., 2013) and reduced regenerative capacity (Gumy et al., 2008), apoptosis (Wu et al., 2012) de-myelination and de-differentiation (Wu et al., 2012).

SC injury has also been paralleled in electrophysiological studies in DPN. DPN has been shown to be characterized by hyperalgesia which is attributed to the impairment of small myelinated and unmyelinated fibers (Courteix et al., 1993; Ohsawa et al., 2008).Towards the more advanced stages of DPN progression, nociception is reduced concomitant with reduced recorded nerve conduction velocities (Ishii, D. N. 1995), demyelination, dysregulated axonal transport (Juranek et al., 2013), axonal atrophy and degeneration (Fross, R. D., & Daube, J. R. 1987) as well as altered sensory and motor potentials (Becker et al., 2014). This biphasic nerve malfunction and modifications are thought to reflect SC injury (Fukunaga et al., 2005).

Throughout the course of DPN, peripheral nerve injury affects the peripheral neural network of neurons comprised of the ensheathing SCs and the surrounding vascular endothelia. Moreover, research has focused on the involvement of neurons in myelin and nerve destruction (Vincent et al., 2005; Cheng, C., & Zochodne, D.W. 2003; Kamiya et al., 2005), but the mechanisms underlying myelin injury and the cell type that is primarily damaged remains to be elucidated. Also, the impact of each and the order of events leading to the pathology of DPN are not well understood. In fact, several studies indicated that SCs are highly vulnerable to glucotoxicity and are targeted in DPN (bedarith et al., 2009; Chan et al., 2011).

D. The Role of Diabetes and Oxidative Stress in Diabetic Complications

1. The significance and the sources of Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen that are formed as a byproduct of cellular metabolic reactions. They comprise hydrogen peroxide (H_2O_2), superoxide free radicals ($O_2^{\cdot -}$) as well as nitrogen based free radical species such as nitric oxide, peroxynitrite and singlet oxygen. ROS are significant cellular entities by cause of their

contribution to cellular proliferation, signal transduction, host defense, homeostatic preservation and gene expression (Turpaev, K. T. 2002). ROS are under homeostatic, regulatory control by antioxidant defense mechanisms (Dröge, W. 2002). In diabetes, it has been well recognized that oxidative stress plays a key role in the development of complications.

Studies involving antioxidant therapy in diabetic animals showed a preventative potential or possible delay of complications. However, antioxidant therapy was shown to be of limited benefit in diabetic patients (Vincent, A.M. 2011; Pop-Busui et al., 2013). More importantly, the total blockade of ROS has been reported to be fatal (De Zeeuw et al., 2013) indicative of the prominent role of ROS in cellular physiology. Thus, identifying the cellular sources of ROS altered in a disease specific manner is essential to better comprehend the pathobiology of diabetes and its complications.

Extensive research has identified the cellular sources of ROS to include nicotinamide adenine dinucleotide phosphate oxidase (NOXs), cytochrome P450 monoxygenase, nitric oxide synthase, lipoxygenase, cyclooxygenase, and xanthine oxidase (Niedowicz, D. M., & Daleke, D. L. 2005). Many of these sources have been linked to intracellular glucose metabolism via glucose autoxidation, mitochondrial oxidative phosphorylation, and the production of advanced glycation end products (AGEs). However, when ROS production overwhelms the cellular defense mechanisms, injury ensues in the form of lipid and protein oxidation, altered metabolism, activation of intracellular signaling and transport pathways, and eventually programmed cell death (Baynes, J.W., & Thorpe, S.R. 1999).

2. *Oxidative stress in Diabetes and Diabetic Neuropathy*

During the onset and progression of diabetic complications, the elevated systemic and cellular oxidative stress associated with persistent hyperglycemia is currently thought to be the final common pathway leading to cellular injury (Baynes, J.W., & Thorpe, S.R. 1999; Feldman et al., 1997; Giugliano, D., & Ceriello, A. 1996; Kowluru, R.A., & Kennedy, A. 2001). Indeed, the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy, and cardiomyopathy has been firmly linked to the accumulation of advanced glycation end products (AGEs) (Berner et al., 2012; Oldfield et al., 2001; Wada et al., 2001; Ma et al., 2009). Other studies showed implications of NOXs (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013a,b,c ; Eid et al., 2016; Gray et al., 2013; Ago et al., 2004), cytochrome P450 monoxygenase (Eid et al., 2009; - Eid et al., 2014; Wang et al., 2011), lipoxygenase (Suzuki et al., 2015 ;Obrosova et al., 2010), cyclooxygenase (Retailleau et al., 2010 ; Kellogg et al., 2008), and xanthine oxidase (Miric et al., 2013;Romagnoli et al., 2010) in hyperglycemia-induced oxidative stress in various diabetic complications. ROS attenuation through the specific inhibition of distinct sources was observed in renal and cardiovascular pathologies (Eid et al., 2009; Eid et al., 2010; Lambeth, J. D., Krause, K. H., & Clark, R. A. 2008; Wu et al., 2012). However the mechanisms by which oxidative stress influences the progression of diabetic complications remain inconclusive and have yet to be elucidated.

Growing evidence points to nerve dysfunction as a result of oxidative stress as well (Vincent et al., 2009; Hichor et al., 2016). Recently, the association between elevated ROS production and myelin injury or nerve dysfunction has been reported in different studies (Obrosova et al., 2005; Hamilton et al., 2013; Oh et al., 2012). Oxidative stress was shown to induce PMP22 carbonylation, one of the most stern and irreversible oxidative complications, and

aggregation which is linked to reductions in sciatic nerve NCV and myelin thickness (Hamilton et al., 2013). Another study further showed that even in the absence of a metabolic dysfunction, oxidative stress is sufficient to impair myelination in the sciatic nerve, alter myelin gene expression and cause PMP22 aggregation, which in turn led to severe locomotor and sensory defects in mice (Hichor et al., 2016). Current efforts are fixated on identifying the sources of ROS associated with the pathophysiology of the peripheral nerve and its associated SCs along with the involved mechanisms (Nayernia et al., 2014; Li et al., 2016). Ongoing work in our laboratory among other studies identified NOX enzymes to be pivotal sources of ROS altered in the diabetogenic peripheral nerve and to be key mediators of injury in DPN.

3. *The NOXs: Modulators of Diabetogenic Peripheral Nerve Function*

NADPH oxidases are a family of proteins whose function is the generation of (ROS) across biological membranes. In humans, the Nox family includes seven members: Nox1, Nox2, Nox3, Nox4 and Nox5, DUOX1 and DUOX2, each with different activation mechanisms and different expression levels in numerous tissues**.** These transmembrane proteins share certain structural properties: a NADPH-binding site, a flavin adenine dinucleotide (FAD)-binding region, six conserved transmembrane domains, and four highly conserved heme-binding histidines (Bedard, K., & Krause, K.H. 2007). NADPH oxidase enzymes are characterized by a relatively low expression with sufficient activity in normal physiological conditions. However, this activity can be triggered intensely and chronically in response to stimuli such as cytokines (Rustenhoven et al., 2016), growth factors (Brandes et al., 2001), hyperlipidemia (Miller et al., 2010), and hyperglycemia (Eid et al., 2010) and leads to homeostatic disruption and a wide range of disorders. For the scope of this study, the focus will be on Nox1.

Nox1 is described to be a 55–60 kDa protein that necessitates p22phox, NoxO1 (Nox Organizer 1), NoxA1 (Nox Activator 1), and the small GTPase Rac to be activated. It has been reported that Nox1-derived ROS production is involved in various physiological processes. These include cell growth (Ibi et al., 2006), differentiation and apoptosis, cell signaling, blood pressure regulation (Gavazzi et al., 2006), as well as angiogenesis (Arbiser et al., 2002). However, studies have shown that Nox1- dependent ROS generation also contributes to the pathophysiology of diabetes and diabetic complications.

Indeed, beta cell function in diabetic mice and humans was conserved upon selective Nox1 inhibition (Weaver et al., 2015). Similarly, pharmacological inhibition or silencing of Nox1 both led to the renoprotection of the diabetic kidney in nephropathy (Gorin et al., 2015; Zhu et al., 2015). Additional studies showed the significance of Nox1 levels in vascular dysfunction in the aorta of diabetic animals (Wendt et al., 2005), and contributed to diabetes–induced atherosclerosis (Gray et al., 2013). Recently, mounting evidence shows NOXs to play a key role in pain sensitization, where hypoalgesia was detected in inflammatory pain mice models lacking Nox1 (Ibi et al., 2008).

Importantly, no studies have reported a specific inhibition of the NADPH oxidases subfamilies in diabetic neuropathy. However, general inhibition of NADPH oxidases was shown to prevent the progression of diabetic complications in *in vitro* and *in vivo* studies (Ahmad et al., 2012; Cotter, M. A. & Cameron, N. E. 2003; Sonta et al., 2004). The administration of apocyanin, a NOX inhibitor, in an STZ-induced type 1 diabetic rat model was able to reestablish nerve conduction velocity and improve blood flow deficit and vascular conductance proposing an injurious role of the Nox family in neurovascular deviations initiated by diabetes (Cotter, M. A. & Cameron, N. E. 2003) and thus attenuate DPN. However, these studies utilized

compounds that are nonspecific NOX inhibitors, by cause of their ability to inhibit other enzymes as well (Vincent, A.M. 2011). Hence, this restriction limits the ability to clarify the direct functional contribution of NADPH oxidases in these effects. Collectively, these data suggest a pathogenic feature of Nox1/Nox4 that has yet to be better understood in DPN and necessitate the importance of using compounds that selectively inhibit NOX isoforms, such as GKT, from the pyrazolopyridine family of chemicals.

E. The mTOR Pathway and PNS Myelination

The mammalian/mechanistic target of rapamycin (mTOR) pathway is one that integrates various environmental signals and translates them into cellular processes, controlling cellular growth, survival and metabolism (Laplante, M., & Sabatini, D.M. 2012). mTOR is a highly conserved serine/threonine protein kinase that interacts with several protein associations to form two complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2). These mTOR complexes differ in their sensitivity to rapamycin, their upstream inputs and downstream targets; mTORC1 signaling is repressed upon acute rapamycin treatment. However, long term rapamycin treatment may diminish mTORC2 function through compromising complex assembly to variable degrees depending on cell type (Sarbassov et al., 2006).

Upon phosphorylation and activation by mTORC1, the downstream effectors, p70S6 Kinase (P70S6K) and 4E-binding protein 1 (4E-BP1), promote mRNA biosynthesis as well as translational initiation and elongation (Ma, X.M., Blenis, J. 2009). Consequently, mTORC1 influences protein synthesis and cell size, controls numerous anabolic processes and suppresses autophagy. Yet, the negative regulation of mTOR activity is controlled by the heterodimeric complex consisting of tuberin (TSC2) and hamartin (TSC1). Furthermore, the phosphorylation of

tuberin by AMP-activated protein kinase (AMPK) maintains its tumor-suppressor activity and prevents the activation of mTORC1 (Eid et al., 2013a). However, compared to mTORC1, very little is known about the mTORC2 physiology and pathophysiology, but mTORC2 signaling is thought to respond to growth factors such as insulin but is insensitive to nutrients unlike mTORC1 (Zinzalla et al., 2011). mTORC2 phosphorylates its downstream effector Akt (a serine/threonine kinase and a key effector) fully activating it (Sarbassov et al., 2005), and modulating cellular processes such as metabolism, cytoskeletal organization, survival, apoptosis, growth, and proliferation by phosphorylating several downstream effectors (Wullschleger et al., 2006).

1. mTOR in Diabetic Complications

In the pathophysiology of diabetes, several injurious pathways have been linked to the persistent activation of mTORC1 signaling (Zoncu, R., Efeyan, A., & Sabatini, D. M. 2011).Emerging evidence has described an amplified basal mTORC1 activity in both genetic and diet-induced animal models of obesity and pre-diabetic disorders (Khamzina et al., 2005; Turdi et al., 2011). In fact, hyperglycemia is thought to be the major player in activating mTORC1 signaling in diabetes and its complications. Indeed, studies have suggested that the blockade of mTORC1 via rapamycin may have protective effects on the diabetic kidney in both type 1 and type 2 diabetic animal models (Eid et al., 2013a; Lloberas et al., 2006; Yang et al., 2007). Besides, *in vivo* studies in type 2 diabetic mice have shown that rapamycin treatment prevents cardiac dysfunction, attenuates oxidative stress and adjusts the expression of antioxidant and contractile proteins (Das et al., 2014).

