Mechanism of insulin/IGF-1 regulation of mitochondrial function and nerve repair in diabetic neuropathy

By

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Abstract

Background: There is impaired neurotrophic growth factor signaling, AMP-activated protein kinase (AMPK) activity and mitochondrial function in dorsal root ganglia (DRG) of animal models of type 1 and type 2 diabetes exhibiting diabetic sensorimotor polyneuropathy (DSPN). We hypothesized that loss of direct insulin or insulin-like growth factor 1 (IGF-1) signaling in diabetes drives depression of AMPK activity and mitochondrial function, both contributing to development of DSPN.

Methods: Age-matched control Sprague-Dawley rats and streptozotocin (STZ)-induced type 1 diabetic rats with/without IGF-1 therapy or insulin implants were used for *in vivo* studies. For *in vitro* studies, adult DRG neurons derived from control or STZ-diabetic rats were cultured under defined conditions and treated with/without IGF-1 or insulin. Activators or inhibitors targeting components of the insulin/IGF-1 signaling pathways were used to unravel the mechanism of insulin/IGF-1 action in DRG neurons.

Results: Insulin increased Akt phosphorylation and neurite outgrowth, and augmented mitochondrial function in DRG cultures derived from control or type 1 diabetic rats. In STZ-diabetic rats insulin implants reversed thermal sensitivity, increased dermal nerve density and restored the expression/activity of respiratory chain proteins in DRG. Decreased expression of mRNAs for IGF-1, AMPKα2 and ATP5a1 (subunit of ATPase) occurred in DRG of diabetic vs. control rats. IGF-1 up-regulated mRNA levels of these genes in cultured DRG neurons from control or diabetic rats. IGF-1 elevation of mitochondrial function, mtDNA and neurite outgrowth was suppressed by inhibition of AMPK (via siRNA). IGF-1 therapy in diabetic rats reversed thermal hypoalgesia, raised corneal nerve density and prevented tricarboxylic acid (TCA) pathway metabolite build-up in the sciatic nerve. Endogenous IGF-1 gene expression in

neurons of the DRG was suppressed by hyperglycemia and rescued by treatment with IGF-1 or the aldose reductase inhibitor, sorbinil. Transcription factors NFAT1 and CEBP-β bound to the IGF-1 promoter in DRG tissue at higher levels in control vs diabetic rats. Inhibition of endogenous IGF-1 down-regulated Akt S473 phosphorylation and background neurite outgrowth in cultured DRG neurons.

Conclusions: Insulin/IGF-1 therapy elevates mitochondrial function via AMPK to drive axonal repair in DSPN. Downregulation of endogenous IGF-1 in DRG neurons in diabetes may contribute to the pathogenesis of DSPN.

Key words: AMPK; axon regeneration; bioenergetics; diabetic neuropathy; dorsal root ganglia; endogenous IGF-1; IGF-1 therapy; insulin implant; mitochondrial function; neurite outgrowth.

Contributions

The results presented in this thesis, and full name and contributions of all authors for generating data are arranged in manuscript format (Chapters 2-4). I, MohamadReza Aghanoori (M.R.A.), would like to acknowledge that the contributions of each author for the respective manuscripts are described below:

Chapter 2:

M.R.A. performed the majority of work including primary neuron cell culture, Western blotting, real-Time PCR and immunocytochemistry. D.R.S. and N.A.C. maintained, treated, and performed behavioral, electrophysiological and biochemical assays on groups of rats. M.R.A., S.K.R. and M.G.S. performed Seahorse analysis of mitochondrial function and cell counting. M.R.A. and S.K.R. carried out the mitochondrial enzymatic activities. M.R.A., N.A.C. and D.R.S. analyzed data. D.R.S. and M.R.A. designed experiments. M.R.A. contributed to write the first draft of paper. P.F. designed experiments, analyzed data and wrote and edited the paper.

Chapter 3:

M.R.A. designed and performed the in vitro studies and analyzed all tissues from the in vivo study. He aided in the metabolomic analysis and thermal sensitivity testing, performed data generation and analysis and contributed to writing of the manuscript. D.S. directed maintenance of diabetic rats, performed thermal sensitivity testing, supervised IGF-I therapy and aided in data analysis and statistics. A.A. performed in vitro studies identifying IGF-1 as a regulator of AMPK. M.A. and S.S. performed metabolomic analysis and bioinformatics. C.A.J. and F.D. maintained and treated diabetic mice and performed imaging studies on the cornea. A.N. performed terminal analysis of corneal confocal measurements. X.Z. performed IENF analysis.

N.A.C. supervised skin histopathology and corneal confocal microscopy studies and aided in writing of manuscript. P.F. supervised all in vitro and in vivo work and wrote the manuscript.

Chapter 4:

M.R.A. designed and performed the in vitro studies and analyzed all tissues from the in vivo study. He performed data generation and analysis and wrote the first draft of the manuscript. V.Y. and D.M. performed nanoparticle assembling of siRNAs. P.A. and V.D. performed bioinformatic screening of IGF-1 promoter. P.F. supervised all in vitro and in vivo work and edited the manuscript.

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Dedication

I would like to dedicate this thesis to my incredible wife Ghazaleh for her love and support.

I also dedicate my thesis to my supportive and beloved dad and mom, and my siblings.

