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NEW MECHANISTIC PATHWAYS IN THE DEVELOPMENT OF SCHWANN CELL INJURY IN DIABETES: ROLE OF THE NADPH OXIDASES, LXR AND MTOR PATHWAYS

by
MOHAMED HASSAN EL MASSRY

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submitted in partial fulfillment of the requirements
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"In the end, though, maybe we must all give up trying to pay back the people in this world who sustain our lives. In the end, maybe it's wiser to surrender before the miraculous scope of human generosity and to just keep saying thank you, forever and sincerely, for as long as we have voices."-Elizabeth Gilbert

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Thank you my amazing family for always pushing me throughout my rise and fall. You're the backbone that I'll always lean on and carry in every step of my path. Thank you!

AN ABSTRACT OF THE THESIS OF

Mohamed Hassan El Massry for Master of Science
Major: Neuroscience

Title: New Mechanistic Pathways in the Development of Schwann Cell Injury in Diabetes: Role of the NADPH Oxidases, LXR AND mTOR Pathways.

Background: Diabetic neuropathy (DN) is the most common debilitating complication of diabetes affecting more than 50% of patients. It is associated with impaired nerve conduction, abnormal thermal perception, axonal atrophy, demyelination, blunted regenerative potential, and loss of nerve fibers. However, the exact mechanisms underlying such complications are still not known. Although reactive oxygen species have been established as the main pathway of cellular injury in diabetic neuropathy, the mechanisms by which they cause their effects need to be more elucidated.

Aim: We will investigate the role of ROS generated by the NADPH oxidases family of enzymes in mediating biological responses in Schwann cells including phenotypic changes such as deregulation of myelin gene expression (P0 and PMP22) and apoptosis. We will study the role of ROS production in the alteration of the signaling pathways involving LXR and mTOR and the crosstalk among these pathways in the mediation of cell injury.

Methods: Raised beam walking test was used to assess behavioral malfunction in diabetic animals. Dihydroethidium (DHE) and 2', 7'-dichlorodihydrofluorescin (DCF) diacetate were used for the detection of intracellular ROS in sciatic nerves and Schwann cells (MSC80) respectively, while NADPH oxidase activity assay helped in the specification of the source of ROS. RT-PCR allowed the measurement of mRNA levels of Nox3, Nox4, PMP22, P0, and LXR-β. Western blots were used to assess the protein expression levels of NADPH oxidases and myelin proteins as well as mTOR, P70S6K and LXR- β. DNA fragmentation/ELISA test was used to study apoptosis in Schwann cells.

Results: Hyperglycemia causes motor defects at the level of the peripheral nerves and is associated with an alteration in P0 and PMP22 levels. NADPH oxidases levels and activity are increased and result in the production of ROS. High glucose induces the activation of mTOR/P70S6K suggested to play a role in Schwann cell injury. LXR expression is decreased which might contribute to ROS production. The final outcome of these variations combined is Schwann cell injury and apoptosis, which is reversed by T0901317 treatment.

Conclusion: Our study indicates that hyperglycemia alters the normal functioning of Schwann cells through a multitude of mechanisms including increased ROS production, decreased LXR pathway activation and increased mTOR activation. Injury and apoptosis of Schwann cells result and lead to alterations in P0 and PMP22 levels. The final consequence is an abnormal motor behavior and defects in motor coordination.

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LIST OF ABBREVIATIONS

DPN:	Diabetic Peripheral Neuropathy
ROS:	Reactive Oxygen Species
LXR:	Liver X Receptor
LXR dKO	LXR$\alpha,\beta^{-/-}$ double knockout
mTOR:	Mammalian Target of Rapamycin
P0 or MPZ	Myelin Protein Zero
PMP22:	Peripheral Myelin Protein 22
Nox family:	NADPH Oxidase Family
PNS	Peripheral Nervous system
T0901317 or T0:	N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide
MSC80:	Mouse Schwann Cells 80
HG:	High Glucose
STZ:	Streptozotocin
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
SC:	Schwann cell
NOD:	Non-Obese diabetic
DN:	Diabetic Neuropathy

CHAPTER I

INTRODUCTION

Diabetes Mellitus (DM) is a group of metabolic diseases caused by the inability of the body to metabolize glucose properly, either due to defects in insulin production and secretion (Type I Diabetes Mellitus), or insulin resistance (Type II Diabetes Mellitus), or both. Diabetes Mellitus can result in a series of microvascular and macrovascular complications affecting a wide range of organs, including the nerves, eyes, kidneys and heart (American Diabetes Association, 2010). One of the most common and debilitating complications associated with diabetes is Diabetic Neuropathy (DN); it affects about 10% of patients newly diagnosed with diabetes and more than 50% of patients with longstanding diabetes (Shakeel, 2014). DN results in a number of sensorimotor disorders including pain, loss of proprioception and motor function and infectious ulcers in the feet and legs that often require limb amputation (Edward et al., 2008). Additionally, DN can lead to autonomic dysfunction, which manifests as orthostatic hypotension, fainting, arrhythmias, gastrointestinal dismotility, bloating, diarrhea, etc (Vinik et al., 2003).

DN can be classified as proximal, focal, autonomic and peripheral, each of which affecting different parts of the body in various ways (Callaghan BC. et al, 2012). The most common form of DN is the distal symmetric polyneuropathy, also known as diabetic peripheral neuropathy (DPN), which is the focus of this study. DPN is associated with impaired nerve conduction, abnormal thermal perception, axonal atrophy, demyelination,

blunted regenerative potential and loss of nerve fibers, resulting in motor dysfunction (Boulton et al., 2014). Patients diagnosed with DN also experience an increased sensitivity to pain (hyperalgesia), as well as an increased responsiveness to non-painful stimuli (allodynia) (Dworkin et al., 2007a; Dworkin et al., 2007 b; Dworkin et al., 2005; Jensen et al., 2006a; Jensen et al., 2006b). With the progression of the disease, pain is replaced with complete numbness followed by serious foot problems, ultimately resulting in ulcerations and leading to foot amputation (Feldman, 1999; Feldman, 2005; Feldman, 2002b). Although DPN has long been viewed as neurocentric, it is now widely accepted that the rate of peripheral nervous system deterioration is intimately correlated with the significant pathological interactions between neurons, Schwann cells, and microvascular endothelium (Dyck and Giannini, 1996; Vincent et al., 2009; Vincent et al., 2013). In the last decade, much attention has been focused on the role of hyperglycemia in the progression of DPN. Clinical studies have established that intensive glycemic control and improved blood glucose levels reduce the incidence and slow the progression of DPN, thus clearly implicating hyperglycemia in the initiation of distal neuropathy (Obrosova I. 2009; Writing Team for the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group, 2002; Stratton I. et al, 2000; Boulton A., 1998). However, the underlying mechanisms leading to diabetic peripheral neuropathy are not well described and need further investigations.

A. The myelination process and myelin injury in the peripheral nervous system

1. Schwann cells and myelination:

The process of myelination is carried out by Schwann cells in the peripheral nervous system. The importance of the myelin sheath resides in the ability to create an insulating covering of the axons, rich in lipids (70-80%) and proteins (20-30%), in order to allow a fast propagation of the action potential in specific regions on the axonal membranes (Garbay et al., 2000). This fast propagation mode is known as "saltatory conduction" and depends largely on the development of well-organized, compact and resistant myelin folds around the axons. The protein content of the myelin sheaths can be described in terms of glycoproteins, since they provide more than 60% of the proteins in myelin. The major glycoproteins are myelin protein zero (P0), peripheral myelin protein 22 (PMP22), myelin associated glycoprotein (MAG) and periaxin, each of which involved in a different stage of the myelination process. The remaining 40% of proteins include basic proteins (20-30%), incorporated in the Schwann cell's membranes, and diverse proteins, each of which contributing to less than 1% of the total protein count (Garbay et al., 2000).

A 28 kDa integral membrane glycoprotein, Myelin Protein Zero (P0), has been described as the most abundant protein in the PNS accounting for 50-70% of the total myelin proteins (Kitamura et al., 1976; Roomi et al., 1978; Greenfield et al., 1973; Wiggins et al., 1975; Smith et al., 1979). This protein, expressed exclusively by myelinating Schwann cells, is essential for the compaction of PNS myelin through homophilic interactions (Kirschner and Ganser, 1980; Lemke and Axel, 1985; Lemke et al., 1988;

D'Urso et al., 1990; Filbin et al., 1990). P0 deficiency in mice (Giese et al., 1992; Martini et al., 1995) or P0 overexpression (Wrabetz et al., 2000) results in hypomyelination and peripheral neuropathy (Xu W. et al, 2001). Also, mutations in the human P0 gene are associated with muscle weakness, atrophy, and sensory loss (Nelis et al., 1999). P0 appears to play a role in the regulation of the expression of multiple myelination genes as well. Numerous studies have shown that $P0^{-/-}$ mutant mice have an abnormally high level of NCAM and NGFR, two early markers of Schwann cell differentiation during the myelination stage (Martini and Schachner, 1986; Jessen et al., 1990). Therefore, any deviation in the expression of P0 would affect the normal myelination process essentially mediated by SCs and result in behavioral abnormalities in the diabetic subject.

PMP22 is another glycoprotein that represents 2-5% of the peripheral myelin proteins (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). In parallel to its key function in myelination, PMP22 is involved in the regulation of cell proliferation (Zoidl et al., 1995; Hanemann et al., 1998; Fabbretti et al., 1995; Brancolini et al., 1999) and may play a role in controlling cellular morphology (Brancolini et al., 1999). Thus, an aberrant change in PMP22 expression is expected to have effects on myelination, cellular proliferation and apoptosis.

2. Schwann cell injury in diabetes

Demyelination accompanied by axonal atrophy has been suggested as one of the pathological changes induced by diabetes in the PNS (Yagihashi et al., 1990; King et al., 1989). The etiology of demyelination in DPN is still unclear. However, emerging

experimental literature points to a key role of Schwann cells in the pathogenesis of DN. Schwann cells are considered a primary target of hyperglycemia (Yu T et al, 2014; Taiana MM et al, 2014; Cinci L et al, 2015), and when exposed chronically to high blood glucose levels, they undergo oxidative, metabolic and apoptotic changes. In vivo studies suggest that hyperglycemia causes reduced nerve conduction velocity, axonal atrophy, and impaired axonal regeneration (Dyck & Giannini, 1996). The mechanisms by which hyperglycemia exerts its damaging effects on myelination remain unclear.