As for the involvement of mTORC2 in diabetic complications pathogenesis, our group has previously validated that rictor/mTORC2 pathway may induce podocyte apoptosis *in vitro* and *in vivo* and increase NADPH-dependent oxidative stress in diabetic nephropathy (Eid et al., 2016). Similarly, it has been recently reported that mTORC2 mediates mesangial cell hypertrophy in the diabetic kidney, proposing a therapeutic potential of mTORC2 inhibition (Das et al., 2016). In a similar context, prolonged low dose of rapamycin treatment inhibiting mTORC1 while preserving mTORC2 signaling succeeded in preventing cardiac dysfunction in type 2 diabetic mice, suggesting a cardioprotective role of mTORC2 in diabetes (Das et al., 2014; Das et al; 2015). Additional extensive research revealed the role of mTORC2 effectors in diabetic retinopathy. mTORC2 effectors have further been show to be associated in the pathogenesis of retinal, endothelial and neuronal cell death induction in response to a diabetic milieu (Huang et al., 2015; Park et al., 2014; Huang, Q., & Sheibani, N. 2008; Yadav et al., 2012).

2. mTOR, Schwann Cells and Diabetic Neuropathy

Currently, little is known about the role of mTOR in DPN. This pathway has been shown to be crucial for the initiation and the extent of the myelination process (Norrmén et al., 2014) such that in another study, it was reported that an arrest of myelination and reduction in nerve conduction velocities occurred in response to total mTOR deficiency (Sherman et al., 2012).Moreover, a recent study reported alleviation of neuropathic pain after rapamycin administration in STZ-induced type 1 diabetic rats (He et al., 2016). mTORC1 signaling was further shown to influence SC myelination in explants cultures of DRG neurons from neuropathic mice. However, upon treatment with rapamycin, PMP22 aggregation was shown to be reduced, restoring myelin integrity in SCs (Rangaraju, S., & Notterpek, L. 2011). Despite that,

the involvement of mTORC1 in the cellular and molecular cross talks with other pathways and especially mTORC2, and their role in DPN and myelination is not described so far.

In the PNS, emerging evidence described the possible role of mTORC2 effectors in SC myelination (Domènech-Estévez et al., 2016). Actually, the current literature describes Akt to be essential to the survival and progression of the cell cycle in rat SCs, (Li et al., 2001; Monje, P. V., Bartlett Bunge, M., & Wood, P. M. 2006) as well as in the regulation of the migratory potential following peripheral nerve injury (Yu et al., 2015). Neuropathic pain transmission as a result of oxidative stress was reported to be associated with mTORC2 modulation (Guedes et al., 2008). Yet, the precise role of the mTORC2 pathway in PNS myelination is still controversial and emphasizes the significance of additional studies to unravel the cellular functions regulated by this signaling pathway.

F. Hypothesis and Aim of Study

Although strict glycemic control can exacerbate organ damage in diabetic complications, DPN onset is autonomous. Consequently, investigating the mechanisms involved in the pathogenesis of DPN is fundamental to identify novel therapeutic targets. This work is centered to study the mechanisms leading to SCs injury in DPN. The aim of this work is to display the influence of hyperglycemia-induced oxidative stress generated by NOX1 in altering the mTOR pathway (mTORC1 and mTORC2) leading to SC injury and peripheral nerve atrophy and dysfunction. mTOR complexes and their specific roles in PNS myelination are further examined using pharmacological agents that target these mechanistic stations in the pathway and dissect it. Therapeutic potential and effectiveness may be validated via behavioral, functional and molecular assessments of SCs and peripheral nerves.

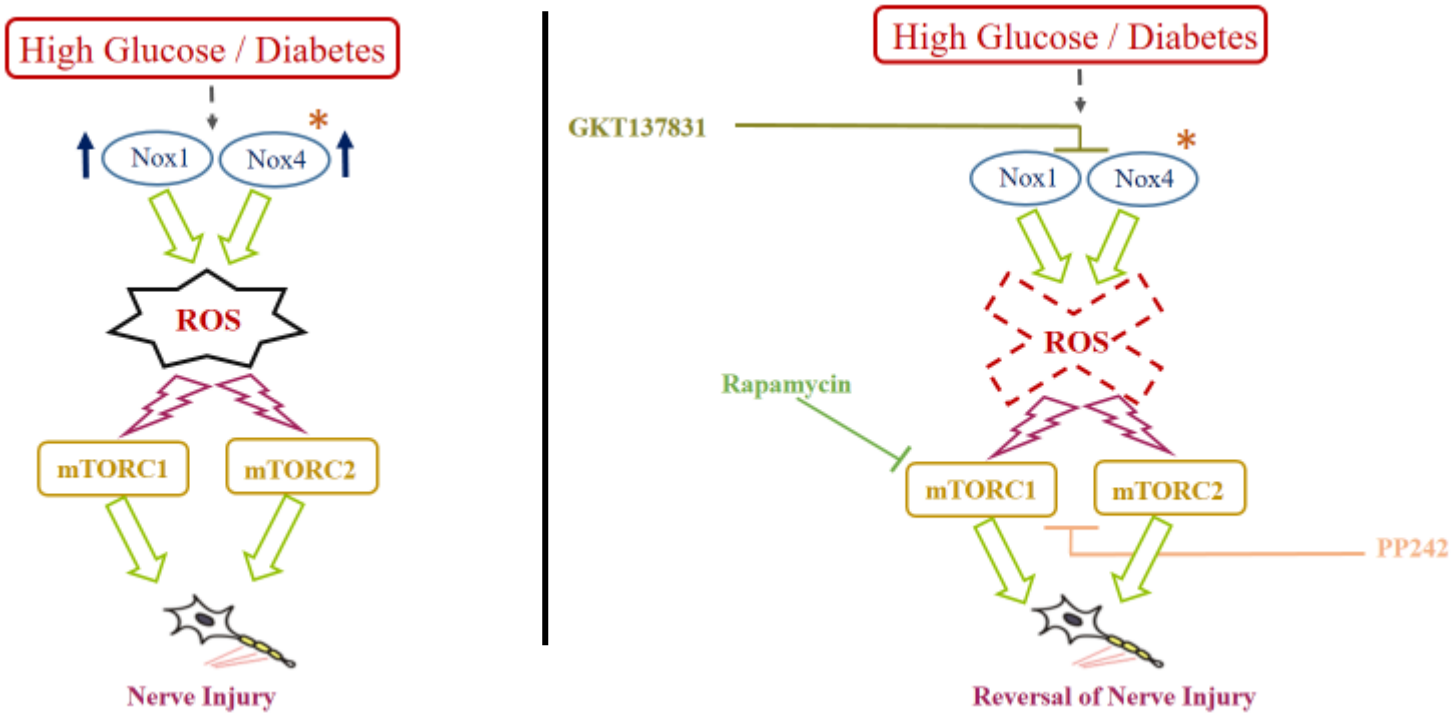

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Figure 1. Hypothesis of the study. Hyperglycemia leads to oxidative stress through activation of NADPH oxidase Nox1. Increased ROS production, causes Schwann cell injury, myelin protein alterations and induces apoptosis through mTORC1/mTORC2 activation.

*Adapted from Eid, S et al., unpublished data.

CHAPTER II

MATERIALS AND METHODS

A. Animal Models

All animal procedures were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the institutional animal care and use committee at the American University of Beirut and by the Regional Ethic Committee (authorization CE2-04) at Paris Descartes University. Three animal models of diabetes were used in this study: Streptozotocin (STZ) -induced swiss webster mice, Streptozotocin (STZ) -induced C57/BL6 mice and non-obese diabetic (NOD) (The Jackson Laboratory, Bar Harbor, ME) mice.

Eight- week old Swiss-Webster male mice (weighing around 40 g) and C57BL/6 male mice (weighing around 25 g) received a single 100 mg/kg body weight intraperioteneal injection of STZ (Sigma-Aldrich, Steinheim, Germany) dissolved in sodium citrate buffer (0.01 M, pH 4.5). Controls received similar injections of citrate buffer. Glucose measurement was performed one week after the STZ injection and blood was obtained via tail vein punctures and a glucometer (Accuchek, Roche). Mice with a fasting blood glucose ≥ 250 mg/dl were considered diabetic. Blood glucose levels were monitored weekly and were significantly different in diabetic animals relative to control littermates. The STZ-model is widely used in DPN studies because it displays an early neuropathic phenotype as well as alterations in neurologic functions (O'Brien et al., 2014)

Twelve-week old FVB and NOD mice on FVB background and weighing 23-25g, were purchased from Jackson Laboratories (Bar Harbor, ME). NOD mice were deemed diabetic after

two consecutive days of glucosuria and with blood glucose levels $\geq$250 mg/dl. NOD mice exhibit an early development of thermal hypoalgesia, which mimics the sensory loss observed in diabetic patients (Obrosova et al., 2005; O'Brien et al., 2014).

Diabetic animals were given one of the following treatments:

- 40mg/kg body weight of GKT137831, a specific Nox1/ Nox4 inhibitor administered three times a week by oral gavage.
- 5 mg/kg/week of PP242, a dual mTORC1/2 inhibitor administrated three times a week by intraperitoneal injection.
- 0.5 mg/kg/body weight of rapamycin (Rapa), an mTORC1 inhibitor administrated three times a week by intraperitoneal injection.

For every treatment, the animals were grouped into three subsets: I, control mice; II, diabetic mice; III, diabetic treated mice.

All animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and had standard chow and water access. Before euthanizing the animals, sensorimotor dysfunction was assessed using the Raised Beam Walking Test and Hind paw Withdrawal Test and the extent of nerve dysfunction was assessed using nerve conduction velocity.

B. Behavioral and Functional Tests

Thermal hyperalgesia. The sensitivity to radiant heat was assessed using the Hind paw withdrawal test (Dirig et al., 1997). The IITC plantar Analgesia meter was used and set up according to the manufacturer's protocol (IITC model 336 TG combination tail-flick and paw algesia meter; IITC Life Science). The test is characterized by a heating beam set at an idle

intensity of 2% and active intensity of 25% with a cut-off time set at 20 seconds. The platform onto which animals were placed for acclimation was set at 32 °C. The heating beam was targeted at the hind paw of animals and the time to sense the heat and withdraw their paws was recorded for analysis. At least six readings per animal were taken at 15 min intervals, and the average was calculated.

Raised beam walking test. Mice were placed on a platform with a rod of 3 cm diameter, 70 cm length and around 30cm above a flat surface (Hichor et al, 2016). At one end of the rod we set a secure platform to house the animal. First, the mouse was allowed to adapt and then trained to cross the beam after which the time taken to traverse, the speed, the number of stops and the number of left or right hindpaw faults/slips were recorded for analysis (Muller et al., 2008). The animals were recorded for three trials per session on week 8 post-STZ injection. The behavior task was recorded using a high definition digital camera.

Nerve conduction studies. Measures of nerve conduction velocity (NCV) were performed in anaesthetized mice at 32–34°C using a heating pad. Motor Nerve Conduction Velocity (MNCV) was determined by measuring compound muscle action potentials using supramaximal stimulation distally at the ankle and proximally at the sciatic notch. The MNCV was calculated by dividing the distance between the cathode positions by subtracting the distal latency from the proximal latency. Sensory Nerve Conduction Velocity (SNCV) was recorded behind the median malleolus in the digital nerve to the second toe by stimulating with the smallest current that resulted in a maximal amplitude response (Muller et al., 2008; Obrosova et al., 2005; Vincent et al., 2007).