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Abbreviations

Acetyl-coA carboxylase (ACC)

Adenylate energy charge (AEC)

Advanced glycation end-products (AGEs)

Aldose reductase (AR)

Aldose reductase inhibitors (ARIs)

AMP-activated protein kinase (AMPK)

Amyotrophic lateral sclerosis (ALS)

Angiotensin converting enzyme (ACE)

Angiotensin-converting enzyme inhibitors (ACEIs)

ATP-sensitive K (K_{ATP})

Auto-inhibitory domain (AID)

Auto-inhibitory sequence (AIS)

BioBreeding/Worcester (BB/Wor)

Brain-derived neurotrophic factor (BDNF)

β-subunit interacting domain (β-SID)

Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ)

Calcitonin gene related-peptide (CGRP)

Carbohydrate-binding module (CBM)

Carnitine palmitoyltransferase 1 (CPT1)

CCAAT/enhancer-binding protein (C/EBP)

Chemotherapy-induced peripheral neuropathy (CIPN)

Central nervous system (CNS)

Corneal confocal microscopy (CCM)

Corneal nerve fiber density (CNFD)

Corneal nerve fiber length (CNFL)

C terminus domain (CTD)

Cystathione β-synthase (CBS)

Diabetes Control and Complications Trial (DCCT)

Diabetic sensorimotor polyneuropathy (DSPN)

Diacylglycerol (DAG)

Dorsal root ganglia (DRG)

Dynamin-related proteins (DRPs)

Dynein-related protein 1 (DRP1)

Electron transport system (ETS)

Eukaryotic elongation factor 2 kinase (eEF2K)

Fibroblast growth factors (FGFs)

Forkhead box O3 (FOXO3)

Genome wide association studies (GWAS)

Gestational diabetes mellitus (GDM)

Glial cell line-derived neurotrophic factor (GDNF)

Glycogen synthase (GS)

Goto-Kakizaki (GK)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Growth hormone (GH)

Hepatocyte nuclear factors (HNF)

High-density lipoproteins (HDLs)

High-density lipoprotein-cholesterol (HDL-C)

Human leukocyte antigen (HLA)

Hydrogen peroxide (H_2O_2)

IGF-1 binding proteins (IGFBPs)

IGF-1 receptor (IGF1R)

Inner membrane (IM)

Inorganic phosphate (Pi)

Insulin-dependent diabetes mellitus (IDDM)

Insulin-like growth factors (IGFs)

Insulin receptors (IRs)

Insulin receptor substrate (IRS)

Intra-epidermal nerve fiber (IENF)

Intermembrane space (IMS)

LDL and triglycerides (TGs)

Liver-kinase-B-1 (LKB1)

Low-density lipoproteins (LDLs)

Low-threshold mechanoreceptors (LTMRs)

Mammalian target of rapamycin complex 1 (mTORC1)

Matrix (M)

Maturity-onset diabetes of the young (MODY)

Messenger RNA (mRNA)

Metabotropic glutamate receptor (mGluR)

Mitochondrial carrier family (MCF)

Mitochondrial DNA (mtDNA)

Mitochondrial inner membrane organizing system (MINOS)

Mitochondrial targeting sequence (MTS)

Mitogen-activated protein kinase (MAPK)

Mitofusin 1 (MFN1)

Mitofusin 2 (MFN2)

Myoclonic epilepsy with ragged red fibers (MERRF)

Myristoylated (myr)

Nerve conduction velocity (NCV)

Nerve growth factor (NGF)

Neural stem cells (NSCs)

Neuro2a (N2A)

Neurotrophin-3 (NT-3)

Nitrite oxide (NO)

Non-insulin dependent diabetes mellitus (NIDDM)

Non-obese diabetic (NOD)

Nuclear factor κB (NF-κB)

Nuclear respiratory factors 1 (NRF-1)

Nuclear respiratory factors 2 (NRF-2)

Optic atrophy 1 (OPA1)

Oxidative phosphorylation (OxPhos)

Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1)

Phosphoinositide 3-kinase (PI3K)

Poly (ADP)-ribose polymerase (PARP)

Polymerase gamma (PolG)

Protein gene product 9.5 (PGP 9.5)

Protein import machinery (PAM)

Protein kinase C isoform beta (PKC-β)

Quantitative sensory testing (QST)

Reactive oxygen species (ROS)

Receptor for AGE (RAGE)

Recombinant human NGF (rh) NGF

Ribosomal RNA (rRNA)

Silent mating type information regulation 2 homolog 1 (SIRT1)

Sorting and assembly machinery (SAM)

Spinal muscular atrophy (SMA)

Streptozotocin (STZ)

Sub-basal nerve plexus (SNP)

Subunit-binding sequence (SBS)

Superoxide dismutase (SOD)

Superoxide ion (O_2^-)

TBC domain family, member 1 (TBC1D1)

Thioredoxin (Trx)

Thioredoxin-interacting protein (TXNIP)

Trafficking kinesin-binding protein 1 (TRAK1)

Trafficking kinesin-binding protein 2 (TRAK2)

Transfer RNA (tRNA)

Transforming growth factor-β (TGF-β)

Translocase of the inner membrane (TIM)

Translocase of the outer membrane (TOM)

Trigeminal ganglia (TG)

Triglyceride (TG)

Ubiquitin proteasome system (UPS)

UDP-5-diphosphate-N-acetylglucosamine (UDP-GlcNac)

Unc-51-like autophagy-activating kinase 1 (ULK1)

Vascular endothelial growth factor (VEGF)

Very low-density lipoproteins (VLDL)

Zucker diabetic fatty (ZDF)

Chapter 1: Background and literature review

1.1 Diabetes

Diabetes is a chronic hyperglycemic condition characterized as a metabolic disorder in which insulin signaling is impaired. Fasting plasma glucose of ≥ 7 mmol/L and glycated hemoglobin (HbA1C) of ≥ 6.5% are considered main criteria for diagnosis of all types of diabetes (Diabetes Canada Clinical Practice Guidelines Expert et al., 2018). High blood sugar can damage nerves and organs leading to morbidity and mortality. The global prevalence of diabetes has multiplied from 108 million in 1980 to 422 million in 2014. In 2014, the number of young adults aged over 18 who had diabetes reached 8.5% (Loke, 2018). As of 2018, 11 million Canadians are living with diabetes or prediabetes and another Canadian is diagnosed every three minutes (CanadianDiabetesAssociation, 2018). Based on death certificates in 2015 in the U.S., diabetes was the seventh leading cause of death, although underreported since persons with diabetes often die due to health-care complications such as kidney failure and cardiovascular disorders which may not be recorded as diabetes in the death certificates (Stokes and Preston, 2017). Economic costs of diabetes in the U.S. in 2017 were estimated to be $327 billion (AmericanDiabetesAssociation, 2018, Stokes and Preston, 2017).