DPN is described in terms of nervous, vascular, and Schwann cell lesions. (Dyck and Giannini, 1996 and Vincent et al., 2009c). Hypoxia, hyerglycemia, and increased oxidative stress contribute directly and indirectly to Schwann cell dysfunction (Eckersley, 2002). Early studies considered that Schwann cells are the primary site of injury in diabetic neuropathy. Accumulation of lipid droplets, enlarged mitochondria as well as basal lamina thickening were observed in human Schwann cells from nerve biopsies of diabetic patients prior to demyelination (Bischoff, 1979). Also, in recent studies, cultured Schwann cells exposed to high levels of glucose undergo caspase dependent and caspase independent apoptosis (Wu et al., 2012; Sun et al., 2012; Yu et al., 2015).

B. Diabetes and Oxidative Stress

High glucose/hyperglycemia is associated with increased systemic and cellular oxidative stress, now considered as a common pathway of cellular injury leading to diabetic complications. Antioxidant treatment prevents or slows the development of neuropathy in animal models of diabetes, suggesting a major pathogenic role of reactive oxygen species (ROS) in the pathology of DN (Cameron et al., 1994; Coppey et al., 2003; Sayyed et al.,

2006). The alteration in several mechanistic pathways known to be involved in diabetes has been shown to contribute to ROS formation in the peripheral nervous system (Edward et al., 2008). Different sources of ROS are reported to be altered in the diabetic milieu. Recent studies indicate a major role for the NADPH oxidase (Nox) family as a source of ROS in the pathology of diabetic nephropathy and cardiomyopathy (Eid et al., 2009; Zhao et al., 2015). However NADPH oxidase involvement in DN is not elucidated.

The NADPH oxidases are a family of enzymes whose only function is the generation of reactive oxygen species (ROS) across biological membranes. The Nox family includes five members: Nox1, Nox2, Nox3, Nox4 and Nox5, in addition to DUOX1 and DUOX2, each of which having a different level of expression in diverse tissues. All the members of the family share certain features including an NADPH binding domain at the COOH-terminus of the oxidases, an FAD binding region near the COOH-terminus, six transmembrane domains and four highly conserved heme-binding histidines located on the third and fifth transmembrane domains (Bedard & Krause, 2007). Nox2, the well-characterized respiratory burst oxidase, consists of two membrane-bound subunits, $gp91^{phox}$ (also designated as Nox2) and $p22^{phox}$. The activation of Nox2 requires the interaction of the membrane subunits with cytosolic subunits, $p47^{phox}$ (the organizer subunit), $p67^{phox}$ (the activator subunit) and $p40^{phox}$, as well as the small GTPase Rac (Clark, 1999; Leusen et al., 1996). The interaction between the different subunits results in the production of oxygen radicals and oxidative stress.

C. NADPH and PNS Injury

NADPH-dependent ROS generation is increased in the dorsal root ganglion neurons following hyperglycemia (Vincent et al., 2009). ROS, lipid peroxidation and protein nitrosylation, and diminished levels of reduced glutathione and ascorbate (anti-oxidants) were all observed in dorsal root ganglia and peripheral nerves in animal models of type 1, type 2 and pre-diabetes. Treatment of STZ-induced diabetic rats with anti-oxidants, comprising α-lipoic acid and γ-linolenic acid, and aldose reductase inhibitors blocks many of the indices of neuropathy (Fernyhough et al., 2010). Nonspecific inhibition of NADPH oxidases using apocyanin or diphenylene iodonium (DPI) reduces diabetes-induced ROS generation in many organs and prevents the development of complications in cells exposed to high glucose and in animal models of diabetes (Cotter & Cameron, 2003; Sonta et al., 2004; Matsushima et al., 2009). Nox 1, 2 and 4 were all shown to be expressed in the central nervous system and to contribute to disease development (Bedard & Krause, 2007; Lambeth et al., 2008). The role and function of the NADPH oxidase family in DN will be investigated in this study.

D. The involvement of LXR Pathway in the Pathology of Diabetes

The oxysterol/LXR pathway majorly carries the metabolism of cholesterol in the cells. Oxysterols are oxidized forms of cholesterol that are known to modulate cholesterol homeostasis by activating cholesterol transporters like ABCA1 (Faulds et al., 2010; Baranowski, 2008). There are several types of oxysterols, among them 24-hydroxycholesterol (24S-OH) and 25-hydroxycholesterol (25-OH), which are synthesized by Schwann cells. They bind and activate LXR. 7-keto cholesterol (7KC) is predominantly

generated from auto-oxidation. This oxysterol is at high levels in the nerve (10^{-5}M) (Makoukji et al., 2011). Oxysterols bind to two isoforms of nuclear receptor LXR in order to regulate their target gene expression (Faulds et al., 2010; Baranowski, 2008): LXR α, which is highly localized in in the liver as well as in the kidneys, intestine, adipose, lungs and in the cerebellum, while LXR β is ubiquitously expressed (Janowski et al., 1996). LXRs form heterodimers with retinoic X receptor (RXR), the nuclear receptor for 9-*cis* retinoic acid. In the nucleus, they regulate gene expression by binding to responsive elements. In the absence of ligands, LXR-RXR heterodimers bind to corepressors (Hörlein et al., 1995). In response to the binding of oxysterols, those dimers detach from corepressors, interact with coactivators (Huuskonen et al., 2004) and activate target genes expression. Transgenic mice studies revealed important roles of LXRβ within the nervous system (Andersson et al., 2005). LXRβ-/- transgenic mice show amyotrophic lateral sclerosis associated with neuronal loss. Axonal atrophy and astrogliosis, also linked to lipid accumulation and nervous system defects in those mice, are very close to what is observed in double LXRα,β-/- (LXR dKO) mice (cholesterol homeostasis related neurodegenerative disorders: lipid accumulation in specific brain regions, neuronal loss, astrocytes proliferation and myelin sheaths disruption) (Andersson et al., 2005; Kim et al., 2008). Makoukji *et al.*, have previously shown that LXRs play a key-role in the myelination process (2011). The knockout of LXR causes demyelination, dysregulation of myelin genes (MPZ and PMP22) and formation of thinner myelin sheaths around the axons (Makoukji et al., 2011). Recently emerging experimental literature reveals an involvement of LXR in the maintenance of glucose homeostasis. T0901317 and GW3965, synthetic agonists of LXR

were shown to normalize glycaemia, improve whole body insulin sensitivity and stimulate insulin secretion in animal models of diet-induced insulin resistance and type 2 diabetes (Cao et al., 2003; Grefhorst et al., 2005). Although LXRs are expressed in human and rodent PNS, data describing its involvement in DPN are still underdevelopment (Cermenati et al., 2012; Cermenati et al., 2010). Activation of LXR using T0901317 protected mice from DPN by increasing neuroactive steroid levels (Cermenati et al., 2010). However, the cellular and molecular roles of LXR in DPN are not characterized.

E. mTOR pathway and its role in the myelination process and cellular injury.

mTOR has been recently associated with the regulation of myelin formation in the peripheral nervous system, but controversial results have been reported regarding its exact role in this process. mTOR has been described as one of the major key players involved in diabetic complications (Eid et al., 2013). Treatment of mice expressing a mutant form of the myelin protein PMP22 with the mTOR inhibitor Rapamycin rescues myelination in dorsal root ganglion cultures, and this has been partly due to the activation of autophagy (Rangaraju et al., 2010). Other studies suggested that Schwann cell- specific mTOR deficient mice have a thin myelin sheath. Schwann cells do not elongate normally in those mice and axon diameter is reduced (Sherman et al., 2012). Slowed nerve conduction velocity (NCV) was also found in raptor mutant mice, in agreement with the observed hypomyelination, shorter internodes, and changes in lipid composition, revealing that raptor/mTORC1 in Schwann cells is crucial determinant of a proper nerve physiology (Norrmén et al., 2014). Although the involvement of mTOR in diabetic injury has been extensively studied, no reports describe the role of mTOR in DPN and specifically in

Schwann cell injury. Our group has shown that mTOR activation reduces kidney epithelial cell survival in the kidneys of an animal model of diabetes, and this increase enhances oxidative stress via the upregulation of NADPH oxidases (Nox4). Inhibition of the mTOR pathway by clinically relevant doses of rapamycin reverses kidney epithelial cell injury (Eid et al., 2009; Eid et al., 2013; Axelsson et al., 2015).

The mammalian target of rapamycin (mTOR) is a highly conserved, nutrient responsive serine/threonine protein kinase and cell growth regulator found in eukaryotes (Wullschleger et al., 2006). mTOR exists in two complexes, both of which activated by hormones and growth factors: mTORC1, the rapamycin sensitive form, responds to nutrients and cellular energy status and mediates its effect through downstream effectors, including p70S6 kinase (p70S6K)/S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) (Um et al., 2006); mTORC2 is largely rapamycin resistant and mediates phosphorylation of protein kinase B (PKB/Akt) at Ser473 (Sarbassov et al., 2005). mTOR is negatively regulated by the heterodimeric complex consisting of tuberin (TSC2) and hamartin (TSC1), which is in turn, positively regulated by AMPK, maintaining the tumor suppressor activity and preventing the activation of mTORC1 (Sabatini, 2006).

F. Hypothesis and aim of the study.

Diabetic neuropathy is associated with impaired nerve conduction, abnormal thermal perception, axonal atrophy, demyelination, blunted regenerative potential and loss of nerve fibers. Schwann cell injury is now recognized as a major feature of DN. It consists of a spectrum of physiological changes mainly affecting the myelination process of axons

in the peripheral nervous system and apoptosis of cells. However, the mechanisms leading to Schwann cell injury in diabetes are not well characterized.

Although strict metabolic control decreases neuropathy in diabetic patients, it is often difficult to achieve this control, which is fraught with complications. Therefore, understanding the mechanism by which high glucose/hyperglycemia exerts its deleterious effects on Schwann cell will help design therapeutic targets to treat DPN.

Our hypothesis is that diabetes-induced oxidative stress, secondary to alteration in the levels and activities of selected NADPH oxidases, contributes to the onset and progression of DPN within the frame of an innate, and possibly adaptive, immune response. The resulting oxidative stress will in turn induce a change in the signaling pathways involving oxysterol/LXR and mTOR leading to Schwann cells injury. Our studies combine behavioral, cellular and molecular investigations aimed to effectively examine the mechanisms of DPN. This will introduce a new paradigm to identifying specific inhibitors or activators for the altered proteins as well as antioxidants to treat peripheral diabetic neuropathy. This project will have a double impact. From a fundamental perspective, it will allow the identification of novel molecular mechanisms involved in DPN. At a clinical level, it paves the way for the development of effective therapeutic targets.