C. mRNA analysis.

mRNA was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method (Eid et al., 2009). Total RNA was isolated from mice sciatic nerves using an RNeasy Mini kit from Qiagen.

mRNA expression was quantified using a Realplex mastercycler (Eppendorf, Westbury, NY) with SYBR green dye and mouse RT^2 qPCR Primers in the table below:

Primers	Sequence
MPZ	F: 5' -GTCAAGTCCCCCAGTAGAA-3' R: 5' -AGGAGCAAGAGGAAAGCAC-3'
PMP22	F: 5' -AATGGACACACGACTGATC-3' R: 5' -CCTTTGGTGAGAGTGAAGAG-3'
Nox1	F: 5' -AAATGAGGATGCCTGCAACT-3' R: 5' -GGGTCAAACAGAGGAGAGCTT-3'
mTOR	F: 5'-AAGCCCGTGATGAGAAGAAG-3' R: 5' -GGGCTGTTCTCATTGCTCTC-3'
Rictor	F: 5' -TGCCTCCCTCAATGAAAAAC-3' R: 5' -GCAATCTTGATGGGRGTGGT-3'
26s	F: 5' -AGGAGAAACAACGGTCGTGCCAAAA-3' R: 5' -GCGCAAGCAGGTCTGAATCGTG-3'

Table 1. List of Primers

D. Western Blot Analysis.

Homogenates from sciatic nerves were prepared in 150 μl of radioimmune precipitation assay buffer containing 20 mmol/l Tris·HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l

Na_3VO_4, 1 mmol/l PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1% NP-40. Homogenates were incubated for two hours at 4°C and centrifuged at 13,000 rpm for 30 min at 4°C.

Cultured mouse Schwann cells were grown to near confluence in 100 mm dishes and serum-deprived for 12 h. All incubations were carried out in serum-free DMEM containing 0.5% FBS at 37^0C for 48h. SCs were then lysed in radioimmune precipitation buffer at 4^0C for two hours. The cell lysates were then centrifuged at 13 000 rpm for 30 min at 4^0C.

Proteins in the supernatants were measured using the Bradford Protein Assay. For immunoblotting, proteins (20-40 μg) were separated on 12-15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with rabbit polyclonal anti-Nox1 (1:500, Santacruz), rabbit polyclonal anti-myelin protein zero (1:1000, Abcam), rabbit polyclonal anti-PMP22 (1:1000, Sigma), rabbit polyclonal anti-pP70S6K^{Thr389} (1:1000, cell signaling), rabbit polyclonal anti-p-mTOR ^{ser2448}, anti-mTOR (1:1000, cell signaling) and anti-p-70S6K (1:1000, cell signaling). The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:20000). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using National Institutes of Health Image software.

E. NADPH Oxidase Activity

NADPH oxidase activity was measured in sciatic nerve homogenates. Proteins were extracted from sciatic nerves using cooled mortar and pestle by smashing the frozen nerve and suspending the remnants in the lysis buffer. To start the assay, 20 μg of homogenates were added to 50 mmol/l phosphate buffer, pH 7.0, containing 1 mmol/l EGTA, 150 mmol/l sucrose, 5 μmol/l lucigenin, and 100 μmol/l NADPH. Photon emission expressed as relative light units was measured every 30 s for 10 min in a luminometer. A buffer blank (<5% of the cell signal) was

subtracted from each reading. Superoxide production was expressed as relative light units per milligrams of protein. Protein content was measured

F. Detection of Intracellular Superoxide in Sciatic Nerves Using HPLC

Cellular superoxide production in sciatic nerves was assessed by HPLC analysis of dihydroethidium (DHE)-derived oxidation products. The HPLC-based assay allows the separation of the superoxide-specific 2-hydroxyethidium (EOH) from the nonspecific ethidium, as previously described (Eid et al., 2016). Briefly, homogenates from sciatic nerves are washed twice with Hanks' balanced salt solution (HBSS)-diethylenetriaminepentaacetic acid (DTPA) and incubated for 30 min with 50 μM DHE (Sigma-Aldrich) in HBSS–100 μM DTPA. Tissues were harvested in acetonitrile and centrifuged (12,000 X g for 10 min at 4°C). The homogenate was dried under vacuum and analyzed by HPLC with fluorescence detectors. Quantification of DHE, EOH, and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by UV absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (i.e., initial minus remaining DHE in the sample; μmol).

G. TUNEL Assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) staining using the TUNEL Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) was performed according to the manufacturer's instructions. Five animals were studied per group.

Briefly, sciatic nerves were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS). Next, tissues were incubated with Cytonin (Trevigen), washed with PBS twice, quenched with 0.3% hydrogen peroxide in methanol, washed in PBS, and placed in labeling buffer (Trevigen). Then, samples were incubated in the labeling reaction mixture containing TdT deoxynucleotide triphosphate mix (Trevigen), cobalt, TdT enzyme, and labeling buffer. The reaction was quenched in Stop Buffer, washed in PBS, treated with strep-horseradish peroxidase solution, washed in PBS, and placed in diamidobenzidine solution. Finally, the samples were washed several times in PBS, counterstained in Methyl Green, and visualized by light microscopy

H. PMP22 aggregation

Cultured mouse Schwann cells were grown in serum-free DMEM media containing 0.5% FBS at 37^{0}C for 48h to near confluence, in 100 mm dishes. Cells were then serum-deprived for 12 h. Sciatic nerves were then lysed in immunoprecipitation buffer (10 mM Tris–HCl [pH 7.5], 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, and 150 mM NaCl) that was supplemented with protease inhibitors (Fortun et al., 2003). The lysates were microcentrifuged, the supernatant was removed, and the insoluble phase was incubated with 10 mM Tris–HCl, 3% SDS for 10 min at room temperature. After a brief sonication, total protein concentrations were measured using the Bradford Protein Assay. Equal amounts of samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes and blocked with 5% BSA/TBS-0.1% Tween and incubated with the indicated PMP22 primary antibody. They were then incubated at room temperature for 1 h with the appropriate secondary antibody, followed by ECL Plus. Protein bands were visualized with an enhanced chemiluminescence detection system. Western blots were quantified by means of NIH Image J Software.

I. Cell Culture and Transfection

Mice Schwann cells (MSC80) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) containing 5 mM glucose normal glucose (NG) or treated with 25 mmol/L glucose (HG) for 48 h in the presence or absence of 20 μM GKT137831 (Nox1/Nox4 inhibitor), in the presence or absence of rapamycin (mTORC1 inhibitor) (20 nmol/L) or), in the presence or absence of PP242 (mTORC1/2 inhibitor) (1 umol/L). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO2.

For the RNA interference experiments, a SMARTpool consisting of small interfering RNA (siRNA) duplexes, specific for Nox1 or mTORC1 were purchased from Dharmacon. siRNA (100 nmol/L) was introduced into the cells by a single transfection using Oligofectamine or Lipofectamine 2000, as previously described (Eid et al., 2013a). Scrambled siRNAs (nontargeting siRNA, Scr; 100 nmol/L) were used as controls. In addition, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas9) system for rictor was purchased from Santa Cruz Biotechnology, Inc. rictor CRISPR/Cas9 KO plasmid (0.1 ug/uL) was introduced into the cells by a single transfection using Oligofectamine or Lipofectamine 2000, as previously described (Eid et al., 2013a). Control CRISPR (nontargeting CRISPR, 0.1 ug/uL) was used as a negative control.

J. Statistical analysis

All statistical analysis was performed using Prism 6 software (GraphPad Software). The results are expressed as means ± standard errors (SE) from multiple independent experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post-test when more than two variables were analyzed. Two group comparisons were performed by student's unpaired t-

test. Statistical significance was determined as a p-value <0.05. When comparing control and diabetic, one asterisk (*) is used when $p<0.05$. The (#) symbol is used when comparing untreated diabetic groups vs. treated diabetics at $p<0.05$.

CHAPTER III

RESULTS

A. Hyperglycemia induces ROS production through an NADPH oxidases-dependent mechanism leading to PNS injury in diabetes.

Reactive oxygen species (ROS) production is currently believed to be the final common pathway that is involved in hyperglycemia-induced diabetes pathophysiology. Numerous sources of ROS have been identified to be altered in response to high glucose such as the NADPH oxidase family of enzymes. The specific isoform Nox4 has been studied and shown to be involved in diabetic complications such as nephropathy (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013a; Eid et al., 2016) and cardiomyopathy (Zhao et al., 2015). Additionally, unpublished data from our lab have shown the involvement of Nox4 in DPN pathogenesis. Yet, little is known about the involvement of Nox1 in the peripheral nervous system (PNS) and diabetic neuropathy.

Consequently, we first assessed ROS production and superoxide production using high performance liquid chromatography (HPLC) and DHE (Dihydroethidium), STZ-induced type 1 diabetic and Non- obese type 1 diabetic (NOD) mice were treated with either vehicle or GKT137831 at 40 mg/kg/day, administered by oral gavage for 8 weeks while mice in the control groups (Ctr and FVB) received vehicle treatment. Sciatic nerves were isolated from six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with GKT and FVB control mice, NOD mice, and NOD mice treated with GKT. Our data show an increased superoxide production in STZ-induced diabetic and NOD mice compared to their control littermates (**Figure 2A**). This was accompanied by an increase in NADPH oxidase activity, indicative of the NADPH

oxidase-dependent mechanism which was evident through elevated Nox1 mRNA levels and protein expression (**Figure 2B-E**). Interestingly, the use of GKT137831, a specific inhibitor of Nox1/4, decreased NADPH oxidase activity (**Figure 2B**), Nox1 mRNA levels and protein expression (**Figure 2C-E**) and reduced ROS overproduction (**Figure 2A**). These data show the direct role of NADPH oxidases in hyperglycemia-induced ROS production.

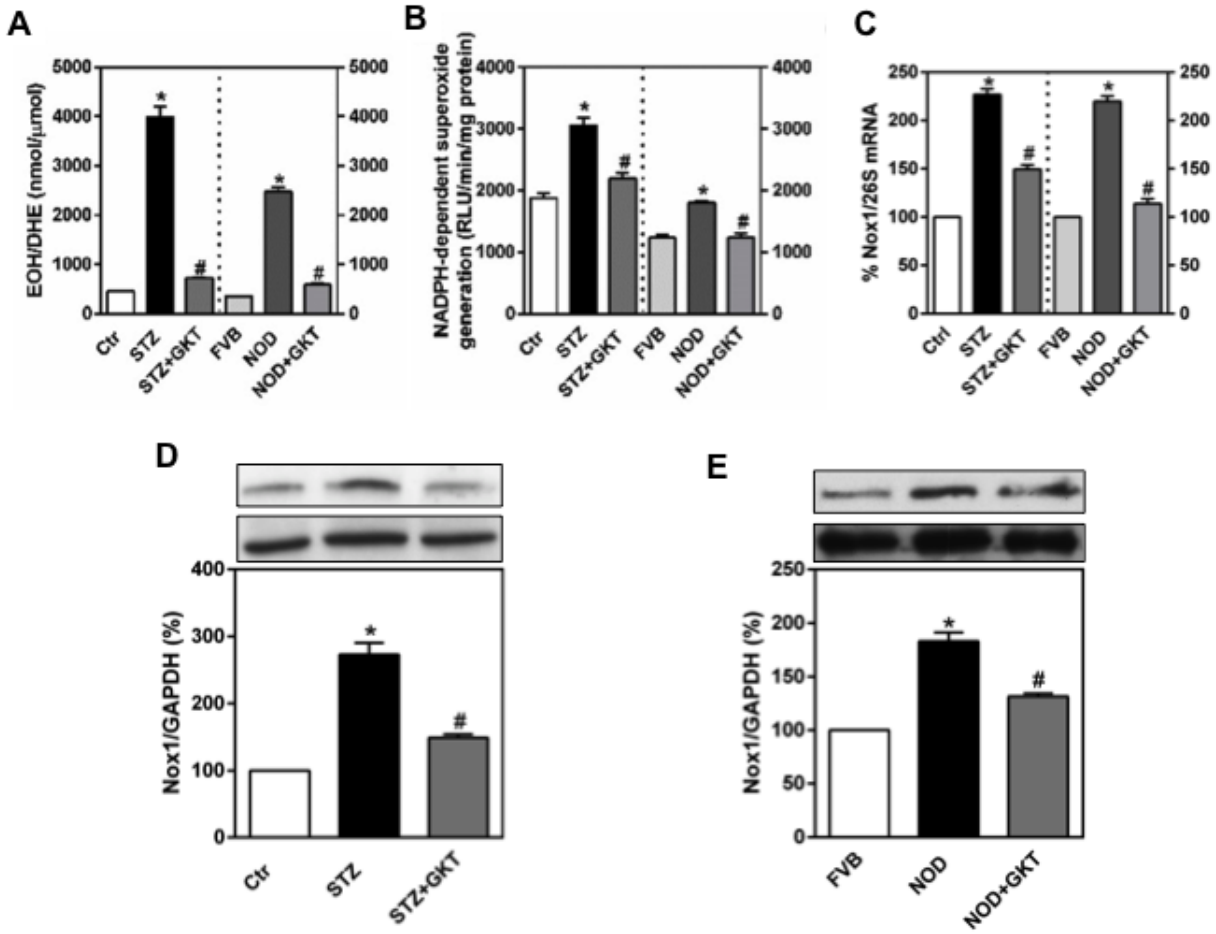

***Figure 2*. Hyperglycemia upregulates Nox1 and enhances NADPH oxidase activity and superoxide production in sciatic nerves of type 1 diabetic mice.** *(A) Superoxide generation evaluated using DHE and HPLC. (B) NADPH-dependent superoxide generation. (C) Relative mRNA levels. (D) Representative Western blot of Nox1 and GAPDH levels with the respective densitometric quantification in sciatic nerves of control, STZ-induced type 1 diabetic mice and STZ-induced type 1 diabetic mice treated with GKT. (E) Representative Western blot of Nox1 and GAPDH levels with the respective densitometric quantification in sciatic nerves of FVB, NOD mice and NOD mice treated with GKT. Values are the mean ± SE from 5 different mice in each group (n=5). *p < 0.05 versus vehicle-treated Ctr or FVB mice; #p < 0.05 versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.*