1.2 Types of diabetes

There are four broad classes of diabetes: type 1 diabetes, type 2 diabetes, gestational diabetes and secondary types of diabetes. Although the normal fasting insulin level is < 174 pmol/L among healthy individuals, its range is highly variant and does not follow the same trend in all classes of diabetes (Buppajarntham et al., 2019). In type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), beta cells of the pancreas are immunologically damaged and can no

longer produce sufficient insulin. T cell-mediated autoimmune destruction of insulin-producing beta cells appears to be triggered by environmental factors in individuals predisposed to genetic factors. Two human leukocyte antigen (HLA) class 2 haplotypes, DR3 and DR4-DQ8, are linked to half of type 1 diabetes heritability (DiMeglio et al., 2018). Type 1 diabetes accounts for 5-10% of all diabetic cases, and is distinguishable from type 2 diabetes by detection of autoantibodies against glutamate decarboxylase, insulin, islet antigen 2, tetraspanin-7 and zinc transporter 8 (Daneman, 2006, DiMeglio et al., 2018).

Type 2 diabetes, also known as non-insulin dependent diabetes mellitus (NIDDM), makes up about 90% of all persons with diabetes and is caused by insulin resistance in target organs rather than beta cell dysfunction (initially). Inability of cells primarily in the liver, muscle and adipose tissues, to respond to normal insulin levels results in hyperinsulinemia and hyperglycemia in most persons with type 2 diabetes. The impairment of each component of the insulin signaling cascade starting from insulin/insulin receptor to effector proteins in the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways play a part in insulin resistance (Blackman and Cooke, 2013). For instance, mutations in insulin or insulin signaling give rise to rare forms of diabetes such as type A insulin resistance syndrome, Rabson-Mendenhall syndrome and maturity-onset diabetes of the young (MODY) where the latter is a result of a heterozygous insulin gene mutation (Molven et al., 2008, Mihai et al., 2012). In general, sedentary lifestyle, obesity, energy-dense diet and genetics are the main contributing factors to the pathogenesis of type 2 diabetes (Chatterjee et al., 2017). Based on genome wide association studies (GWAS), more than 300 loci are robustly associated with type 2 diabetes (Ingelsson and McCarthy, 2018).

Gestational diabetes mellitus (GDM) is characterized as a hyperglycemic condition which develops between the 24^{th} and 28^{th} weeks of gestation in pregnant women. Obesity, maternal age and lack of exercise are the key drivers of GDM. GDM is as multifactorial as type 2 diabetes, and insulin production and insulin sensitivity is impaired (Johns et al., 2018, Coustan, 2013).

Patients with different pathophysiology may also develop diabetes which can be classified as secondary types of diabetes. These conditions include cystic fibrosis, congenital rubella and cytomegalovirus infections, pancreatitis, hemochromatosis, drug (e.g. glucocorticoid)-induced diabetes, endocrinopathies, genetic syndromes such as Down syndrome, mitochondrial disorders and a wide variety of other disorders (Forlenza et al., 2018).

1.3 Major complications

Long-term complications of diabetes include neuropathy, retinopathy, nephropathy, cardiovascular disease and cognitive impairment, which can appear after about 10 years of diabetes (Johns et al., 2018). Diabetic retinopathy is associated with lesions, hard exudates, macular edema and cataract, and is the leading cause of blindness among young people. Diabetic nephropathy affects less than 30% of diabetic patients and is associated with proteinuria which in the long term can lead to renal failure. Associated components of metabolic syndrome such as hypertension and dyslipidemia, particularly in type 2 diabetic patients, are major risk factors for peripheral and cerebrovascular disease (Nathan, 1993, Johns et al., 2018). The majority of diabetic patients die of concurrent cardiovascular complications. However, the most common non-fatal complication of diabetes is neuropathy affecting up to 50% of persons with diabetes. This lifelong complication causes pain and later loss of sensation confers patients to foot ulcers which eventually may lead to limb amputation (Nathan, 1993, Johns et al., 2018).

There are two main types of diabetic neuropathies: generalized neuropathy and focal/multifocal neuropathy that are listed in detail in Table 1.1 (Nathan, 1993, Llewelyn, 2003). The most common form of diabetic neuropathy is diabetic sensorimotor polyneuropathy (DSPN), which affects the sensory, motor and autonomic nervous systems in the periphery. DSPN is symmetrical, affects both sides, and has a distal dying-back pattern in peripheral nerves (Kobayashi and Zochodne, 2018). This specific axonal degeneration is also seen in other neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease and spinal muscular atrophy (SMA) (Conforti et al., 2007, Fischer et al., 2004).