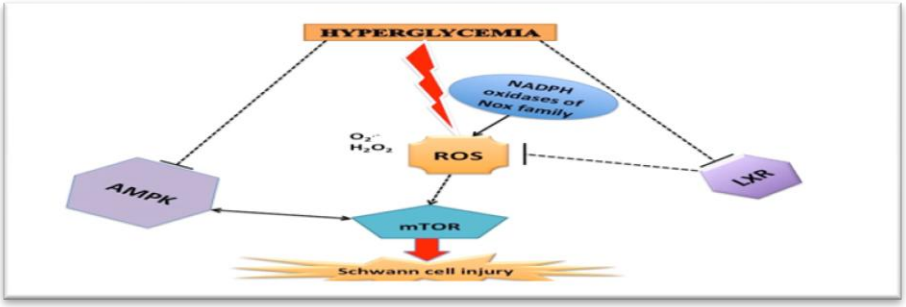


Figure 1. Proposed model of Schwann cell injury in diabetes

CHAPTER II

MATERIALS AND METHODS

A. Animal models.

Animal models of type I diabetes were used for this study. The first model is a Streptozotocin (STZ) induced diabetic model. Swiss-Webster mice, 4-6 weeks old and weighing around 40 g, received intravenous injection of STZ (Sigma-Aldrich, Steinheim, Germany) dissolved in sodium citrate buffer (0.01 M, pH 4.5) for 5 successive days, starting with 100 mg/kg of body weight on the first day and progressively decreasing the dose to 85, 75, and finally 65 mg/kg of body weight for the last two days. Controls received five 100 mg/kg of body weight, injection of citrate buffer (saline solution). 10 days later, the mice developed diabetes, as the glucose level of the STZ injected animals rose to 250-300 mg/dL.

Another model of type I diabetes was also used. The non-obese diabetic NOD mouse model is a well-established animal model of spontaneous T1D that shares many susceptibility loci and phenotypic features with human T1D after 3–5 weeks of diabetes, similar to the pathology described in diabetic human. Studies were performed on these animals starting at the age of 2 months (body weight around 22 g). FVB/NJ of similar age were used as a control strain for this animal model.

LXR $\alpha/\beta^{-/-}$knock-out mice and their wild-type (WT) controls are a kind gift from our collaborator Dr. Charbel Massaad in Paris Descartes (France). Animals are 8-week-old males. To reduce the effect of stress, the elapsed time between the capture of a mouse and

its death by decapitation was under 30 s. Sciatic nerves were collected and frozen in liquid nitrogen.

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 were extracted. Biochemical analysis was done on the nerves to assess the defects at the molecular level.

B. Raised Beam walking Test.

Raised beam walking test is a behavioral test used to assess motor dysfunction in animals. The set up 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.

C. Cell line, culture conditions.

Mouse Schwann cells (MSC80) were constantly grown in a Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany), to which 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (P/S) (Sigma-

Aldrich) were added. The cells were allowed to adhere to the surface of a 100 mm dish. The cell line was incubated under standard conditions at a temperature of 37ºC and 5% CO2. At high confluence, cells were harvested by trypsin and reseeded for different experimental procedures. Cell treatments included glucose at concentration of 25 mM (HG) and 10 μM T0901317 (LXR agonist).

D. Detection of intracellular ROS.

The peroxide-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescin (DCF) diacetate (Molecular Probes) was used to measure intracellular ROS in MSC80s. Cells were grown in 12-well tissue culture plates until 70-80% confluence and serum-deprived for 24 hours, then incubated in 25 mM glucose for 48 and 72 hours. Immediately before the experiments, cells were washed with PBS containing $Ca^{2+}$and Mg^{2+} and then loaded with 5 μM DCF diacetate dissolved in PBS for 30 min at 37°C. DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured in a multiwell fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific)

E. Western Blot Analysis.

MSC80 cells 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 Bradford Protein Assay. For immunoblotting, 30-40 μg of proteins were separated on 10-12% Polyacrylamide gel

Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk or BSA in Tris-buffered saline and then incubated with rabbit polyclonal anti-Nox1 (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-Nox 4 (1:250, Santa Cruz Biotechnology), rabbit polyclonal anti-mTOR Ser2448 (1:500, abcam), rabbit polyclonal anti-P0 (1:50, abcam), rabbit polyclonal anti-PMP22 antibody (1:250, abcam). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). 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 Schwann cells 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 cell 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 3, Nox4, PMP22, P0 and LXR- β* . 26S was used as internal reference gene.

Primers	Sequence	Annealing TºC
Nox3	**F: 5'-ATGCCGGTGTGCTGGATTCTGAAC-3'** **R: 3'-CTAGAAGTTTTCCTTGTTGTAATAGAA-5'**	**62ºC**
Nox4	**F: 5'-TTCGGGTGGCTTGAAGAAGT-3'** **R: 5'–TGGGGTCCGGTTAAGACTGA-3'**	**62ºC**
PMP22	**F: 5'-AATGGACACACGACTGATC-3'** **R: 5'-CCTTTGGTGAGAGTGAAGAG-3'**	**62ºC**
LXR-β	**F: 5'-CTTGGTGGTGTCTTCTTGA-3'** **R: 5'-TGTGGTAGGCTGAGGTGTA-3'**	**62ºC**
P0	**F: 5' -GTCAAGTCCCCCAGTAGAA-3'** **R: 5'-AGGAGCAAGAGGAAAGCAC-3'**	**62ºC**
26S	**F: 5'-AGGAGAAACAACGGTCGTGCCAAAA-3'** **R: 5'-GCGCAAGCAGGTCTGAATCGTG-3'**	**62ºC**

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

G. NADPH oxidase assay.

NADPH oxidase activity was measured in sciatic nerves and in Schwann cells grown in complete medium (10% FBS, 1% P/S). 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). Cultured

MSC80 cells were washed twice with ice-cold phosphate-buffered saline and scraped from the plate on ice using the lysis buffer as well. To start the assay, 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. Detection of ROS in animal tissue.

Dihydroethidium (DHE), which is relatively specific for superoxide anion measurement, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophoreethidium bromide. DHE staining for superoxide was carried out as previously described (Maalouf et al., 2012). Briefly, frozen sciatic nerves were cut into 4 µm thick sections and placed on glass slides. DHE (20 µmol/l) was applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescent images of ethidium-stained tissue were obtained with a laser-scanning confocal microscope (Zeiss, LSM 710) at t=30 mins. Fluorescence was detected at 561 nm long-pass filter. Superoxide generation was demonstrated by red fluorescent labeling. The average of four areas per section stained with DHE was taken as the value for each animal. Quantification was done using Zen light Software.

I. Cellular DNA Fragmentation ELISA.

The cellular DNA fragmentation ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect BrdU-labeled DNA fragments in MSC80 cells. The cells were grown in 12-well tissue culture plates until 70-80% confluence. They were serum deprived for 24 hours and then treated for 48 and 72 hours according to the experimental conditions. In the first step of the apoptosis assay procedure, an anti-DNA coating solution containing anti-DNA antibody was adsorptively fixed in the wells of a microplate. 7 µL of BrdU were added to the cultured cells 12 hours before stopping the treatment. Then, BrdU-labeled DNA fragments were added to the microplate after collection using the incubation buffer. In the third step, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed on the surface of the microplate by microwave irradiation, in order to improve the accessibility of the antigen BrdU for detection by the anti-DNA antibody. As a final step, anti-BrdU-peroxidase conjugate reacted with the BrdU incorporated into the DNA. The amount of peroxidase bound in the immune complex was photometrically determined, after the removal of unbound peroxidase conjugates and the addition of the substrate solution. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (Multiskan Ex).

J. Statistical analysis.

Results are expressed as mean ± SE from multiple independent experiments. Statistical significance is assessed by student's unpaired t-test. Significance is determined as probability (p) <0.05.

CHAPTER III

RESULTS

A. Type 1 diabetes results in defects in motor coordination.

Diabetes can result in alteration of motor skills in mice. Fine motor coordination and balance are assessed by the raised beam-walking assay (Luong et al., 2011). This test examines the ability of the animal to remain upright and to walk on an elevated (50 cm above the table top) and relatively narrow beam (12 mm width). Peripheral nerve injury tends to induce defects in motor functions, which can cause the rodent to slip to one side. Non-obese diabetic (NOD) (figure 2.A) and STZ-induced diabetic mice (figure 2.B) were used in this assay. Our preliminary results show a significant decrease in mice speed while crossing the beam when compared to control littermates. Also, the number of foot slips (faults) of the diabetic animals is significantly increased by many folds when compared to controls.

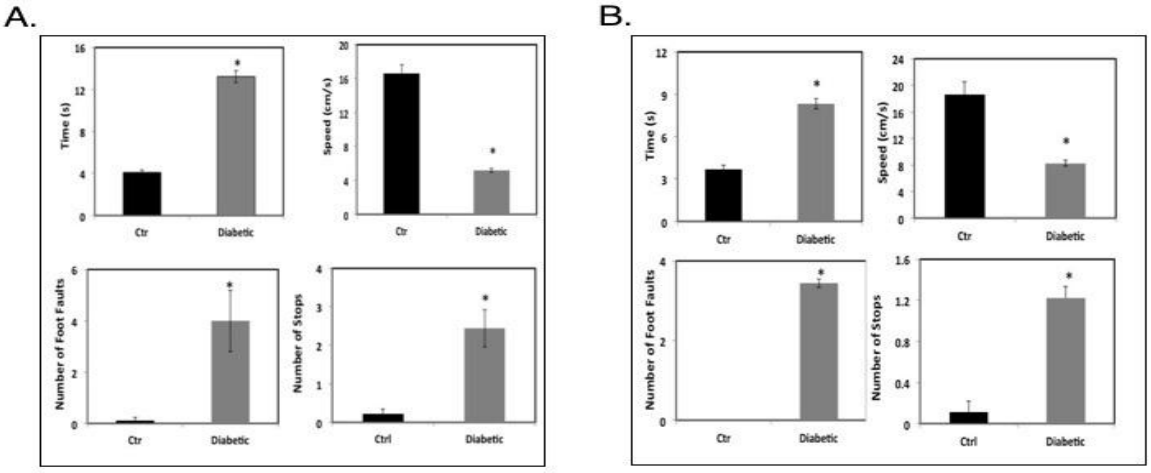


Figure 2. The effect of hyperglycemia on motor coordination.
Barograms representing the average of the time, speed, faults and stops of 2-4 months old diabetic animals vs. control animals assessed by the raised beam-walking test in A. STZ-induced (n=3) and B. NOD mice (n=5). Values are the means ± SE. *P<0.05, diabetic vs. control.

In order to correlate the behavioral damage seen in diabetic mice with changes in the signaling pathways, a series of biochemical and molecular experiments were performed on STZ- injected mice.