To further examine the role of a NADPH oxidase mediated DPN pathogenesis, the behavioral alterations were studied in STZ-induced type 1 diabetic animals and NOD mice upon treatment with GKT. The extent of nerve dysfunction and sensorimotor coordination were assessed by the Nerve Conduction Velocity (NCV) test, the hind paw withdrawal test and the raised beam-walking test (**Figure 3**). The first test conducted was NCV which is indicative of earliest injurious stage in DPN. The data show an overall reduction in NCV of diabetic mice relative to their controls. However, treatment with GKT reversed the neurophysiological defects in diabetic mice, similar to the control levels (**Figure 3A-B)**.

Diabetes has been previously reported to be associated with sensorimotor deficits that manifest as a heightened sensitivity (hyperalgesia and allodynia) in the early stages followed by a nearly complete loss of sensation and motor function in the late stages. Consequently, the hind paw withdrawal test that assesses thermal perception and pain was performed. The results reported a significantly longer period of time for the diabetic mice to respond to the heat of the beam and retract their paws relative to their control littermates and GKT-treated diabetic mice. These results suggest that GKT administration restored nociception and thermal algesia (**Figure 3C**). Similarly, fine motor coordination and balance, were assessed by the raised beam-walking test (Luong et al., 2011) (**Figure 3D-G)**. The recorded measurement from this test reported a significantly longer time frame with an elevated tendency of foot slips and stops while diabetic mice crossed the beam (**Figure 3D - F)** in comparison to their controls that seemed to cross with minimal setbacks. More importantly, GKT-treated diabetic animals behaved similar to the control mice (**Figure 3D-G)**. Collectively, these results indicate that hyperglycemia-induced

NADPH oxidase upregulation mediated neurophysiological and behavioral defects in type 1 diabetic animals.

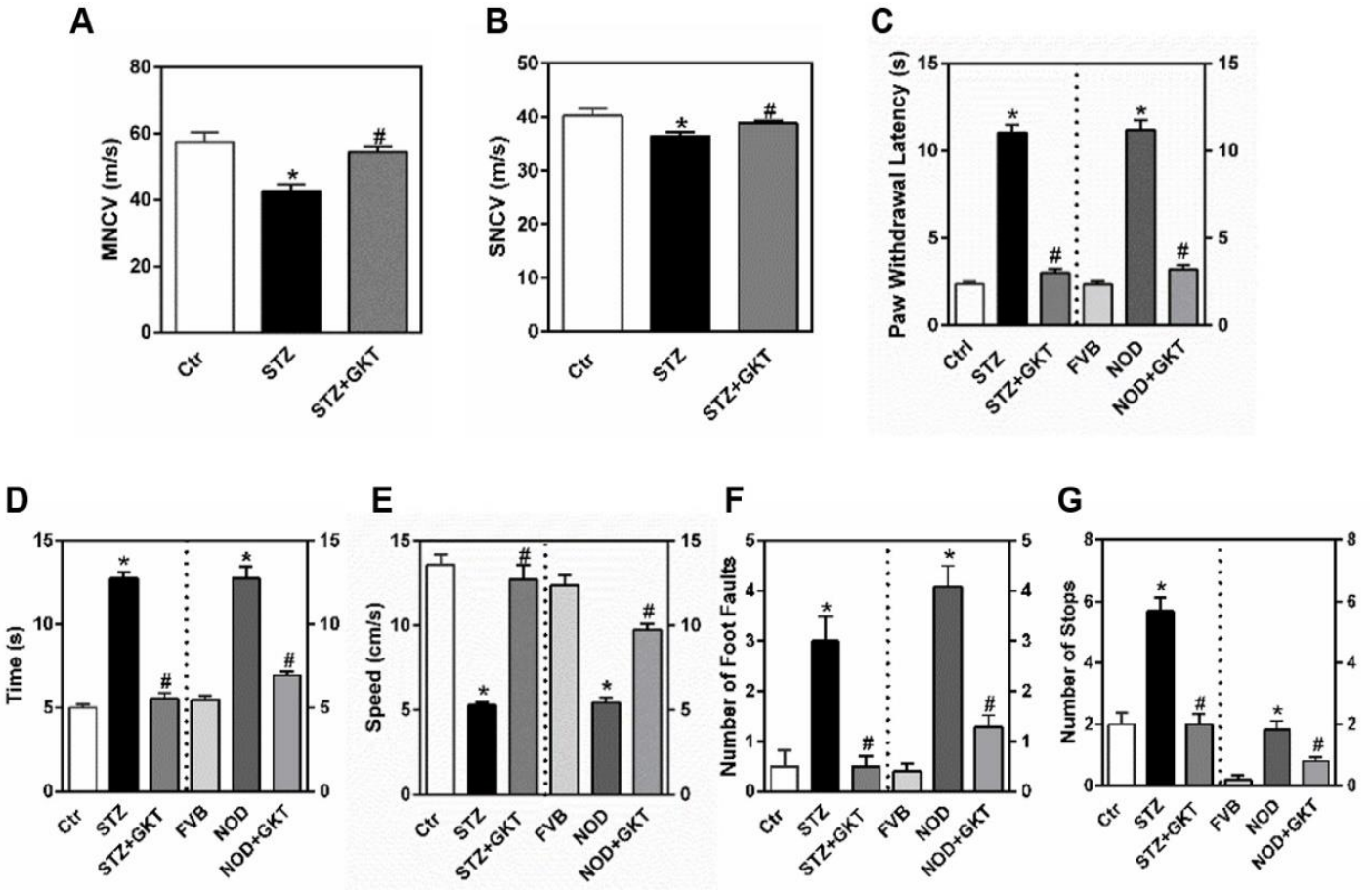

Figure 3. **Nox1 upregulation mediates hyperglycemia-induced neurophysiological and sensorimotor coordination defects in type 1 diabetic mice.** STZ-induced type 1 diabetic and NOD mice were treated with either vehicle or GKT137831 at 40 mg/kg/day, administered by oral gavage for 8 weeks while mice in the control groups (Ctr and FVB) received the vehicle. Behavioral and functional tests were performed on six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with GKT and FVB control mice, NOD mice, and NOD mice treated with GKT. Assessment of (A) MNCV and (B) SNCV after 8 weeks of diabetes in ctr, STZ-induced type 1 diabetic mice and STZ-induced diabetic mice treated with GKT. The histograms confirm nerve dysfunction in STZ-induced type 1 diabetic mouse. This effect was reversed in diabetic mice treated with GKT. (C) Histograms representing thermal sensitivity in response to a heating stimulus of the control/FVB, STZ-NOD and STZ+GKT/ NOD+GKT mice. Assessment of fine motor coordination by the raised beam walking test. The histograms represent the average time (D), speed (E), faults (F), and stops (G). Values are mean ± SE from 5 different mice in each group (n=5). *$p < 0.05$ versus vehicle-treated Ctr or FVB mice; #$p < 0.05$ versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.

In order to correlate the behavioral results with the *in vivo* changes brought about by diabetes, further molecular tests were conducted to investigate the role of Nox1 in myelin injury (**Figure 4**). Sciatic nerves harvested from controls, diabetic and GKT-treated diabetic animals were subjected to a variety of molecular assessments. The results show that hyperglycemia induced myelin injury by altering myelin integrity as seen by increased MPZ and PMP22 at the mRNA level (**Figure 4A-B)** and protein expression level (**Figure 4C-F)** with the concurrent upregulation of Nox1 expression and activity. In addition internucleosomal DNA fragmentation has been a major advance in the detection of apoptosis. TUNEL-positive cells also show some features of apoptotic morphology, such as condensed nuclei. Our data also show a significant TUNEL-positive staining of sciatic nerves derived from STZ-induced diabetic animals (**Figure 4G-H)** in parallel to the upregulation of Nox1 mRNA levels and protein expression. Finally, GKT administration was shown to significantly ameliorate and reverse myelin injury providing further evidence that peripheral nerve injury progressed through a NADPH-oxidase, and potentially Nox1-dependent mechanism.