Table 1.1. Diabetic neuropathy classification (Llewelyn, 2003, Nathan, 1993)

Main types	Subtypes
Generalized neuropathy	Hyperglycemic neuropathy
	Acute painful sensory neuropathies
	Diabetic sensorimotor polyneuropathy (DSPN): The most common form of diabetic neuropathy
Focal/multifocal neuropathy	Cranial neuropathies
	Focal limb neuropathies such as carpal tunnel syndrome
	Thoracolumbar radiculoneuropathy
	Lumbosacral radiculoplexus neuropathy (Bruns-Garland syndrome)
Diabetic autonomic neuropathy	Most frequently coexisting with other neuropathies

1.4 Diabetic sensorimotor polyneuropathy (DSPN)

DSPN is a major health issue that is associated with debilitating pain, morbidity and mortality, and thus is a major economic and psychological burden to families and society (Kuntzer and Ruiz, 2014). Neurological signs and electrophysiological measurements are the diagnosing tools for DSPN (Pirart, 1977, Mulder et al., 1961, Kambiz et al., 2015, Tuncer et al., 2011) and the only current treatments are glycemic control and pain management (Llewelyn, 2003). Despite its common prevalence, DSPN is under diagnosed or not reproducibly diagnosed due to variable endpoint measurement methods, variable DSPN definitions and variable types of patients studied. Therefore, it limits early diagnosis, prevention and treatment options (Dyck et al., 2010).

1.4.1 Epidemiology

In an early epidemiologic study, the comparative prevalence of DSPN among persons with diabetes versus subjects who had impaired glucose tolerance versus control subjects was 25.8%, 11.2% and 3.9%, respectively (Franklin et al., 1990). Other investigators have reported DSPN in 13.3% (Ziegler et al., 2009), 50% (Lee et al., 2015), 66% (Dyck et al., 1993) and 28.5% (Young et al., 1993) of persons with diabetes. Up to 26% of persons with diabetes have chronic painful DSPN, which makes up almost half of DSPN subjects (Ziegler et al., 2014). DSPN is also quite common in those with prediabetes affecting up to 49% (Lee et al., 2015). In the EURODIAB IDDM study, over 3000 persons with diabetes from 16 countries were screened, and the rise in the prevalence of DSPN was due to several risk factors including duration of diabetes, dyslipidemia, age, poor glycemic control, high blood pressure, smoking and obesity (Tesfaye et al., 2005).

1.4.2 Clinical presentation

Typical clinical manifestations of DSPN include tingling, numbness and weakness that start from lower extremities and progress length-dependently in a stocking-glove distribution. The symptoms are predominantly sensory and symmetrical (Kuntzer and Ruiz, 2014). On the other hand, the symptoms of painful DSPN are associated with pain, electric/stabbing sensation and burning. Patients can also develop hyperalgesia and allodynia, many of which are treated with painkillers (including opioids) (Daousi et al., 2004). Other signs of DSPN at the time of diagnosis are absent ankle reflexes, weakness of small foot muscles, impaired peripheral pulses, and retinopathy and nephropathy signs (Llewelyn, 2003). Severe DSPN confers patients to foot ulceration (occurs in 15% of DSPN patients) and may eventually lead to limb amputation. Diabetes is the leading cause of limb amputation worldwide accounting for 80000 amputations each year in the USA (Margolis et al., 2011).

1.4.3 Pathology

There are pathological structures and functions in the nerves of DSPN patients that are correlated and can be attributed to clinical signs of DSPN. Most studies on the pathology of DSPN have used biopsied or autopsied materials of sural nerve obtained from patients with established DSPN. Animal models of DSPN show similar but not the exact same abnormal nerve features (Yagihashi et al., 2007). The pathogenetic factors in DSPN patients and animal models, and how the abnormalities in the nerves lead to symptoms are discussed in detail below.

1.5 Types and features of DRG sensory neurons

The somatosensory system in organisms perceives and controls position, touch, temperature, pain, pressure and other feelings by the receptors in the nerve endings originating from dorsal root ganglia (DRG) in the trunk and trigeminal ganglia (TG) in the head region. There are

different subpopulations of primary sensory neurons in DRG and TG that mediate processing of information from noxious to innocuous stimuli including thermal, mechanical or chemical stimuli and transmit them to second-order sensory neurons in the central nervous system (CNS) (Amir and Devor, 2003). Neural crest cells in the dorsal root of the spinal cord migrate to ganglia in three waves of neurogenesis during early embryonic period and generate various DRG neurons. Then, these DRG neurons undergo morphological stages starting from a spindle-shaped bipolar, eccentric bulged bipolar, bell-shape bipolar, short-stem pseudo-unipolar to eventually give rise to mature long-stem pseudo-unipolar. The long-stem pseudo-unipolar sensory neuron has the distal process that terminates in peripheral tissues, is activated and depolarized by stimuli and transmits the action potential to the spinal cord or brain stem where the proximal process ends (Nascimento et al., 2018, Amir and Devor, 2003).

Early classification of somatosensory neurons based on anatomical and electrochemical features divided them into four general fiber subtypes: unmyelinated small diameter C fibers, thinly-myelinated medium diameter Aδ, myelinated large diameter Aβ, and myelinated large diameter proprioceptors Aα (Figure 1.1) (Kandel, 2013). C fibers are the most abundant sensory neurons in DRGs with 10-30μm diameter and 2m/s conduction velocity that respond to thermal, chemical and even mechanical stimuli. C fibers can be peptidergic and express calcitonin gene related-peptide (CGRP) or substance P, or non-peptidergic and express isolectin IB4 (Averill et al., 1995, Le Pichon and Chesler, 2014). Aδ fibers, on the other hand, are larger (30-50μm) and conduct faster (30m/s) than C fibers and respond to force, pain and innocuous temperature. Aβ and Aα fibers have a diameter of more than 50μm and conduct as fast as 30-70m/s. Aβ fibers express low-threshold mechanoreceptors (LTMRs) and are responsive to vibration, hair reflection and touch. Aα fibers respond to muscle twitch, tension and contraction (Kandel, 2013,

Le Pichon and Chesler, 2014). Although a simplified classification of DRG neurons is explained here, there is accumulating literature reporting different subpopulations for DRG neurons based on unbiased single-cell RNA sequencing studies (Usoskin et al., 2014) followed by molecular and neurochemical classification which are reviewed in detail elsewhere (Wood et al., 2018).