B. Type 1 diabetes induces myelin genes up-regulation in the sciatic nerves.

Streptozotocin (STZ)-induced type 1 diabetic mice were euthanized after 1 month of initiation of diabetes. The expression of myelin proteins MPZ and PMP22 was significantly increased in the sciatic nerves isolated from diabetic mice when compared to control littermates (figure 3).

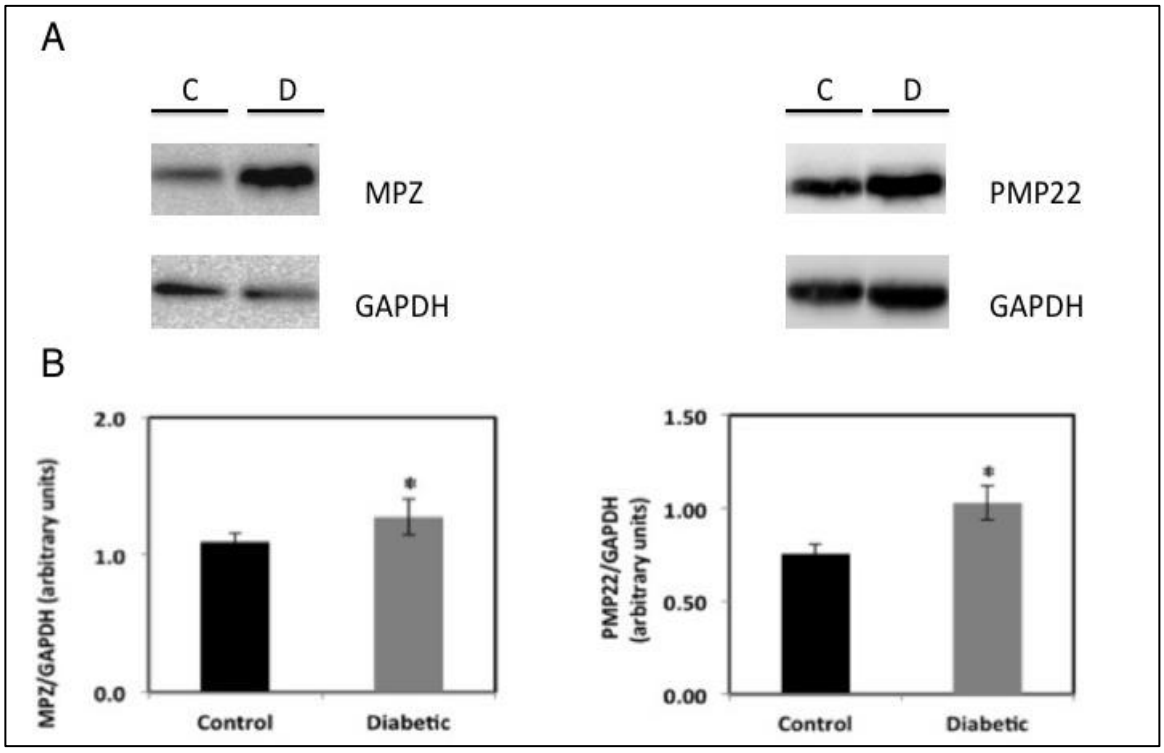


Figure 3. The effect of diabetes on myelin genes P0 and PMP22 expression.
The level of expression of P0 and PMP22 were studied in sciatic nerves extracted from mice that either received an injection of saline solution (control group) or STZ injections to induce diabetes. A. represents illustrative western blots of MPZ or P0 (left) and PMP22 (right) protein expression in diabetic vs. control animals. GAPDH was used as a loading control. B. represents the densitometric quantification of MPZ (left) and PMP22 (right). Values are the means of three animals ± SE. *P<0.05, diabetic vs. control.

C. Hyperglycemia induces oxidative stress in diabetic mice

Our group has previously shown that high glucose concentration and oxidative stress are the main potential mediators of the complications seen during the development and onset of type 1diabetes. ROS production was assessed by DHE (dihydroethidium) staining. The results show that ROS was significantly increased (more than 3 folds) in the sciatic nerve of diabetic animals when compared to their control littermates (figure 4.A&B).

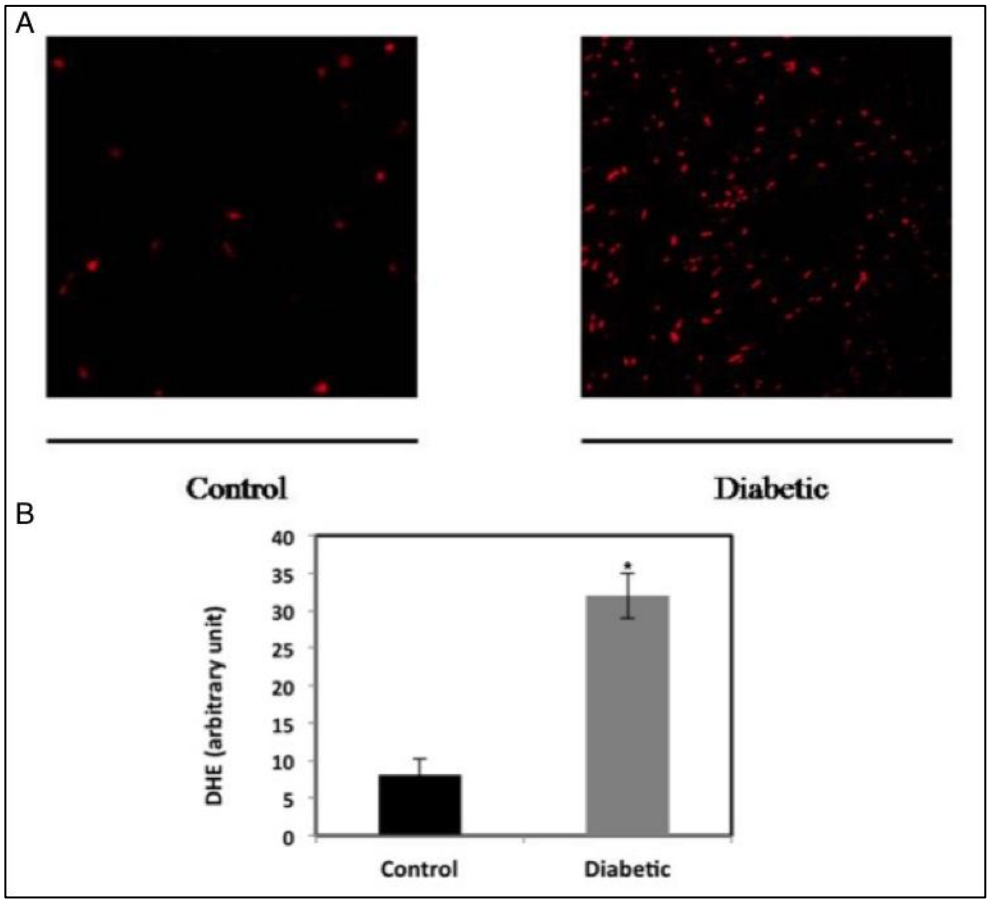


Figure 4. Hyperglycemia induces ROS production assessed by DHE staining.
A. Superoxide production assessed by DHE staining. B. Quantification of ROS in sciatic nerves using the Image-Pro Plus 4.5 software. The barograms represent mean ± SE of four different areas taken from four individual mice in each group.

D. Increased ROS production is mediated by an up-regulation in NADPH oxidases levels and activity.

To further investigate the increase in ROS production seen in the diabetic animals, the NADPH dependent superoxide generation (NADPH oxidase activity) and the protein levels of the electron chain transporters of the Nox family, Nox1, Nox2 and Nox4 were assessed in the sciatic nerves. Our results show an increase in the NADPH oxidases activity in

diabetic animals as compared to their control littermates (figure 5.A). This was paralleled by an up-regulation of the Nox1, Nox2 and Nox4 protein expression (figure 5.B & C)