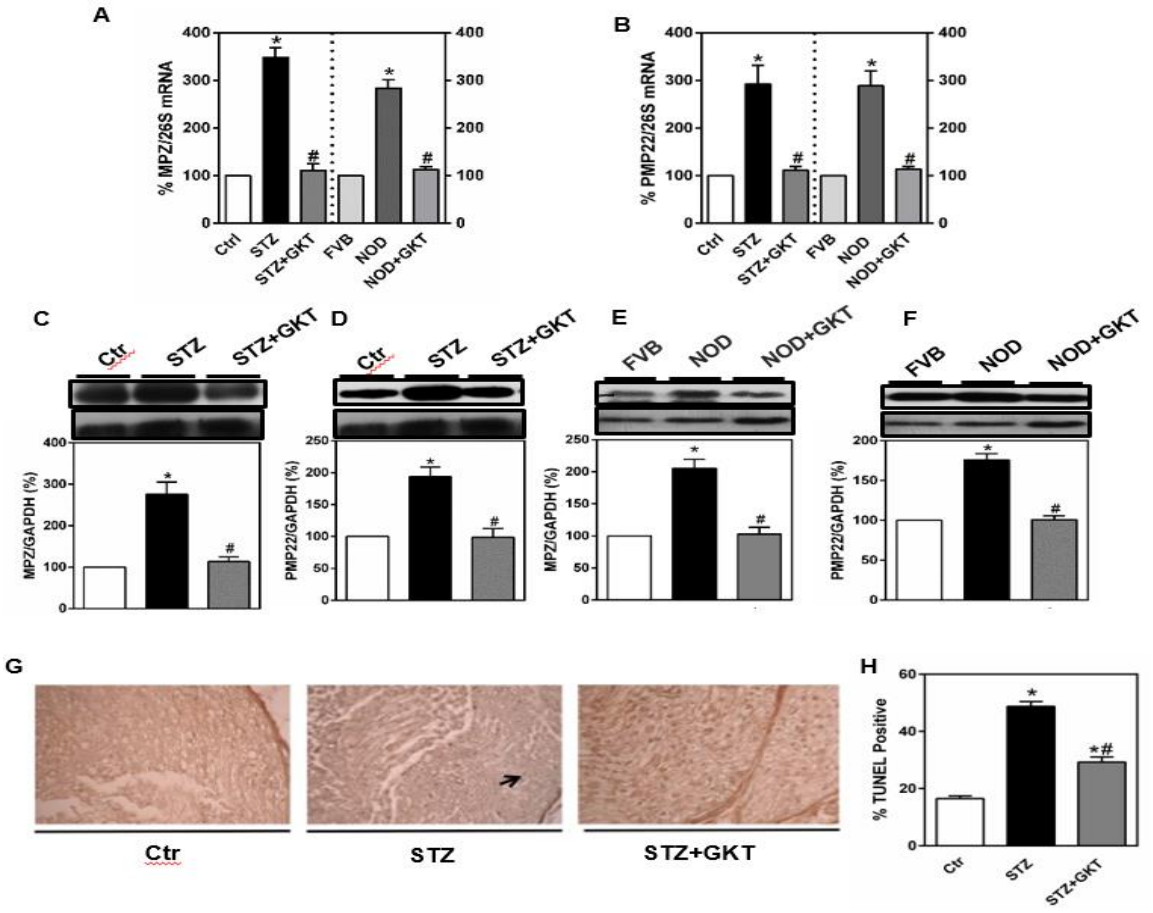

Figure 4. **Nox1 regulates peripheral myelin gene expression and PNS phenotypic changes in type 1 diabetic mice.** STZ-induced type 1 diabetic and NOD mice were treated with either vehicle or GKT137831 at 40 mg/kg/day, administered by oral gavage for 8 weeks while mice in the control groups (Ctr and FVB) received the vehicle. Sciatic nerves were isolated from six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with GKT and FVB control mice, NOD mice, and NOD mice treated with GKT. (A) Relative mRNA levels of MPZ. (B) Relative mRNA levels of PMP22. Representative Western blot of (C) MPZ, (D) PMP22 and GAPDH levels with the respective densitometric quantification in sciatic nerves of control, STZ-induced type 1 diabetic mice and STZ-induced type 1 diabetic mice treated with GKT. Representative Western blot of (E) MPZ, (F) PMP22 and GAPDH levels with the respective densitometric quantification in sciatic nerves of FVB, NOD mice and NOD mice treated with GKT. (G) Sections of sciatic nerves of Ctr, STZ-induced type 1 diabetic, and STZ-induced type 1 diabetic mice treated with GKT stained by TUNEL and (H) percent of TUNEL-positive cells. Values are the mean ± SE from 5 different mice in each group (n=5). *$p < 0.05$ versus vehicle-treated Ctr or FVB mice; #$p < 0.05$ versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.

B. mTORC1/mTORC2 signaling regulates Nox1-induced peripheral nerve injury in diabetes.

Our previous results highlight the role of NADPH oxidase in DPN pathogenesis as well as other complications. Similarly, the mTOR signaling pathway has been reported to play a role in diabetic nephropathy by our laboratory (Eid et al., 2016). Previous reports describe mTOR to contribute to myelination in the PNS. However, to our knowledge, no investigations have been set out to investigate the correlation between mTORC1/2 activation and NADPH oxidases alteration in DPN. Thus, we next wanted to examine alterations of mTOR pathway *in vivo*. Sciatic nerves from controls, STZ-induced type 1 diabetic animals and GKT-treated diabetic animals were used. As we previously described, GKT administration attenuated hyperglycemia-induced up-regulation of Nox1 and NADPH-dependent superoxide generation (**Figure 2)**. We next demonstrated that the attenuation of NADPH-induced ROS generation was accompanied by a decrease in the activation of the mTORC1/p70S6K axis (**Figure 5A-D)** in addition to a decrease in rictor levels (the mTORC2-activating subunit) **(Figure 5E-F)**. These results indicate that diabetes-induced NADPH oxidase Nox1 upregulation is associated with activation of the mTOR pathway in DPN.

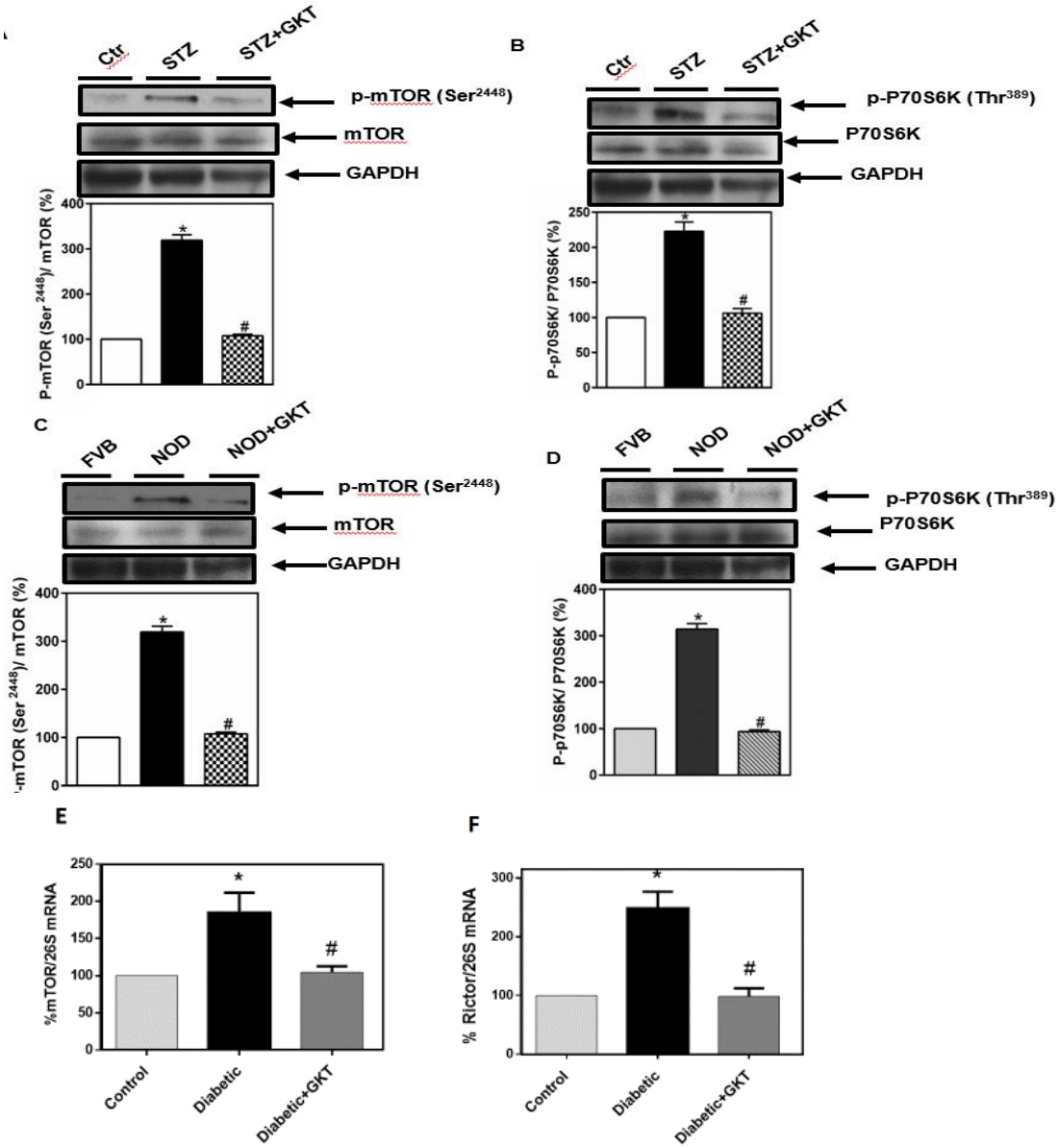

Figure 5. Upregulation of NADPH oxidase Nox1 activates mTOR pathway and leads to peripheral nervous system (PNS) injury in type 1 diabetes. *STZ-induced type 1 diabetic and NOD mice were treated with either vehicle or GKT137831 at 40 mg/kg/day, administered by oral gavage for 8 weeks while mice in the control groups (Ctr and FVB) received the vehicle. Sciatic nerves were isolated from six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with GKT and FVB control mice, NOD mice, and NOD mice treated with GKT. Representative Western blot of (A) p-mTOR/mTOR (B) and p-p70S6K/p70S6K and GAPDH levels with the respective densitometric quantification in sciatic nerves of control, STZ-induced type 1 diabetic mice and STZ-induced type 1 diabetic mice treated with GKT. Representative Western blot of (C) p-mTOR/mTOR (D) and p-p70S6K/p70S6K and GAPDH levels with the respective densitometric quantification in sciatic nerves of FVB, NOD mice and NOD mice treated with GKT. (E), (F) Relative mRNA levels. Values are the mean ± SE from 5 different mice in the different groups (n=5). *p < 0.05 versus vehicle-treated Ctr or FVB mice; #p < 0.05 versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.*

(Same gel was used to detect mTOR/p-mTOR, and the same for P70S6K/p-P70S6K).

C. mTORC1/mTORC2 mediates sciatic nerve injury in DPN

To further understand the effect of mTORC1/mTORC2 inhibition on peripheral nerve injury, behavioral tests were performed (**Figure 6**). STZ-induced type 1 diabetic animals were treated with PP242, a dual mTORC1/mTORC2 inhibitor, for 8 weeks. The extent of nerve function and sensorimotor coordination were assessed by, hind paw withdrawal test and the raised beam walking test.

Hind paw withdrawal test data showed a significantly poor response by the diabetic mice. Whereas PP242 treated animals showed a significantly lower latency compared to diabetic animals, and similar to the control littermates suggesting that the treatment restored thermal algesia (**Figure 6A)**. Moreover, data from the raised beam walking test showed a relatively longer period of time for the diabetic mice to cross the beam (**Figure 6B)** with an increased tendency to slip (**Figure 6D)** and stop (**Figure 6E)** in comparison to their controls that seemed to cross with minimal setbacks. Interestingly, diabetic mice treated with PP242 behaved similar to the control non diabetic animal. Collectively, these results indicate that hyperglycemia-induced mTORC1/mTORC2 activation mediates neurophysiological and behavioral defects in peripheral nerve injury in type 1 diabetes.