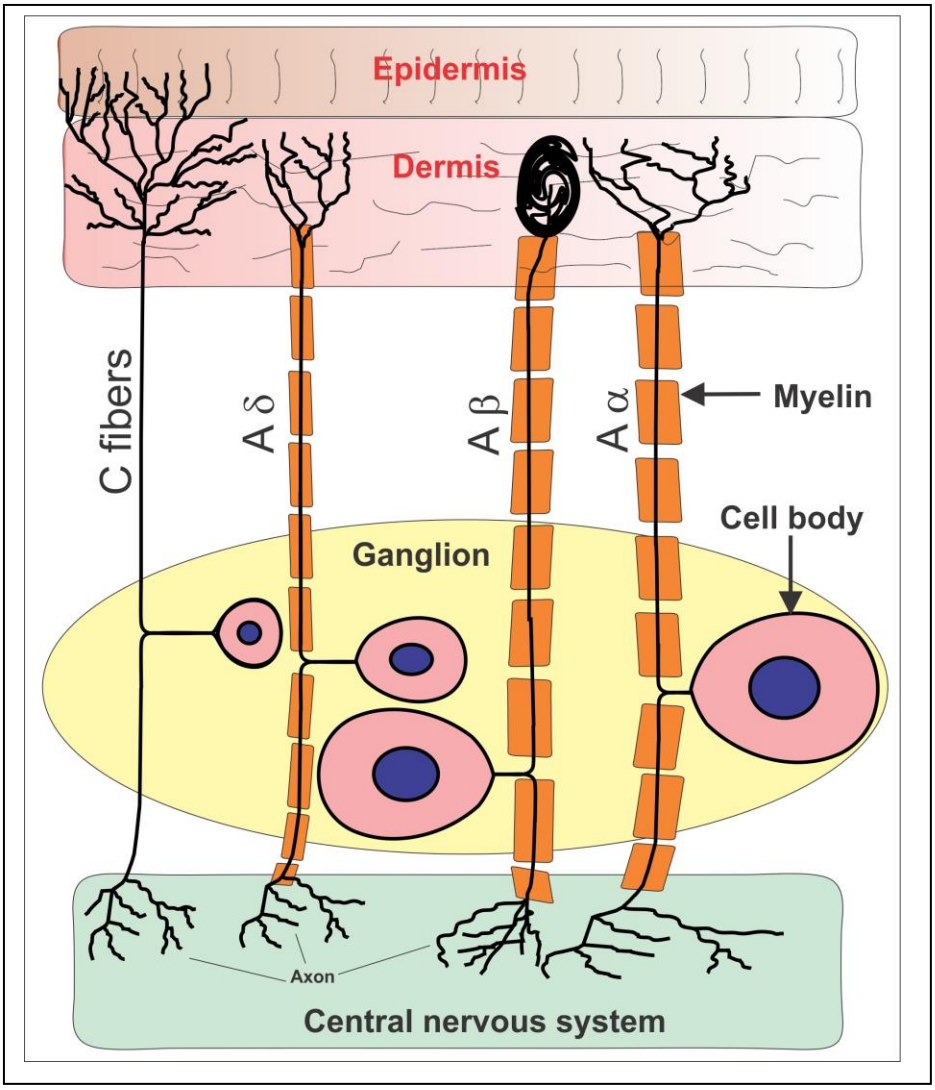

Figure 1.1. Sensory neuron subtypes. Early classification of somatosensory neurons based on anatomical and electrochemical features divided them into four general fiber subtypes: unmyelinated small diameter C fibers, thinly-myelinated medium diameter Aδ, myelinated large diameter Aβ, and myelinated large diameter proprioceptors Aα. Their dendrites and receptors reach the dermis and epidermis of the skin. Sensory neurons have their axons and synapse in central nervous system. This figure is original and was prepared by myself.

1.6 Nerve and DRG abnormalities in human DSPN

Distal dying-back nerve fiber degeneration, endoneurial microangiopathy, segmental demyelination and remyelination, and axonal loss are the most prominent structural features of pathologic nerves in DSPN patients (Malik et al., 2005, Yagihashi and Matsunaga, 1979). There is a controversy as to whether the microvascular injury in the nerves is the primary cause of nerve fiber loss or unknown mechanisms inside the DRG and/or nerves lead to nerve fiber loss. Microangiopathy and its primary involvement in nerve degeneration is supported by various studies showing that the subjects who had microangiopathy at first clinical assessment developed DSPN in the subsequent follow-up assessments (Thrainsdottir et al., 2003, Dyck et al., 1986, Dyck et al., 1984). However, despite nerve fiber loss in sural nerve of DSPN patients, no endoneurial microvessel injury was observed in one study thus questioning the causative role of microangiopathy (Llewelyn et al., 1988).