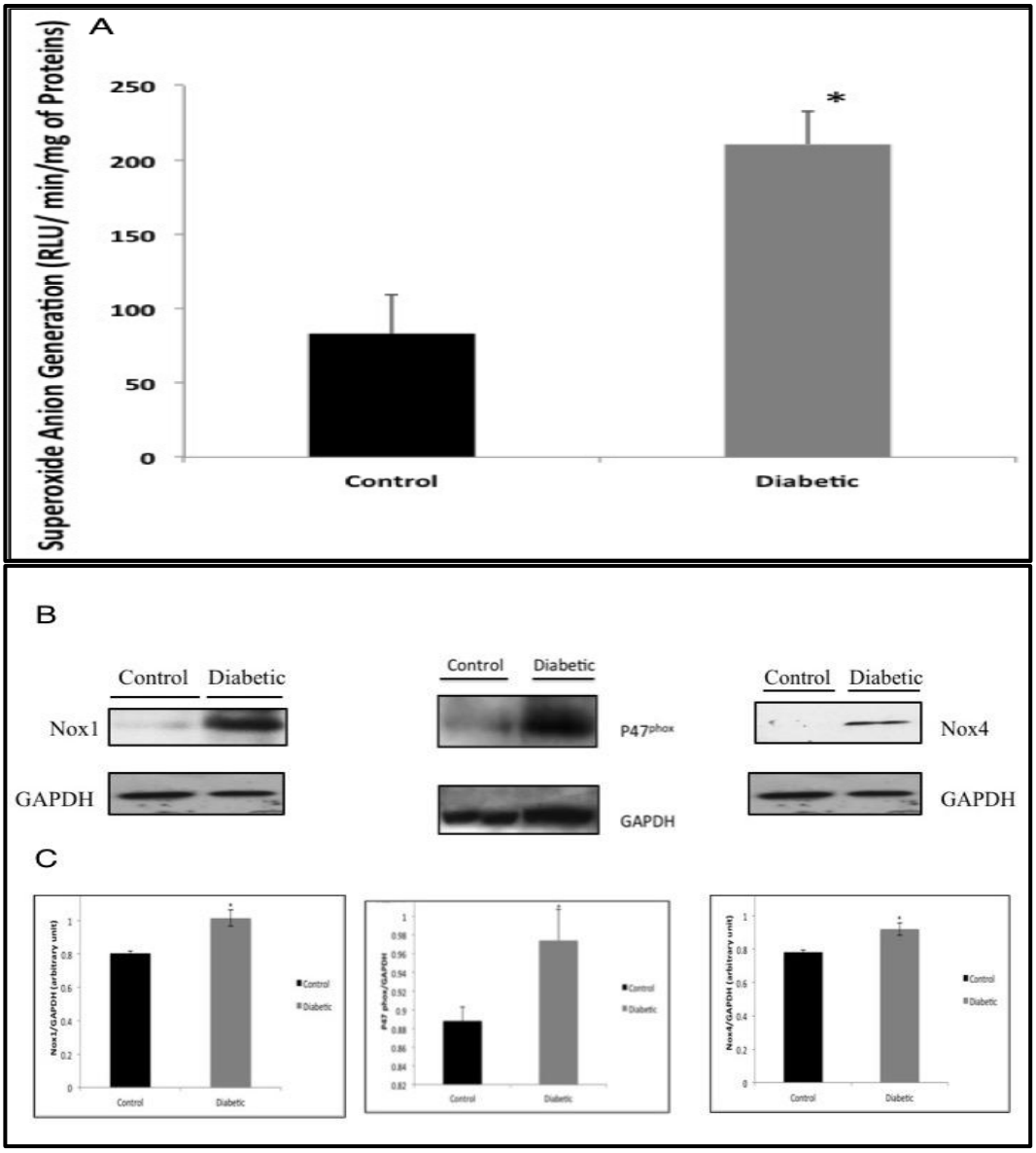


Figure 5. Hyperglycemia induces NADPH dependent superoxide generation and NADPH oxidase Nox1, Nox2 and Nox4 protein expression.
NADPH oxidase level and activity were measured in this study. Figure A. represents the average superoxide generation in diabetic animals vs control animals (n=4). Figure B. represents the protein expression of Nox1, 2 and 4 (from left to right) in diabetic sciatic nerves as compared to control levels (n=3). GAPDH was used as loading control. Figure C. represents the densitometric quantification of Nox1, Nox2 and Nox4 (from left to right).Values are the means ± SE. *P<0.05 diabetic vs. control.

One of the major aims of our study is to indentify the signaling pathways that are altered during the development of diabetic neuropathy. LXR and mTOR have been shown by our group and others to play a role in the physiology and pathophysiology of the peripheral nerves. However, the involvement of these pathways in DPN and their

E. Hyperglycemia induces LXR inactivation.

Our preliminary results show a significant decrease in LXR protein expression in the sciatic nerves of diabetic animals as compared to their control littermates (figure 6.A). Furthermore, the involvement of LXR in altering NADPH oxidases Nox4-dependent ROS production was assessed by measuring the protein expression of Nox4 in the sciatic nerves of LXR double knockout mice (LXR $\alpha, \beta^{-/-}$). Figure 6.B shows a significant increase in Nox4 levels in LXR dKO as compared to their control littermates. The results may suggest that LXR inactivation in the diabetic sciatic nerves induces ROS production in a NADPH dependent mechanism.

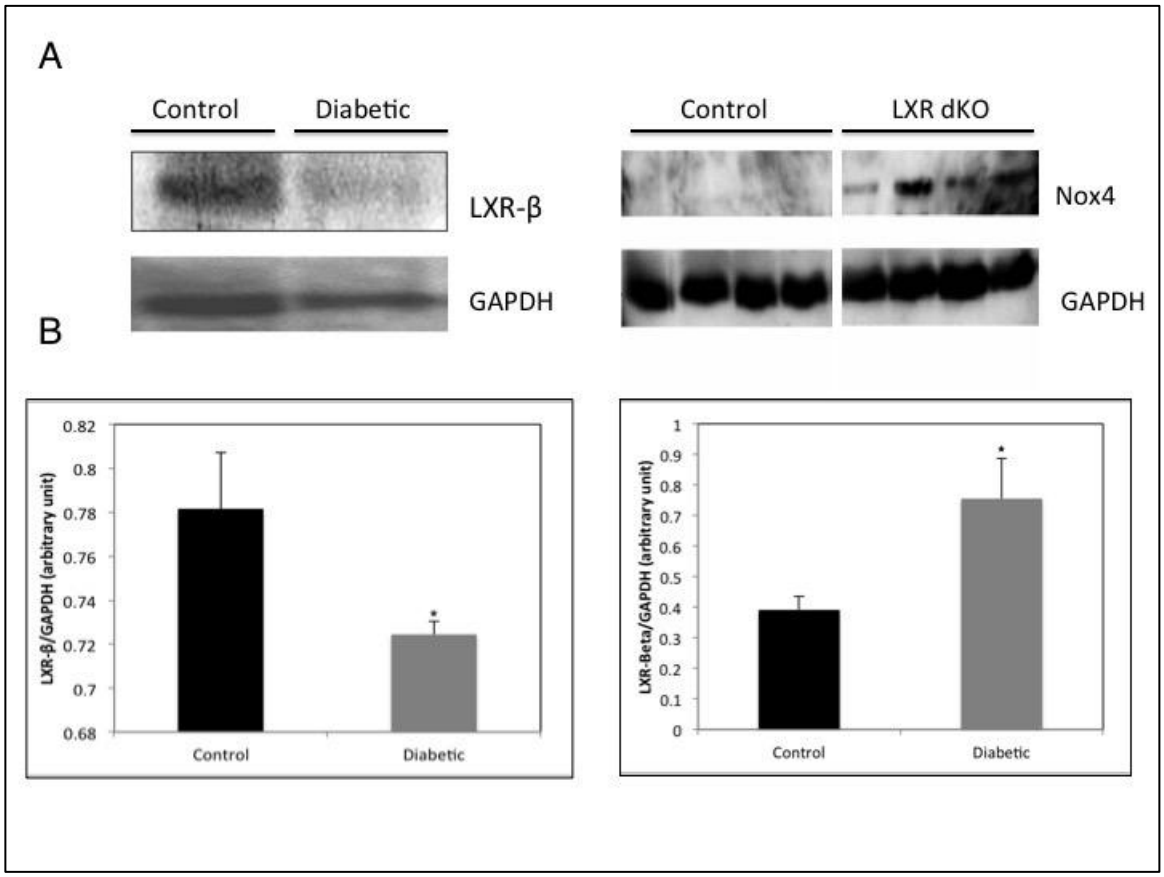


Figure 6. Diabetes results in a down-regulation in LXR activity.
LXR-β expression in the sciatic nerves of diabetic animals and Nox4 expression in sciatic nerves of LXR dKO mice were assessed by western blot. A. representative western blots showing the expression of LXR-β in diabetic vs. control mice (left) and Nox4 in dKO mice vs. control mice (right) along with their loading control (GAPDH). B. Barograms representing the densitometric quantification of the results obtained for LXR-β (left) and Nox4 (right). Values are the means ± SE. *$P < 0.05$ diabetic mice vs. control mice or LXR dKO vs. control mice.

F. mTOR/P70S6K pathway is activated in sciatic nerves of Type 1 diabetes.

The effect of hyperglycemia on mTOR activation was studied in a mouse model of Type 1 diabetes. Phosphorylated (p)-P70S6K^{Thr389} was increased in the sciatic nerves of STZ-injected mice (D) compared to controls (C). P70S6K is a read out of mTOR activation.

Concomitantly, mTOR is shown to be increasingly phosphorylated on its activation residue Ser2448 (figure 7.A&B).

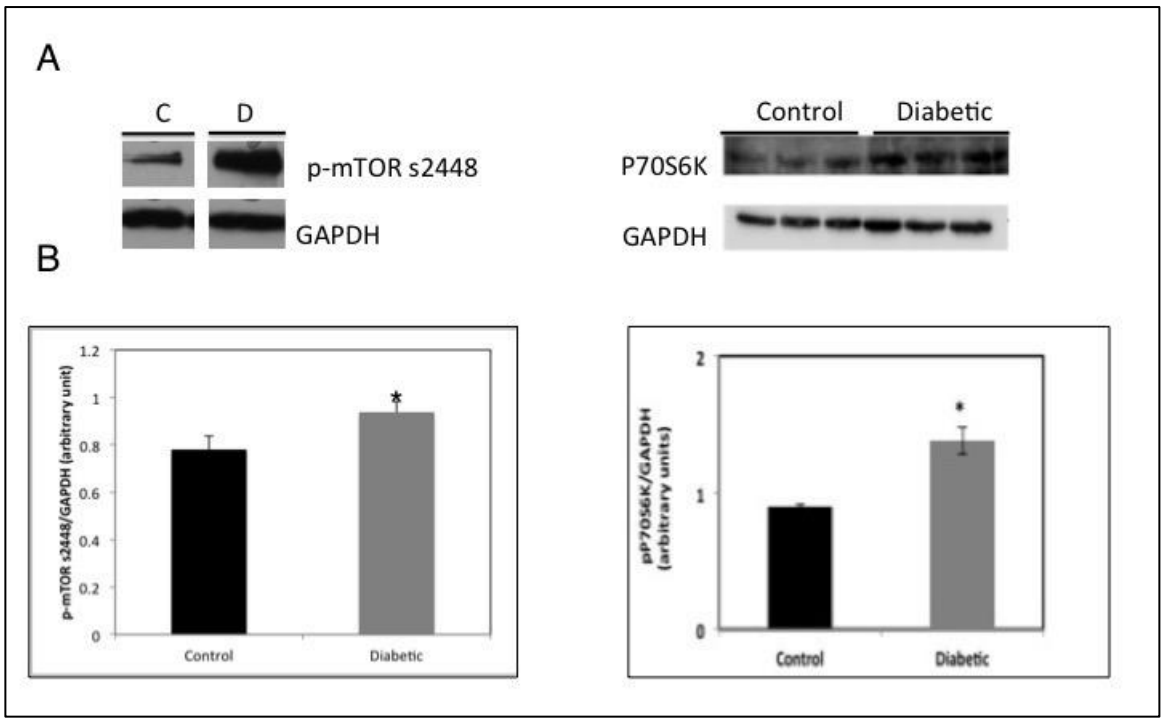


Figure 7. The mTOR/P70S6K pathway is activated by type 1 diabetes.
The activation of mTOR/P70S6K was also assessed in this study. A. represents illustrative western blots of p-mTOR (left) and p70S6K (right) protein expression in diabetic animals vs. control animals. GAPDH was used as a loading control. B. represents the densitometric quantification of the results obtained for mTOR (left) and P70S6K (right). Values are the means ± SE from three animals for each group. **P* < 0.05 diabetic mice vs. control mice.

G. High Glucose results in Schwann cell injury *in vitro.*

In order to further dissect the cellular and molecular mechanisms of injury by which hyperglycemia exerts its deleterious effect on Schwann cells, MSC80 cells were cultured in growth medium with 5 mM (normal glucose) or 25 mM of glucose (high glucose). Schwann cell injury was assessed by altered myelin genes expression and apoptosis.