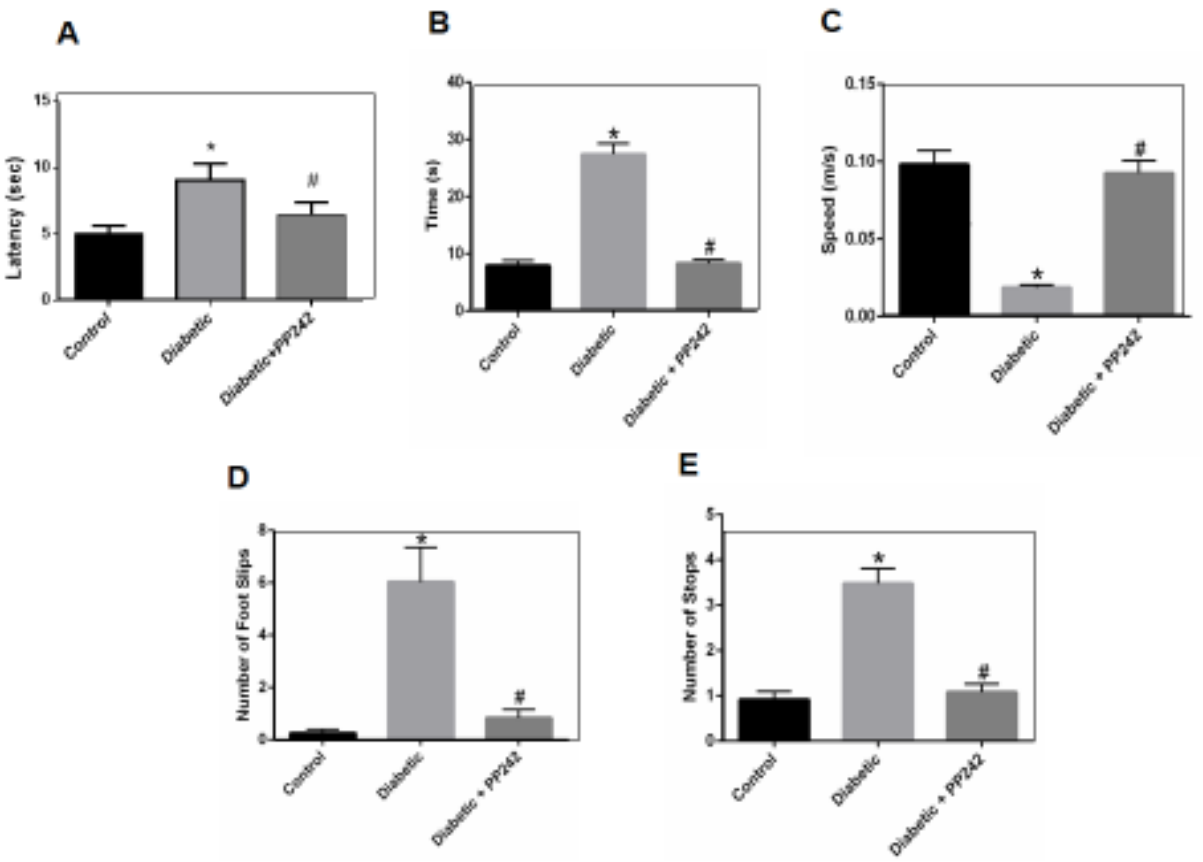

Figure 6: mTORC1/mTORC2 alteration mediates hyperglycemia-induced neurophysiological and sensorimotor coordination defects in Type 1 diabetic mice. STZ-induced type 1 diabetic were treated with PP242 at 5 mg/kg/body weight, administrated three times a week by intraperitoneal injection. Behavioral and functional tests were performed on 3 groups of mice: Control (Ctr)-black mice, STZ-induced diabetic mice, and STZ-induced diabetic mice treated with PP242. The histograms confirm nerve dysfunction in STZ-induced type 1 diabetic mouse. (A) Histograms representing thermal sensitivity in response to a heating stimulus of the control (C57BL/6), STZ and STZ/PP242. Assessment of fine motor coordination by the raised beam walking test. The histograms represent the average time (B), speed (C), faults (D), and stops (E). Values are the mean ± SE from 6 different mice in the different groups (n=6). *$p < 0.05$ versus vehicle-treated Ctr; #$p < 0.05$ versus vehicle-treated STZ-induced type 1 diabetic mice.

In order to correlate the behavioral results with the *in vivo* alterations brought about by diabetes, further molecular tests were conducted. In particular, the involvement of mTORC1/2 pathway in myelin protein expression and myelin injury has been examined in this study (**Figure7**). The results provide evidence that hyperglycemia-triggered myelin injury could be prompted through an increased expression of MPZ and PMP22 at the mRNA (**Figure 7A-B)** and protein levels (**Figure 7C-D)** and preliminary data shows a TUNEL-positive staining in the sciatic nerves of type 1 diabetic animals.

PP242 administration was shown to normalize the physiological alterations of myelin injury reinforcing the key role of mTORC1/mTORC2 in DPN pathogenesis (**Figure 7A**

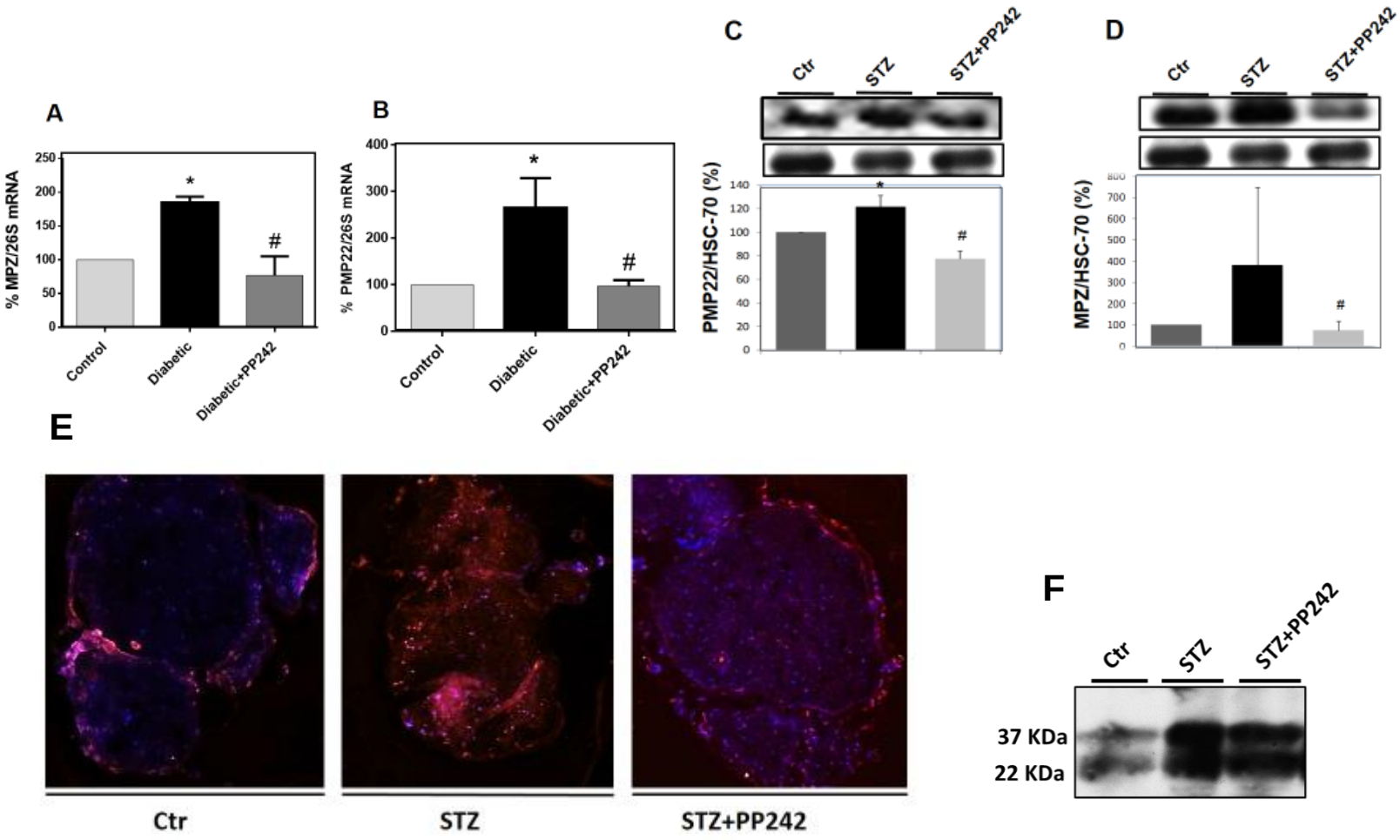

Figure 7. **mTORC1/2 regulates peripheral myelin gene expression and PNS apoptosis in type 1 diabetes.** STZ-induced type 1 diabetic were treated with either vehicle or PP242 at 5 mg/kg/body weight, administrated three times a week by intraperitoneal injection. Sciatic nerves were isolated from three groups of mice: C57BL/6 control mice, C57BL/6 STZ-induced diabetic mice and C57BL/6 STZ-induced diabetic mice treated with PP242. Relative mRNA levels of (A) MPZ and (B) PMP22. Representative Western blot of (C) PMP22, (D) MPZ and GAPDH levels with the respective densitometric quantification in sciatic nerves of control, STZ-induced type 1 diabetic mice and STZ-induced type 1 diabetic mice treated with PP242. (E) Sections of sciatic nerves of Ctr, STZ-induced type 1 diabetic, and STZ-induced type 1 diabetic mice treated with PP242 stained by TUNEL(F) PP242 treatment attenuates diabetes-induced PMP22 aggregation in STZ-induced type 1 diabetic mice. Values are the mean ± SE from 3 different mice in the different groups (n=3). *$p < 0.05$ versus vehicle-treated Ctr; #$p < 0.05$ versus vehicle-treated STZ-induced type 1 diabetic mice.

D. The specific inhibition of mTORC1 via Rapamycin ameliorates hyperglycemia-induced PNS injury

Although dual inhibition of mTORC1/mTORC2 was shown to reverse peripheral nerve injury, the next aim was to examine the effect of mTORC1 inhibition *in vivo* via Rapamycin. STZ-induced type 1 diabetic animal models and NOD mice diabetic mice were treated with Rapamycin for 8 weeks after which the extent of nerve dysfunction and sensorimotor coordination were assessed by NCV, hind paw withdrawal test and the raised beam walking test (**Figure 8)**.

The data show an overall reduction in NCV in sciatic nerves obtained from diabetic mice relative to their controls. Treatment with rapamycin significantly reversed the neurophysiological defects in diabetic mice to levels similar to that of controls (**Figure 8A-B)**. Like previously reported tests, the hind paw withdrawal test showed that diabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws by contrast to the controls. By contrast, a significantly lower latency was recorded in rapamycin treated diabetic mice, indicating its therapeutic potential in restoring thermal algesia and nociception (**Figure 8C)**. Results from the raised beam walking test showed that diabetic animals took a relatively longer period of time to cross the beam (**Figure 8D)** with an increased tendency to slip (**Figure 8F)** and stop (**Figure 8G)** in comparison to their controls. In contrast, rapamycin treated type 1 diabetic mice behaved similar to the controls non-diabetic animals (**Figure 8D-G)**. Collectively, these results indicate that hyperglycemia-induced mTORC1 activation mediates neurophysiological and behavioral defects in type 1 diabetes.

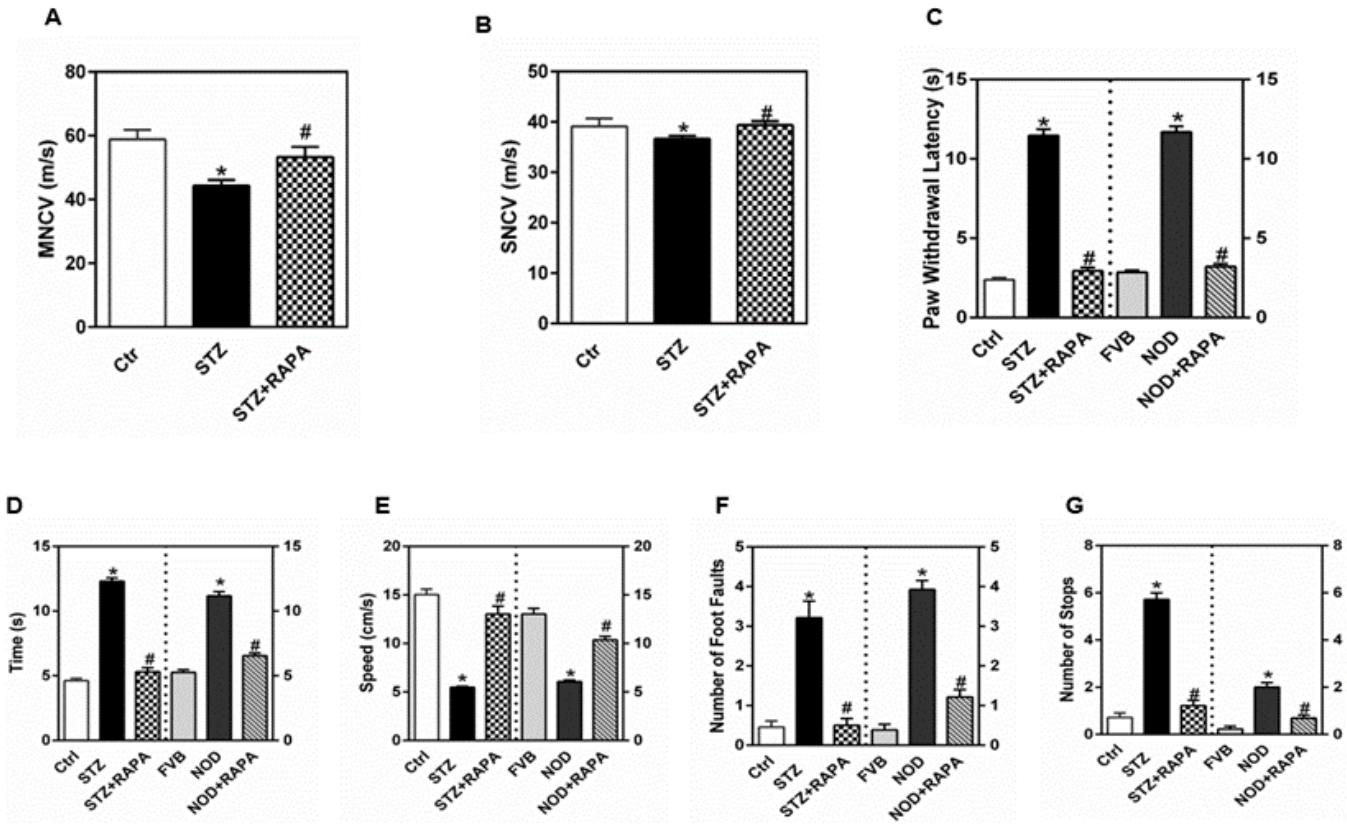

Figure 8. **mTORC1 alteration mediates hyperglycemia-induced neurophysiological and sensorimotor coordination defects in Type 1 diabetic mice.** STZ-induced type 1 diabetic and NOD mice were treated with either vehicle or rapamycin (Rapa) at 0.5 mg/kg/body weight, administrated three times a week by intraperitoneal injection. Behavioral and functional tests were performed on six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with rapamycin and FVB control mice, NOD mice, and NOD mice treated with rapamycin. Assessment of (A) MNCV and (B) SNCV after 8 weeks of diabetes in ctr, STZ-induced type 1 diabetic mice and STZ-induced diabetic mice treated with rapamycin. The histograms confirm nerve dysfunction in STZ-induced type 1 diabetic mouse. This effect was reversed in diabetic mice treated with GKT. (C) Histograms representing thermal sensitivity in response to a heating stimulus of the control/FVB, STZ/NOD and STZ+Rapa/ NOD+Rapa mice. Assessment of fine motor coordination by the raised beam walking test. The histograms represent the average time (D), speed (E), faults (F), and stops (G). Values are the mean ± SE from 5 different mice in the different groups (n=5). *$p < 0.05$ versus vehicle-treated Ctr or FVB mice; #$p < 0.05$ versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.