Some of the methods utilized to assess the structure and function of nerves in order to measure the severity of DSPN in patients are listed in Table 1.2. Nerve conduction velocity (NCV) measurement of peripheral nerve is sensitive and specific in assessing the severity of DSPN. However, NCV assessment can only predict abnormalities in large myelinated nerve fibers and is not able to detect early small fiber neuropathy occurring in prediabetes or diabetes (Dyck et al., 1997, Vinik et al., 2005). Nerve conduction studies are reliable enough to be used as a complementary method for clinical assessment of DSPN signs and symptoms including ankle reflexes (Dyck et al., 2010, Asad et al., 2009). A sensitivity of 80% and 83%, and specificity of 89% and 72% for peroneal and sural nerve conduction velocity, respectively, was reported when assessed in 246 type 1 and type 2 diabetic patients with DSPN (Weisman et al., 2013).

Quantitative sensory testing was developed to assess small fiber neuropathy at early stages where patients exhibited normal NCV and/or a structural assessment of nerve could not be implemented. In this method, they measured response latency either to cold (A delta fibers: small thinly-myelinated fibers) or warm (C-fibers: small non-myelinated fibers) stimuli (Shukla et al., 2005). Newly diagnosed type 1 diabetic patients on insulin for 4-31 days showed elevated heat and cold perception threshold and their associated pain perception in their foot compared to healthy subjects. Cold perception was the most sensitive test for detecting small nerve-related abnormal function in this study (Ziegler et al., 1988). In another study, cold and warm stimuli were perceived by type 1 diabetic patients who had a duration of diabetes from one to 49 years, with higher thresholds compared to control subjects (Navarro and Kennedy, 1991).

Table 1.2. Methods to assess the severity of diabetic neuropathy (Vinik et al., 2013)

Method	Fiber type	Type of assessment	Advantages
Skin biopsy and IENF	Small fibers	Small-caliber sensory nerves including c fibers in epidermis, dermal myelinated and autonomic nerve fibers	Quantitates small IENF density through antibody staining
Nerve conduction velocity	Myelinated fibers	Measures the ability of the nerves to conduct an electrical stimuli	Standardized and well documented universal method
Thermal sensitivity testing	C fibers	Assesses the sensitivity of C fibers to heat stimuli	Noninvasive and conventional
Corneal confocal microscopy	C fibers	Measures small fiber density in the cornea	Noninvasive, reliable and newly developed
Contact heat evoked potentials	Small fibers	Uses nociceptive heat stimulus which is recorded through electroencephalographic readings	Detects small fiber neuropathy in the absence of other indices

Intra-epidermal nerve fiber (IENF) and sub-basal nerve plexus (SNP) density profiles are two parameters to measure structural abnormalities in non-myelinated and thinly myelinated nerve fibers. In this method, the skin biopsies of the foot are obtained from patients, nerves are immunostained for a nerve-specific protein called protein gene product 9.5 (PGP 9.5), and are visualized and quantified (Lauria et al., 2005, Lauria et al., 2010). In a study on 25 diabetic and 23 non-diabetic patients with symptoms of neuropathy and 20 control subjects, IENF was found to be significantly reduced in the distal leg but not proximal leg and forearm when compared to controls (Pittenger et al., 2004). Skin biopsies from 30 type 2 diabetic patients with less than 3 years of duration of diabetes and 23 age- and sex-matched control subjects were tested for IENF measurement in distal leg. A significant loss of intraepidermal nerve fibers was observed in diabetic patients with symptoms of DSPN compared to diabetic patients without DSPN symptoms and control subjects although the annual rate of nerve fiber loss was similar in all diabetic patients (Divisova et al., 2016). IENF loss is correlated with progressive neuropathy symptoms (Quattrini et al., 2008). In 29 diabetic patients with no clinical sign of small or large fiber neuropathy and 84 control subjects, reduced IENF density was observed in ankle but not thigh of diabetic patients. The diagnosing sensitivity and specificity of IENF assessment was calculated to be 72.4% and 76.2%, respectively, for this study group (Umapathi et al., 2007).

In comparison, visualization of small nerve fiber length and density in the cornea using a newly developed technique called corneal confocal microscopy (CCM) is as sensitive and specific as IENF assessment in foot skin biopsies (Alam et al., 2017, Chen et al., 2015). CCM is an objective method, quick and non-invasive and hence a good replacement and candidate tool to monitor DSPN progression and a range of other peripheral neuropathies including chemotherapy-induced peripheral neuropathy (CIPN) and HIV neuropathy (Petropoulos et al.,

2013, Kemp et al., 2017, Ferdousi et al., 2015). CCM and skin biopsy were assessed in 63 patients with type 1 diabetes and 26 control subjects, and corneal nerve fiber density (CNFD) and length (CNFL) were found to be significantly reduced in DSPN patients and positively correlated with IENF density (Chen et al., 2015). In a more recent study on 92 type 1 diabetic patients and 84 control subjects, CNFD and CNFL were significantly reduced in patients with DSPN compared to patients without DSPN proposing CCM as the efficient diagnostic method for DSPN (Chen et al., 2018). In a similar study, CNFL was found to have highest sensitivity (up to 91%) and specificity (up to 93%) among other CCM parameters in diagnosing DSPN (Ahmed et al., 2012). Optical coherence tomography is another method that has been recently developed to assess retinal nerve fiber thickness in DSPN patients although it is not well established and longitudinal studies are required before its universal use (Shahidi et al., 2012).