1- *High glucose (HG) induces an alteration in myelin genes expression in vitro.*

Temporal effect of HG on P0 and PMP22 expression in Schwann cells was studied at both transcriptional and translational levels. The results show that the treatment of MSC80 cells with 25 mM of glucose for 3, 6, 24, 48 and 72 hours resulted in a significant increase in PMP22 mRNA levels at different time points. There was also a slightly significant increase in P0 at the mRNA level at 3h followed by a drop at 72h (figure 8.A). At the translational level, the assessment of the expression of P0 shows a significant increase in its level in cells treated for 72h with 25 mM glucose as compared to the non-treated cells (figure 8.B).

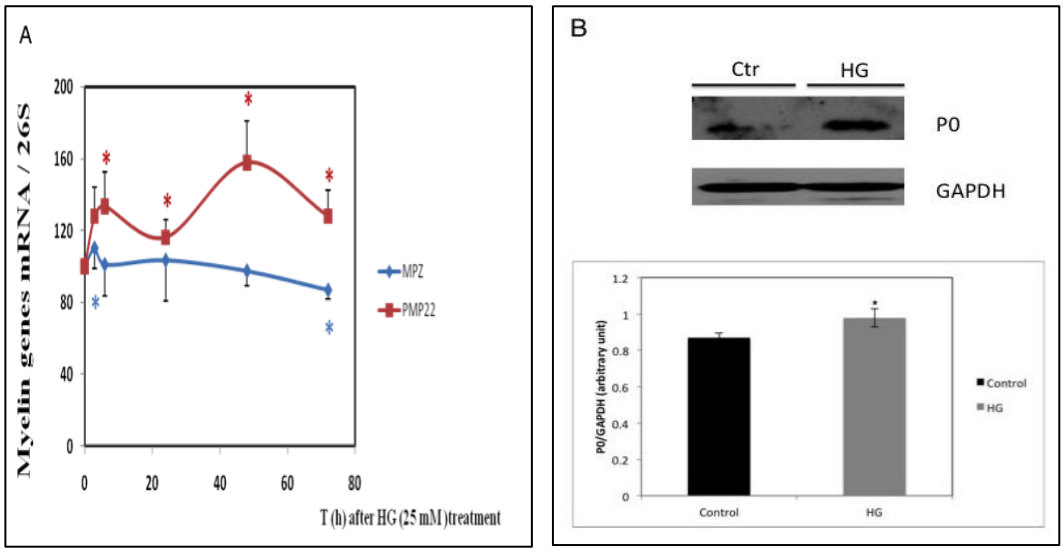


Figure 8. High glucose treatment alters the expression levels of myelin proteins P0 and PMP22 in Schwann cells.
A. represents the temporal effect of high glucose treatment on the mRNA level of P0 and PMP22 measured in MSC80 cells by RT-PCR (n=7) . B. represents P0 protein levels assessed after 72 hours of treatment with glucose along with the densitometric quantification of the results obtained (n=3). GAPDH was used as a loading control. Values are the means ± SE. *P<0.05, high glucose vs. control.

2- ***High glucose triggers Schwann cell apoptosis in vitro.***

Mouse Schwann cells (MSC80) were treated with HG (25 mM) for 48 (figure 9.A) and 72 hours (figure 9.B). Our results show that HG induced SC death (2.3 fold change at 48 hours and 1.3 fold change at 72 hours), as assessed by cellular DNA fragmentation/ ELISA test.

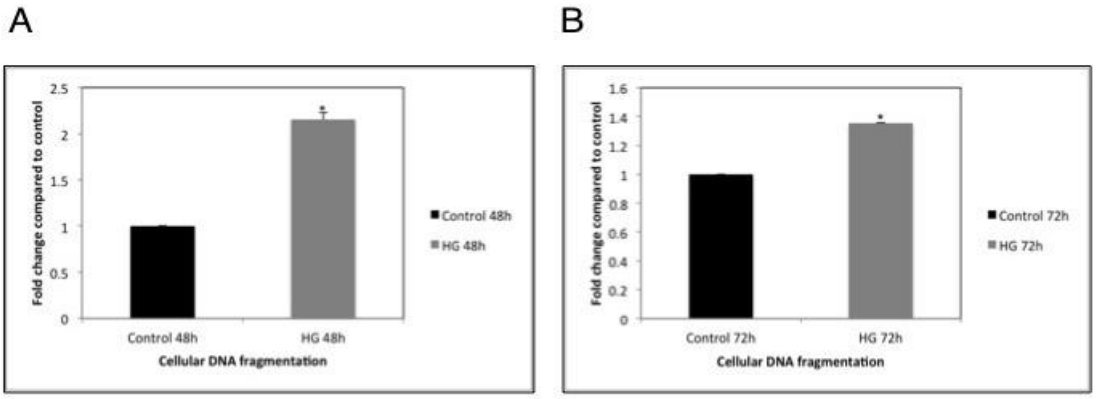


Figure 9. High glucose results in Schwann cell apoptosis *in vitro.*
Figure 9 represents the results of Schwann cells' apoptosis following the treatment with high glucose for 48 (figure 9.A) and 72 hours (figure 9.B), as assessed by DNA fragmentation/ELISA test (n=2). Values are the means ± SE. *P<0.05, high glucose vs. control.

H. Hyperglycemia results in increased ROS production in MSC80.

MSC80 were treated with 5 mM of glucose (normal glucose) or with 25 mM of glucose (high glucose) for 48 (figure 10.A) and 72 hours (figure 10.B). DCF is used to measure intracellular superoxide production. Our results show that HG induces an up-regulation in ROS production when compared to cells incubated in normal glucose. This experiment was done to confirm the results already published (Eid et al., 2013) concerning ROS production in cells

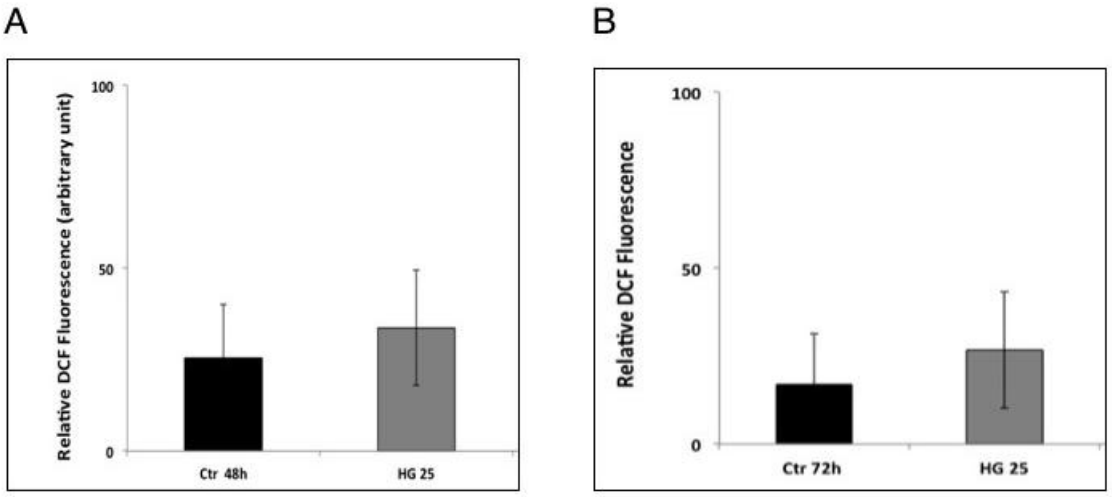


Figure 10. Hyperglycemia is associated with increase ROS production at the level of the Schwann cells.
Figure 10 represents the results of intracellular superoxide generation as assessed by DCF fluorescent probes after 48 hours (figure A) and 72 hours (figure B) of cell treatment with high glucose (25 mM) vs. normal glucose (5 mM). n=1. Values are the means ± SE.

I. High glucose treatment increases NADPH dependent superoxide production and NADPH oxidases mRNA levels and protein expression.

NADPH dependent superoxide production was increased in cells incubated with high glucose for 3 hours (figure 11.A), and the effect was sustained till 72 hours of exposure to glucose (data not shown). This increase was paralleled by a rise in the NADPH oxidases Nox3 and Nox4 mRNA levels (figure 11.B) and Nox1 and Nox4 protein expression (figure 11.C).

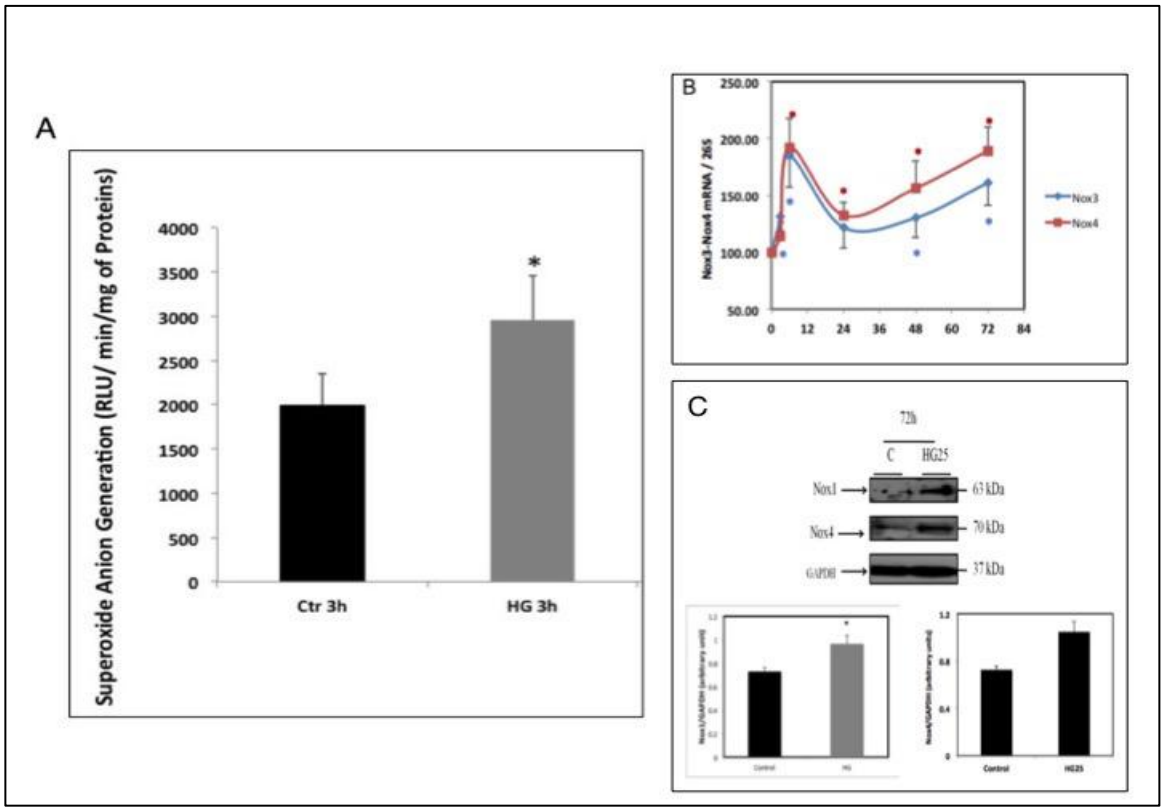


Figure 11. High Glucose triggers an upregulation in the activity and expression of NADPH oxidases.
A. represents the NADPH oxidase activity level in Schwann cells treated with HG for 3 hours (n=3). B. represents the change mRNA levels of Nox3 and Nox4 measured in cells treated with HG as a function of time (n=5). C. represents Nox1 and Nox4 protein expression in MSC80 following the treatment with normal glucose vs. high glucose (n=3 for Nox1 and n=2 for Nox4). GAPDH was used as a loading control. Values are the means ± SE. *P<0.05, high glucose vs. control.