Further molecular tests were done to correlate these behavioral results with the observed *in vivo* results. For that reason, the alterations in myelin injury and myelin protein expression were assessed and the findings provide evidence that hyperglycemia-triggered myelin injury prompted through an increased expression of MPZ and PMP22 at the mRNA (**Figure 9A-B)** and protein levels (**Figure 9C-F)** in the sciatic nerves of type 1 diabetic animals. Myelin alteration were paralleled by an induction of cellular apoptosis as assessed by TUNEL-positive staining of sciatic nerves derived from STZ-induced diabetic animals (**Figure 9G)**. Rapamycin administration reversed peripheral nerve injury and restored myelin morphology and function, supporting the pathogenic role of mTORC1 in DPN (**Figure 9A-G).**

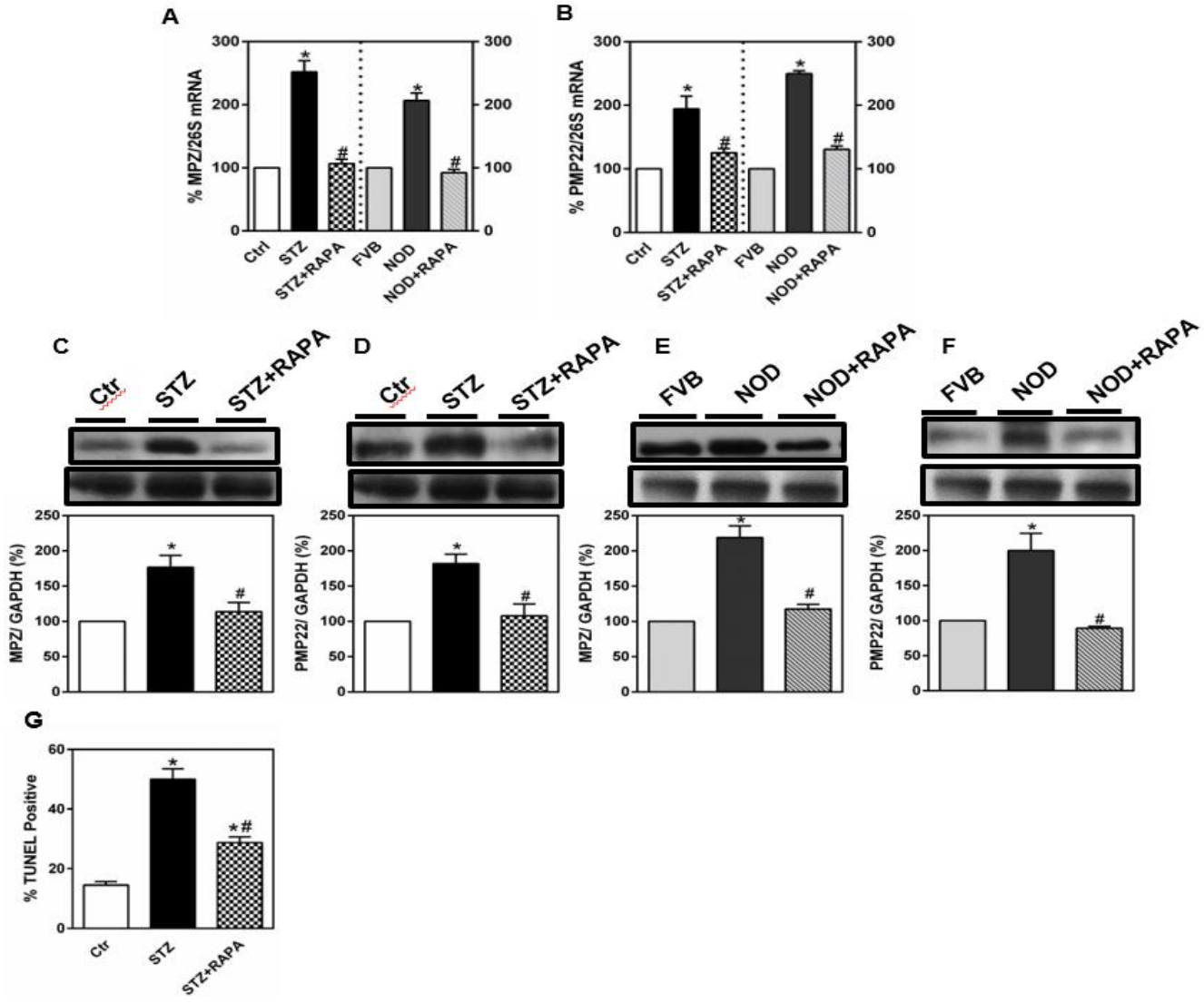

Figure 9. **mTORC1 regulates peripheral myelin gene expression and PNS apoptosis in type 1 diabetes.** STZ-induced type 1 diabetic and NOD mice were treated with either vehicle or rapamycin (Rapa) at 0.5 mg/kg/body weight, administrated three times a week by intraperitoneal injection. Sciatic nerves were isolated from six groups of mice: Control (Ctr) mice, STZ-induced diabetic mice, STZ-induced diabetic mice treated with rapamycin and FVB control mice, NOD mice, and NOD mice treated with rapamycin. (A) Relative mRNA levels of MPZ and (B) PMP22. Representative Western blot of (C) MPZ, (D) PMP22 and GAPDH levels with the respective densitometric quantification in sciatic nerves of control, STZ-induced type 1 diabetic mice and STZ-induced type 1 diabetic mice treated with rapamycin. Representative Western blot of (E) MPZ, (F) PMP22 and GAPDH levels with the respective densitometric quantification in sciatic nerves of FVB, NOD mice and NOD mice treated with rapamycin. (G) percent of TUNEL-positive cells. Values are the mean ± SE from 5 different mice in the different groups (n=5). *$p < 0.05$ versus vehicle-treated Ctr or FVB mice; #$p < 0.05$ versus vehicle-treated STZ-induced or NOD type 1 diabetic mice.

E. *In vitro* silencing of the NOX/mTOR axis ameliorates hyperglycemia-induced Schwann cell injury.

In order to further confirm our *in vivo* findings, *in vitro* studies were performed. Data reported in this paragraph are just preliminary obtained results and further experiments are still ongoing. To investigate the effect of the Nox1/mTOR pathway alterations in Schwann cells (SC), MSC80 cells were transfected with SMARTpool of simTORC1, siNox1, siRictor or with nontargeting siRNA (Scr) prior to treatment with high glucose (HG) (**Figure 10)**. The myelin gene expression of PMP22 was utilized as an indirect assessment of SCs functionality. HG was shown to induce hypermyelination in SCs which manifested as increased mRNA levels of PMP22. Interestingly, cells that were transfected with either siRNA targeting Nox1, mTORC1 or Rictor showed a normalization of myelin protein PMP22 gene expression (**Figure 10)**. Taken together, our in vitro data suggest that the NADPH oxidases Nox1, the mTOR complex 1 and the mTOR complex 2 play a major role in diabetic peripheral neuropathy, and corroborate with the *in vivo* findings. Further experiments are needed to assess if the interaction between the different drugs that causes the total effect of the drugs to be greater than the sum of the individual effects of each drug. More importantly, studies on the effect of each of these pathways will be studies on SCs apoptosis.

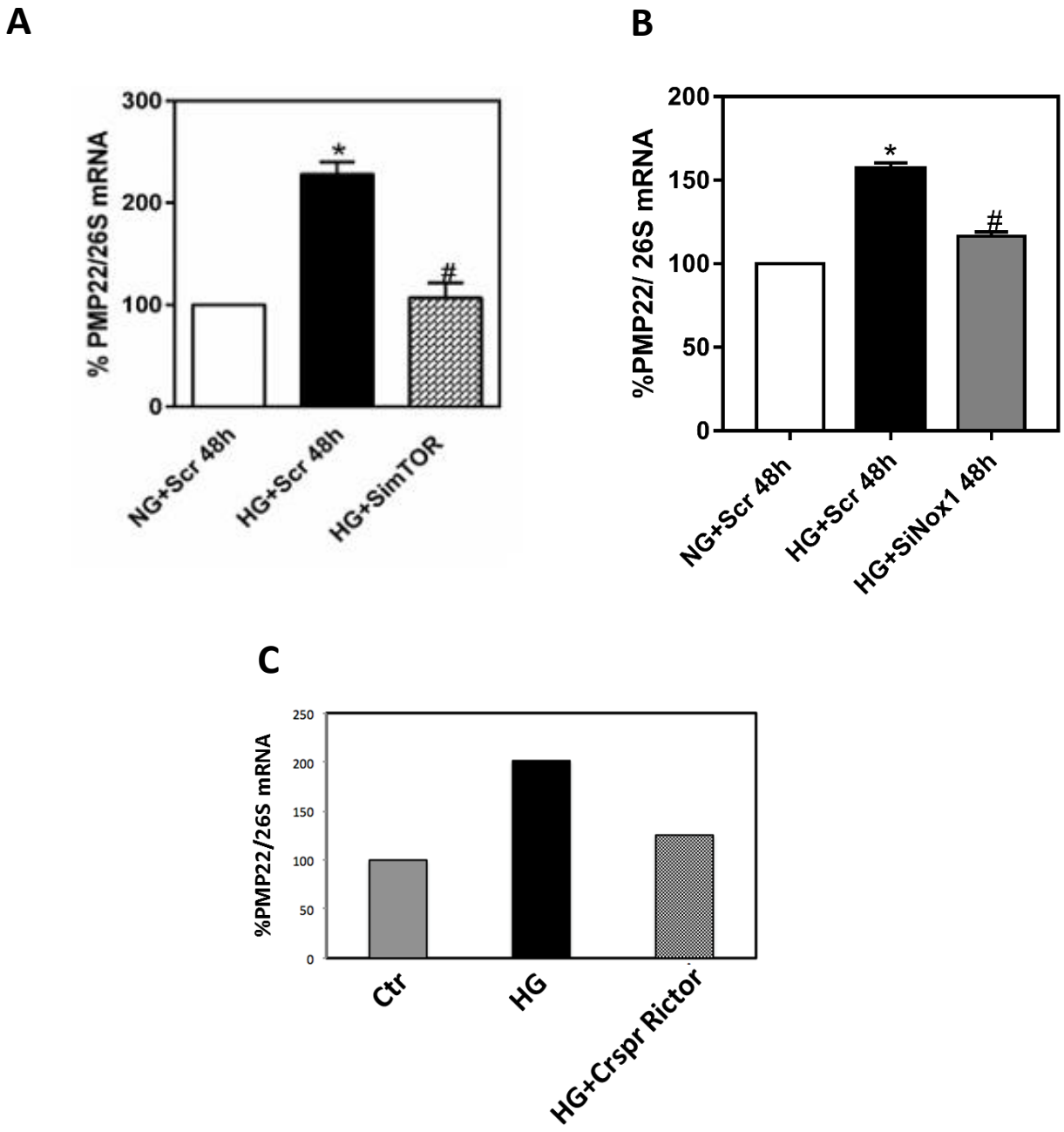

Figure 10. **Silencing of mTORC1 (simTORC1), mTORC2 (siRictor) or Nox1 (siNox1) prevents SC injury in the diabetic milieu.** Mouse Schwann Cells (SCs) incubated in normal glucose (NG; 5 mM) or high glucose (HG; 25 mM) were transfected with nontargeting siRNA (Scr) or with SMARTpool of siRNA targeting mTORC1 (simTORC1), mTORC2 (siRictor) or Nox1 (siNox1). mTOR silencing inhibits HG-induced upregulation of PMP22. PMP22 mRNA levels of cell transfected with (A) SimTOR (n=3), (B) SiNox1 (n=3), (C) CrprRictor (n=1).). Values are the means ± SE. *p<0.05, High glucose vs. Normal glucose. #p<0.05, High glucose vs. Transfected

CHAPTER IV

DISCUSSION

Diabetic Peripheral Neuropathy (DPN) is the most common complication of diabetes, characterized by impairment in neurons, Schwann cells and peripheral vasculature within the nervous tissue. It is widely accepted that hyperglycemia is a major contributor to the pathophysiology of diabetic complications especially DPN. However, the pathogenic mechanism of DPN is unclear hindering the progression in treatment options.