1.7 Nerve and DRG abnormalities in animal models of DSPN

One of the obstacles in studying diabetic neuropathy is the lack of animal models that could represent the same pathological features of DSPN as in human patients. The first and complete animal models of type 1 diabetes and diabetic neuropathy were induced by alloxan and streptozotocin (STZ) injections in 1960s and 1970s, respectively. The BioBreeding/Worcester (BB/Wor) rat and the heterozygous Ins2Akita mouse are two common genetic models of type 1 diabetes that develop hypoinsulinemia and hyperglycemia. The BB/Wor rat develops nerve conduction slowing, sural nerve fiber loss, thermal hyperalgesia, reduced endoneurial blood supply and decreased neuropeptides in DRGs after 4 weeks of diabetes (Kamiya et al., 2009, Stevens et al., 2004). This model reproduces most closely the human disease but cost reduces its use. Also, the onset is indeterminate and so it is difficult to track disease progression from its initiation. In comparison, the Ins2Akita mouse has been reported to display DSPN features

including sensory nerve conduction slowing, mechanical hypoalgesia, thermal hypoalgesia and tactile allodynia after 16 weeks of diabetes (Yang and Chon, 2011, Drel et al., 2011). The inducible type 1 diabetic animals, on the other hand, have become more popular in research than the genetic models due to low cost of maintenance and ease of use while exhibiting similar DSPN representation. The corresponding diabetogenic agents, alloxan and STZ, specifically enter beta cells of the pancreas through the GLUT-2 glucose transporter and cause DNA damage and eventually cell death resulting in low or no insulin secretion by beta cells (Elsner et al., 2000). Both of these experimental rat and mouse models of type 1 diabetes develop impaired nerve conduction velocities, intra-epidermal nerve fiber loss, corneal nerve fiber loss, thermal hypoalgesia and axonal degeneration in the epidermis and dermis (Jakobsen and Lundbaek, 1976, Preston, 1967, Sima and Robertson, 1978).

The reported measures of neuropathy including both structural and functional nerve abnormalities have been variable in different studies on these STZ-induced diabetic animals. The variability could simply be due to various animal sources/strains, STZ dose/route of delivery and animal responses to STZ, duration of diabetes, amount of insulin production and different glucose concentration. In an early study, sural (sensory) nerve conduction velocity in 70 Sprague-Dawley rats that received tail vein injection of 65mg/kg STZ was significantly slowed after 2 weeks compared to controls. In this study, tibial (motor) nerve conduction slowing occurred after 4-6 weeks of diabetes, and the severity of the abnormal nerve functioning was higher in STZ than alloxan-diabetic rats (Moore et al., 1980). In another study on 10 adult male Sprague-Dawley STZ-diabetic rats, Malik et al. (Walker et al., 1999) found that animals displayed a significant reduction in tibial nerve conduction velocities in spite of no significant change in myelinated and unmyelinated nerve fiber density and diameter in tibial sections and

endoneurial microvessel density after 27 days of diabetes induced by single 30mg/kg STZ injection (Walker et al., 1999). Sensory and motor nerve conduction velocities were impaired in one month of diabetes in male Wistar rats that received 40mg/kg STZ injection intravenously (Biessels et al., 1999). Single intraperitoneal injection of 65 mg/kg STZ and induction of diabetes in WAG/RijHsd female rats caused significant decrease in rewarming rate of the plantar skin after cold exposure, heat sensitivity, mechanical sensitivity, nerve conduction velocity and IENF density in hind paw skin biopsies after 4 weeks of diabetes in a more recent study (Kambiz et al., 2015).

Our lab has conducted many studies with type 1 diabetic animal models where intraperitoneal injection of 55 mg/kg STZ was used for male Wistar rats that was associated with impaired motor and sensory nerve conduction velocities after at least 4 weeks of diabetes (Huang et al., 2003, Huang et al., 2002) or intraperitoneal injection of 75-85 mg/kg STZ in Sprague-Dawley rats caused a deficient thermal response after two months of diabetes and reduced IENF density profile after 3-4 months of diabetes (Roy Chowdhury et al., 2012). Taken together, the pathology and endpoints in the Sprague-Dawley STZ-diabetic rat/C57Bl6/J STZ-diabetic mouse does include thermal hypoalgesia, nerve conduction slowing and distal loss of IENF and corneal nerves but no major loss of nerve fibers in the nerve bundles and no demyelination or segmental demyelination, so, does not exactly reproduce the human state.

There are a range of animal models of type 2 diabetes that develop DSPN. Some studies have reported spontaneous diabetes and structural and functional abnormalities in peripheral nerve in cats. Biochemical, electrophysiological and histological studies of the spontaneously occurring type 2 diabetic feline have shown Schwann cell injury, myelin sheath splitting and ballooning (Mizisin et al., 1998), sensorimotor neuropathy as evidenced by nerve conduction slowing and

axonal degeneration (Mizisin et al., 2002), myelinated fiber loss and accumulation of membranous debris in nerve biopsies (Mizisin et al., 2007). In a follow-up study, nerve biopsies from 12 adult diabetic and 7 non-diabetic cats revealed microangiopathy associated with a significant increase in basement membrane size, capillary luminal size and myelinated nerve injury compared to control cats (Estrella et al., 2008). A range of other spontaneous diabetic animal models including diabetic WBN/Kob rat (Yagihashi et al., 1993) and non-obese diabetic (NOD) mouse (Schmidt et al., 2003) were reported, which had either similar polyneuropathy-related structural and functional nerve deficits (in the former one) or no major DSPN signs (in the latter model). In comparison, both the genetic rat model of obesity and obese Zucker rat, and the Zucker diabetic fatty (ZDF) rat model of type 2 diabetes developed insulin resistance after 4 weeks of age, vascular deficiency and motor nerve conduction slowing (Oltman et al., 2005). On the other hand, the nonobese type 2 diabetic animal models such as Goto-Kakizaki (GK) rat also developed peripheral neuropathy from which corneal nerve loss was the most prominent characteristic even before the onset of hyperglycemia (Davidson et al., 2014b, Wang et al., 2012, Murakawa et al., 2002). Leptin-deficient (*ob*/*ob*) mouse and leptin receptor-deficient (*db*/*db*) mouse are two animal models of obese type 2 diabetes that exhibit similar DSPN characteristics that include thermal hypoalgesia, loss of IENF, sensory and motor nerve conduction slowing and tactile allodynia after 9-13 weeks of spontaneous hyperglycemia and insulin resistance (O'Brien et al., 2015, Cho et al., 2014, Nowicki et al., 2012, Drel et al., 2006, Robertson and Sima, 1980). High-fat fed Sprague-Dawley rats or C57Bl6/J mice became diabetic after 4-6 weeks of high-fat (45% kcal) diet, developed insulin resistance and gain weight. However, DSPN features including nerve conduction slowing and thermal hypoalgesia were not evident until they were