J. High glucose results in altered LXR mRNA levels in cultured Schwann cells.

Treatment of MSC80 cells with 25 mM glucose caused an alteration in LXR mRNA level when compared to cells exposed to normal glucose. These variations were significantly pronounced at 48 hours of treatment, followed by a significant decrease at 72 hours (figure 12).

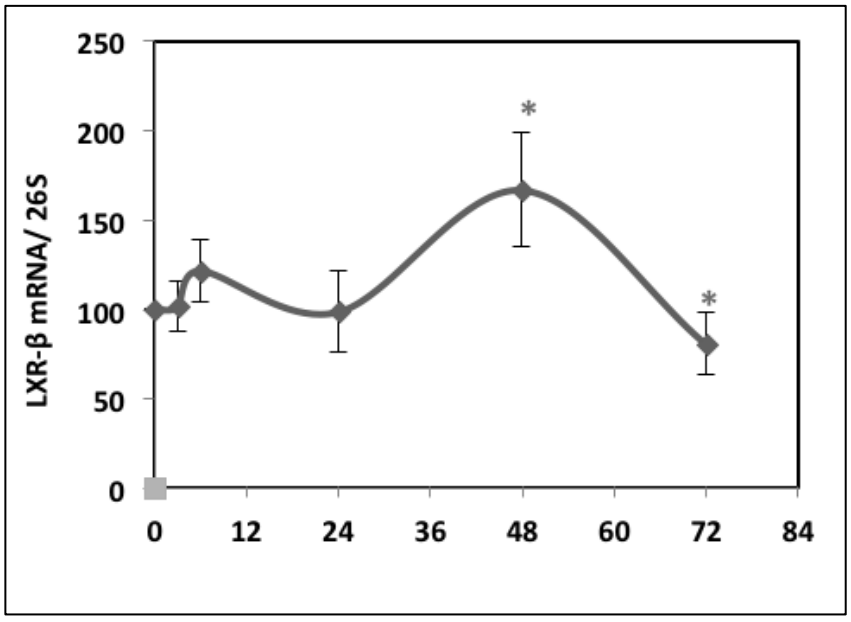


Figure 12. High glucose leads to an alteration in LXR-β mRNA in Schwann cells. LXR-β mRNA levels were measured in MSC80 cells treated with 25 mM of glucose vs. normal glucose for 3, 6, 24, 48 and 72 hours (n=5). Values are the means ± SE. *P<0.05, high glucose vs. control.

K. High glucose induces mTOR signaling pathway activation in Schwann cells.

Schwann cells were incubated in media containing high glucose or 5 mM of glucose for 72 hours (figure 13). HG treatment induced an increased phosphorylation of mTOR on its activation site (Ser2448).

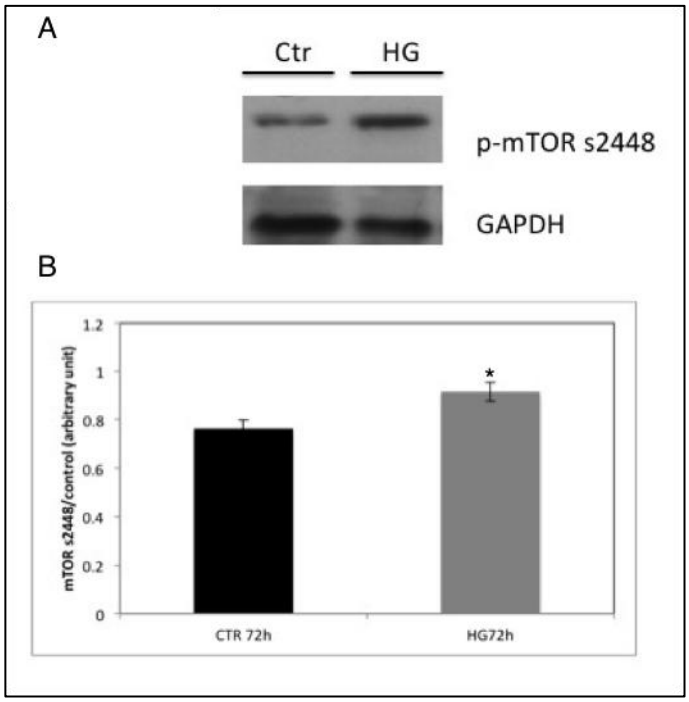
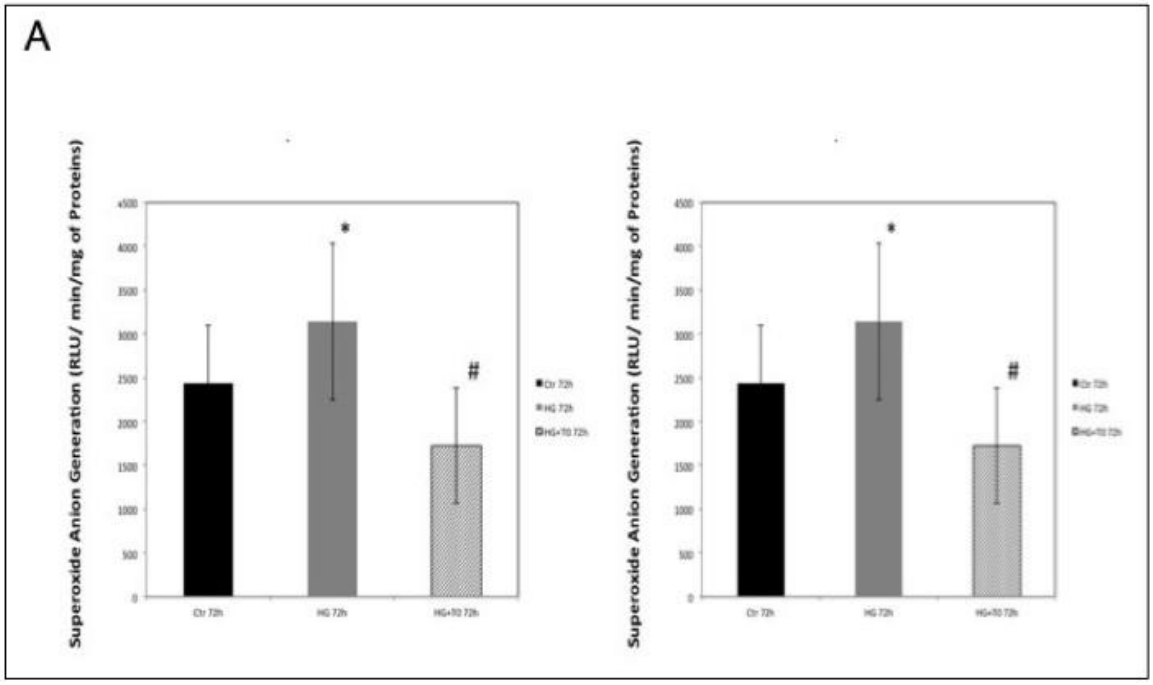


Figure 13. High glucose stimulates an increased activation of the mTOR pathway. Figure 13 represents an illustrative western blot of the phosphorylated mTOR p-mTOR^{Ser2448} in Schwann cell treated with HG vs. normal glucose along with its loading control, GAPDH (figure A) and the densitometric quantification of the results obtained (figure B). n=3. Values are the means ± SE. *P<0.05 high glucose vs. control.

L. T0901317 treatment allows a decreased activation of the NADPH oxidases and reversal of Schwann cell injury shown by the restoration of PMP22 protein levels and decreased apoptotic cell death.

In order to assess the effect of the alteration in different proteins seen in type 1 diabetic animals or in Schwann cells treated with high glucose, different genetic or pharmaceutical inhibitors are being used. However, for the scope of this study, the focus has been diverted to T0901317. The LXR agonist is used to confirm the involvement of LXR in diabetic neuropathy and its cross talk with the ROS generating NADPH oxidases. For this reason, MSC80 cells were treated with high glucose for 48 and 72 hours in the presence or absence of T0901317, while control cells were culture in medium with 5 mM of glucose. As

expected, our results show that T0901317 treatment reverses the effect of high glucose-induced NADPH oxidase dependent ROS production (figure 14.A). T0901317 also reversed the effect of high glucose-induced deregulation in PMP22 levels (figure 14.B). Interestingly, Schwann cells' apoptosis was blocked by T0901317 treatment in cells exposed to high glucose (figure 14.C). Our results suggest that high glucose induces Schwann cell injury by inactivating the LXR pathway, which in turn increases ROS production through a NADPH dependent mechanism. Treatment of SCs with T0901317, LXR agonist, decreased ROS production by decreasing NADPH oxidase activity, reversed myelin gene deregulation and blocked SC apoptosis.