A large body of data including work done in our group (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013; Eid et al., 2014; Eid et al., 2016), show that chronic hyperglycemia triggers the excessive production of reactive oxygen species (ROS) and thus leads to oxidative stress. Several studies pointed to the implication of ROS in peripheral nerve injury, however, the mechanisms by which this occurs are poorly understood. In this study we aim to understand and identify the role of NADPH-oxidases, specifically Nox1, as a source of ROS in the progression of DPN. Our *In vivo* results show an alteration in myelin protein profiles in peripheral nerves of animal models of diabetes, paralleled by altered pain and thermal perception as well as sensorimotor defects at the behavioral level. Inhibition of NADPH oxidases, Nox1 and Nox4, via GKT inhibitor restored peripheral nerve function, supporting that hyperglycemia induced peripheral nerve injury through an NADPH dependent mechanism. This mechanism was also shown to be mTOR dependent whereby hyperglycemia induced the activation of the mTOR complex 1 and mTOR complex 2. Furthermore, mTOR activation was shown to be associated with myelin protein alteration and peripheral nerve defects. Importantly, the dual blockade of the mTORC complexes pathway via PP242 normalized myelin protein expression, and restored functionality

as reported through molecular and behavioral tests. These data lend support to a previously unknown mechanism of DPN progression which entails hyperglycemia-induced NADPH-oxidase ROS production that leads to mTORC1 and mTORC2 activation and the subsequent peripheral nerve injury and malfunction. This mechanism was further validated through *in vitro* findings in Schwann cells (SCs) although preliminary, however promising, and emphasize the significance of this pathway as a therapeutic target for DPN.

Myelin protein homeostatic expression and SC integrity are hallmarks of intact myelination and peripheral nerve function (Makoukji et al, 2011 Meffre et al 2015; D'Urso et al., 1999; Hichor et al., 2016**)**. Alterations in myelin protein expression may be detrimental to the myelin sheath surrounding neuronal axons leading to nerve dysfunction and degeneration. Diabetes and hyperglycemia have been reported in the literature to inflict injury through altering myelin protein expression (Conti et al., 1996; Cermenati et al., 2012; Kawashima et al., 2007). Several studies indicate that PMP22 aggregation and the defected disposal of non-functional myelin protein contributes to nerve demyelinating phenotypes such as in Charcot Marie Tooth type 1A (CMT1A) demyelinating neuropathies (Tobler et al., 2002; Fortun et al., 2003; Fortun et al., 2005) and most recently, in type 2 diabetes (Hamilton et al., 2013). This study is concurrent with the previously reported studies and add to the literature whereby myelin protein aggregation is not only associated with type 2, but type 1 diabetes as well. Yet, the mechanisms by which diabetes alter myelin proteins are not well defined.

As for nerve dysfunction in diabetes, numerous investigations suggest that defects in peripheral nerves are strongly associated with neuronal and SC death (Wu et al., 2012; Sun et al., 2012; Jeong et al., 2009; Liu et al., 2016). Although several studies reported SC apoptosis in response to HG/hyperglycemia (Delaney et al., 2001; Kalichman et al., 1998; Wu et al., 2012;

Sun et al., 2012; Jeong et al., 2009; Liu et al., 2016), other reports showed otherwise (Gumy et al., 2008; Sango et al., 2002). Nevertheless, the results of this study showed a significant increase in apoptosis in sciatic nerves derived from STZ-induced type 1 diabetic mice.

The final common pathway that has been associated with hyperglycemia is currently considered to involve ROS production and oxidative stress, and this was described for numerous diabetic complications including DPN. Strong evidence points to the deleterious and injurious role of ROS in the pathogenesis of diabetic complications (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013a, b; Eid, A. et al., 2013; Eid, S. et al., 2013; Sun et al., 2012; Cameron NE et al., 1993; Stevens MJ et al., 2000; Pop-Busui et al., 2013; Kowluru, R.A., & Kennedy, A. 2001). Additionally, the inhibition of ROS has been investigated as a therapeutic approach in several diseases including diabetes. However, recent research studies showed that a beneficial approach for diabetic complication treatment or to a higher extent other diseases treatment such as cancer is based on cells and tissues specificity. Actually, our group among others has described the involvement of Nox1 and Nox4 in the pathophysiology of diabetic complications through ROS production (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013; Eid et al., 2016; Gorin et al., 2015; Li et al., 2010; He et al., 2013; Weaver et al., 2015). In DPN, ROS amelioration via antioxidant therapy was shown to be beneficial in attenuating neurovascular injury (Cameron, N. E., & Cotter, M. A. 1999; Stevens et al., 2000). Moreover, several lines of evidence indicate an improvement in the nerve conduction velocity, pain, thermal perception as well as perineurium morphology upon the inhibition of ROS production (Greene et al., 1999; Cotter, M. A., & Cameron, N. E. 2003; Li et al., 2016). Still, the mechanism of ROS and specifically the mechanism of NADPH oxidases in DPN are not yet identified. In this study, we provide evidence for the role of NADPH oxidase Nox1 as a major source involved in high glucose-

induced schwannopathy and peripheral nerve dysfunction. More importantly, we show that the administration of GKT, a Nox1 and Nox4 inhibitor, *in vivo* ameliorate nerve injury in DPN. The results showed that Nox1/4 inhibition provides a neuroprotective effect as reflected by elevated NCV, reduced sensorimotor deficits and close to normoglycemic controls, thermal and pain perception. Moreover, the reversal of peripheral nerve injury in response to GKT administration was shown through normalized peripheral myelin protein levels and reduced neuronal apoptosis. These *in vivo* outcomes highlight the role of Nox1 in the structural and functional alterations of PNS injury in type 1 diabetes. These data are consistent with another study conducted in a Nox1 Knockout mice model of neuropathic pain (Masakazu et al, 2008). The study reports that thermal and mechanical hyperalgesia was significantly attenuated in neuropathic pain-induced animals, supporting the role of NOX1/NADPH oxidase in elevating TRPV1 activity and thus, sensitivity to painful stimuli (Ibi M, et al, 2008). In that same spirit, and in unpublished data from our group (Eid S et al. non published data), we show that the NADPH oxidases NOX4 isoform play a major role in DPN. However, the precise mechanism by which NADPH oxidases influence peripheral nerve injury and myelin function are still unknown. In this study, we elucidate the involvement of the mTOR pathway.

The mTOR signaling pathway regulates many major cellular processes and is involved in an increasing number of pathological conditions, including cancer, diabetes, obesity and neurodegeneration (Laplante, M., & Sabatini, D.M. 2012; Zoncu et al., 2011). In the PNS, mTORC1 signaling has been studied (Sherman et al., 2012; Flores et al., 2008; Narayanan et al., 2009), however its exact mechanism on myelination and SC integrity is not well studied. Our work establish the contribution of the mTOR complexes to myelination through *in vivo* studies and its activation in DPN. The data were indicative of mTORC1/mTORC2 significant activation

in sciatic nerves from diabetic animals concomitant with Nox1 upregulation. GKT treatment was shown to reduce Nox1 and total mTOR levels indicating the involvement of mTORC1/mTORC2 pathway in NADPH-induced peripheral nerve injury. These findings corroborate with other reports whereby the involvement of mTOR in pain perception was reported (Obara et al., 2011; Géranton et al., 2009; Xu et al., 2014). Previous studies linked mTOR signaling activation to chronic pain in several experimental models of inflammatory and neuropathic pain (Obara et al., 2011; Géranton et al., 2009; Xu et al., 2014). Similarly, these results lend support to another recent study that described mTOR activation in DRG of type 1 diabetic rats to be associated with hyperalgesia (He et al., 2016). However, the effect of total mTOR inhibition on the behavioral, cellular and molecular alterations brought about by DPN is not yet clarified.

Through this work, we demonstrate the impact of effective inhibition of mTORC1 and mTORC2 via PP242, and the specific inhibition of mTORC1 via rapamycin administration, on the development of neuropathic functional changes in diabetic animals. Our results showed that diabetes-induced mTOR activation is associated with increased PMP22 aggregation and PMP22 in addition to MPZ expression. Furthermore, PP242 or rapamycin were shown to restore P0 and PMP22 transcript and protein levels and attenuated TUNEL-positive staining in the sciatic nerves extracted from STZ-induced type 1 diabetic animals. Likewise, mTORC1 and mTORC2 silencing in cultured SCs restored PMP22 levels, consistent with our *in vivo* findings. These findings resonate with two studies whereby mTOR mutant animals possessed reduced MPZ levels (Sherman et al., 2012), and reduced NCV recordings as well as dysmyelination observed in the absence of mTORC1 (Norrmén et al., 2014). More importantly, data from rapamycin-treated animals corroborate with other studies that reported the crucial role of mTORC1 in the

initiation and regulation of the myelination process (Norrmén, C., & Suter. U. 2013; Sherman et al., 2012).

As for the role of mTORC2, the work for the scope of this study reveals a possible and individualistic role for mTORC2 regarding peripheral nerve function. However, the precise role of mTORC2 has yet to be determined. Studies in the CNS have also found that activated mTORC1 enhances myelin protein levels in addition to the myelin thickness (Flores et al., 2008; Narayanan et al., 2009), but the impact of mTORC2 deficiency on the myelination process in the CNS dint show an important effect (Bercury et al, 2014). Although no investigations have been conducted in the PNS, nor in vitro to examine the specific inhibition of mTOR complexes, we have shown that the targeted silencing of mTORC2 alone in SCs restored myelin protein levels, similar to the inhibition of mTORC1, as well as Nox1. Nevertheless, the sole contribution of mTORC2 to the myelination process remains unclear and necessitates further investigations.

Consequently, having provided evidence of mTOR involvement in myelination in various animal models of diabetes, the future directions of this project aim to further investigate the influence of PP242, dual mTOR inhibition, *in vitro*, to better understand the complex interplay between mTOR signaling and other pathways such as Akt and their role in myelination. Having observed the deleterious role of mTORC2 on SC myelin protein expression, special emphasis will be placed on mTORC2, especially its silencing *in vitro*, and *in vivo*, in order to better understand the individualistic, synergistic, or perhaps, additive contribution alongside mTORC1 to SC and peripheral nerve physiology and function, as well as DPN pathogenesis. This entails designing mTORC2 sense and antisense oligonucleotides for treatments in *in vivo* studies as well as pharmacological agents specific for mTORC2 activation and inactivation to investigate targeted knockin or knockdown effects in SCs and diabetic animal models.

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