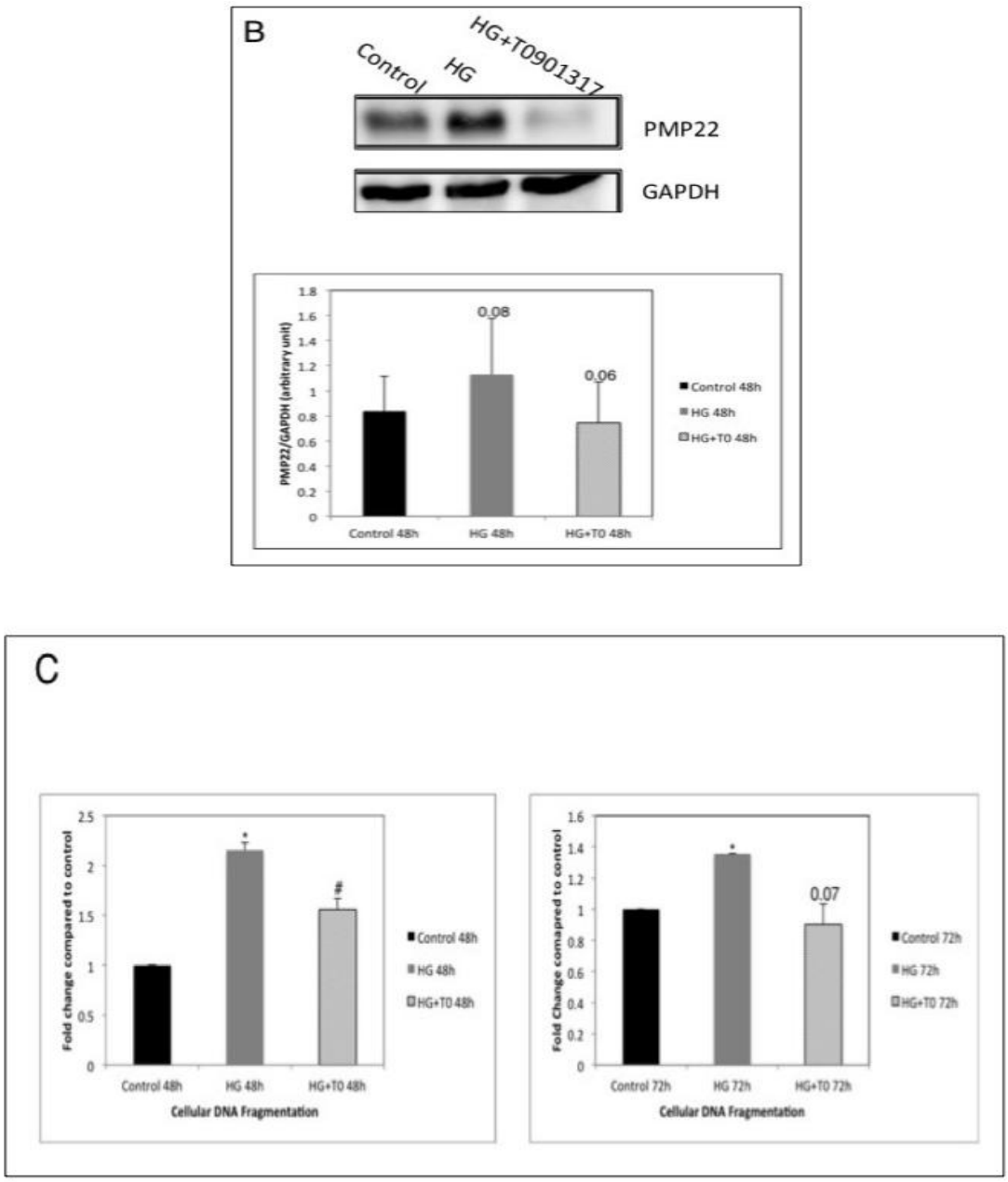


Figure 14. T0901317 reverses Schwann cell injury in the hyperglycemic milieu.
A. represents the change in NADPH oxidase activity in cells treated for 48 (left) and 72 (right) hours with high glucose or with high glucose+T0901317 vs. normal glucose (n=3). B. represents PMP22 protein expression level at 48 hours of treatment with high glucose or high glucose+T0901317 vs. normal glucose (n=3). C. represents Schwann cell apoptosis in media containing normal glucose vs. media with high glucose or high glucose+T0901317 at 48 (left) and 72 (right) hours of treatment, as assessed by ELISA test (n=2). Values are the means ± SE. *P<0.05 high glucose vs. control; #P<0.05 high glucose with T0901317 vs. high glucose.

CHAPTER IV

DISCUSSION

DPN has been shown to cause motor dysfunction as well as cellular and molecular dysfunction. Recent experimental studies focus on the role of Schwann cells in mediating nerve injury in diabetic neuropathy. Demyelination and SC apoptosis have been suggested as one of the major phenotypes that contribute to DPN (Wu et al., 2012; Sun et al., 2012; Yu et al., 2015). However, the exact mechanisms underlying the phenotypic changes seen in Schwann cells are still controversial and yet to be investigated. It has been shown that type 1-diabetes affects motor coordination. Performance on the raised beam is a useful measure of fine coordination and balance and has been validated by previous work. We demonstrate that peripheral nerve injury tends to induce defects in motor coordination.

Myelin dysfunction such as alterations in myelin compaction and aberrant separation of myelin lamellae are all markers of myelin sheath abnormalities occurring during the course of diabetic neuropathy in animal models, and contributing to nerve degeneration (Cermenati et al., 2012). These defects might be attributed to abnormal lipid content of the myelin sheath, knowing that lipids constitute 70-80% of myelin and are required for the saltatory propagation of nervous influx (Garbay et al., 2000). However, the maintenance of myelin proteins' levels is compulsory for the integrity of myelin, although they represent only 20-30% of the myelin composition. P0 is mandatory for the spiraling, compaction and maintenance of the myelin sheath while PMP22 is involved in the initiation of myelination, determination of myelin thickness and stability of the myelin sheath (Garbay et al., 2000).

Any alteration in the expression of these proteins would have a deep impact on the development and preservation of nerve fibers and their myelin sheaths, as observed in Charcot-Marie-Tooth disease (Niemann et al., 2006). Our study shows that P0 and PMP22 expression level significantly increases with diabetes and this alteration in myelin protein levels is causing demyelination of the peripheral nerves. The alteration in P0 and PMP22 was also observed at the mRNA levels, where PMP22 mRNA transcripts were significantly increased with glucose treatment of Schwann cells, while P0 transcripts were decreased. In concordance with our results, Conti et al. show that P0 expression levels were increased in an experimental model of diabetic neuropathy (Conti et al., 1996). However, other investigators showed a decrease in the levels of myelin protein zero in sciatic nerves of diabetic animals (Kawashima et al., 2007; Cermenati et al., 2012). PMP22 and P0 are dosage-sensitive genes and their coordinated expression is necessary for efficient myelination. Increase in PMP22 and MPZ transcripts in our animal model of diabetes may thus result in a less efficient myelination of axons. This observation is not surprising because several studies described a congenital hypomyelination of peripheral nerves associated with P0 overexpression and its consequent mistargeting to mesaxon membranes (Wrabetz et al., 2000; Yin et al., 2000).

Reactive oxygen species are known to function in a tissue and cell specific manner and any deviation in their homeostatic levels could be implicated in the pathogenesis of many diseases including DPN (Maritim et al., 2003; Shakeel, 2014). Treatment with anti-oxidants (resveratrol, α-Lipoic Acid and vitamin E) have been shown to block several manifestations of diabetic neuropathy including thermal hyperlgesia, allodynia and decreased NCV (Kumar, 2007; Sharma, 2006). Many enzymes, such as those in the

mitochondrial electron transport chain, xanthine oxidase, uncoupled NOS, lipoxygenases, cyclooxygenases and cytochrome P450 monooxygenase, produce ROS in different organs of the body including the PNS. We study another major source of ROS, the Nox family of NADPH oxidases. We demonstrate that hyperglycemia triggers an up-regulation in Nox1, Nox2, Nox3 and Nox4 in sciatic nerves of diabetic animals. A significant increase in Nox levels and activity was also detected in Schwann cells treated with high glucose. The activity was also shown to be significantly reduced by treatment with T0901317 (LXR agonist). ROS might mediate their deleterious effect through the down regulation of LXR or the activation of mTOR.

In this study, we also provide in this study a link between ROS production and the alteration of the LXR pathway. The expression of LXR at the transcriptional and translational level was shown to be significantly altered with hyperglycemia. Moreover, LXR dKO (LXRα, β $^{-/-}$) mice showed an increased production of Nox4, raising the levels of oxidative stress in the sciatic nerves of these animals. Hichor et al. (unpublished data from the laboratory of our collaborator Dr. Charbel Massaad's lab) suggest that LXR deficiency induced nerve injury by increasing reactive oxygen species and altering the anti-oxidant machinery. To further highlight the importance of LXR functioning at the level of the nerves, Makoukji et al. correlated the LXR pathway with myelination, where a decrease in LXR expression in a double knockout mouse model (LXR α, β $^{-/-}$) resulted in hypomyelinated sciatic nerves (Makoukji et al., 2011). Later studies proposed that LXR plays a role in the myelination process through myelin lipid regulation (Cermenati et al., 2012). Cermenati et al. also showed that LXR pathway plays a protective role in the pathology of diabetes by increasing the production of neuroactive steroids, which function

in reversing the changes observed in diabetic neuropathy (Cermenati et al., 2010). Therefore, the observed defect in LXR expression in DN exerts its effects through a multitude of mechanisms: an up-regulation in Nox levels and activity, resulting in nerve injury, a decreased ability to regenerate the myelin sheath following nerve damage produced by the diabetic milieu and a decrease in protective mechanisms which, when activated, reverse the detrimental effect of high glucose concentrations on the functioning of the nerve fibers. However, to further confirm the involvement of this pathway in high glucose-induced cell injury, knocking down the LXR gene by siRNA transfection of Schwann cells will be performed. Afterwards, we will compare the alterations observed due to gene knockdown to what is observed in a hyperglycemic milieu.

Our data show an increased phosphorylation of mTOR on its activation site (serine 2448), along with its downstream target P70S6kinase due to high glucose concentrations in the sciatic nerves and mouse Schwann cells (MSC80). mTOR plays a critical role in cellular injury by mediating cellular growth, apoptosis, differentiation and transcription (Yuan et al., 2008). mTOR has also been involved in PNS myelination: while Norrmen et al. associate mTORC1 inactivation with hypomyelinated nerves (Norrmen et al., 2014), other studies indicate that mTORC1 overexpression causes hypermyelinated nerves (Noseda et al., 2013). However, the role of mTOR in mediating injury in DPN hasn't been described. Our findings suggest that high glucose-induced ROS generation is responsible for the activation of mTOR pathways, which might lead in turn to Schwann cell apoptosis. So in our future experiments, we will knock down the mTOR gene and study apoptosis in cells with or without glucose treatment.

Therefore, the development of diabetic neuropathy results from an alteration in multiple mechanistic pathways: hyperglycemia induces a decreased activation of the LXR pathway, a key player involved essentially in lipid and cholesterol homeostasis. Concomitantly, reactive oxygen species levels peaked due to high glucose availability. This increase was associated with the activation of the mTOR pathway, resulting in an alteration in P0 and PMP22 expression and SC apoptosis. Knowing that Schwann cells are the major players in the maintenance of nerve functioning, any deviation from the normal function induces neural abnormalities similar to what is observed in diabetes. Nerve demyelination occurs complemented by a reduced ability to regenerate. This results in loss of function of the peripheral nerves and a poor motor behavior, manifested by detectable deficiencies in motor coordination.

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