maintained on high-fat diet for more than 12 weeks (Yorek et al., 2015, Davidson et al., 2014a, Anderson et al., 2014, Coppey et al., 2011).

1.8 Pathogenesis of DSPN

Early studies on pathogenesis of DSPN focused on hyperglycemia-related pathways. A variety of aldose reductase inhibitors (ARIs), which block abnormal glucose metabolism pathways, were used to prevent/reverse diabetic neuropathy indices in animal models. Failure of the clinical trials with such drugs due to toxicity and adverse effects, and suboptimal method for neuropathy endpoint assessments induced a suspension of all ARI preclinical and human clinical trial work (Oates, 2008). In the meantime, other hypotheses for the etiology of neuropathy have undergone further development including impaired insulin signaling, growth factor deficiency, dyslipidemia, vascular deficiency, neurovascular deficiency and metabolic syndrome.

1.8.1 Impaired insulin signaling

Insulin and IGF-1 share the same signaling pathway. Both IGF-1 and insulin bind to receptors (InsR for insulin and IGF-1R for IGF-1) consisting of two α and two β subunits across the plasma membrane and activate similar intracellular pathways. At higher concentrations of insulin and IGF-1, they cross occupy these receptors (Xu et al., 2018). In neurons, IGF-1R and insulin receptor activates several messengers that include the survival kinase, PI3K, which is activated by IRS-1 and induces Akt (Grote et al., 2013b, Huang et al., 2005, Kim et al., 2011). P70S6K is a serine/threonine kinase that is phosphorylated on the T389 site upon PI3K/Akt activation and triggers protein synthesis via mobilizing S6 ribosomal protein to regulate survival and growth of neurons (Chung et al., 1994). S6 ribosomal protein is also phosphorylated by ERK-activated p90 ribosomal S6 kinases (RSKs) to initiate protein synthesis (Roux et al., 2007).

Insulin signaling abnormalities in DSPN. Neurons are under regulatory control by insulin, and loss of insulin production and insulin resistance in type 1 and type 2 diabetes, respectively, provide a pathogenic mechanism contributing to DSPN in parallel with the damaging impacts of hyperglycemia. A number of studies have demonstrated that loss of direct insulin signaling contributes to diabetic neuropathy and retinopathy (Zochodne, 2016a, Ishii, 1995, Reiter and Gardner, 2003). Direct neurotrophic and neuroprotective actions of insulin were reported in studies showing that neurons expressed the appropriate proteins to respond to insulin exposure. Insulin receptors (IRs), IGF1Rs and receptor substrate scaffolds (IRS1, IRS2) are expressed by sensory neurons and activate signal transduction pathways that modulate neurite outgrowth and axonal plasticity (Fernyhough et al., 1993, Huang et al., 2005, Singh et al., 2012, Grote et al., 2013b). Insulin receptors are widely expressed in neuronal cell bodies of DRG as well as in axons in the periphery (Sugimoto et al., 2002). Insulin receptor levels are increased in response to nerve injury or in diabetic conditions. Insulin supplementation is essential for the growth of human-derived neural stem cells (NSCs) in culture, and withdrawal of insulin confers cells to massive cell death and low growth (Rhee et al., 2013). Insulin and IGF-1, through activation of the PI3K pathway, sensitized and translocated the vanilloid receptor 1 (also called TRPV1) which mediates inflammatory thermal nociception in sensory neurons (Van Buren et al., 2005).

Insulin treatment increased mitochondrial polarization in cultured DRG neurons, and improved motor and sensory nerve conduction velocities in STZ-diabetic rats with no effect on hyperglycemia (Huang et al., 2003). Local insulin injection in the hind paw in C57BL/6J STZ-diabetic mice raised the density of epidermal axons and improved mechanical sensation (Guo et al., 2011). Although insulin is not involved in glucose uptake in most neurons or partially involved in glucose uptake in glial cells, it acts as a neurotrophic factor in neurons and is

essential for their survival and growth (Mielke and Wang, 2011). The impaired insulin signaling in type 1 diabetes (mainly due to insulin production deficiency) and type 2 diabetes (mostly due to insulin resistance) leads to reduced neurotrophic support thus contributing to pathogenesis of DSPN (Mielke and Wang, 2011, Grote et al., 2013a, Hinder et al., 2017).

IGF-1 signaling abnormalities in DSPN. The level of IGF-1 in the circulation of humans and animal models of type 1 and type 2 diabetes is substantially decreased (Zhuang et al., 1997, Palta et al., 2014, Ishii et al., 1994, Ekstrom et al., 1989). Thus, any impairment in IGF-1 and IGF-2 levels and signaling can worsen neurodegeneration in diabetes (Ishii, 1995, Rauskolb et al., 2017, Zochodne, 2016b). In addition, IGF-1 plays an important role during nervous system development and early postnatal growth (Zackenfels et al., 1995) and promotes neurite outgrowth in sensory (Fernyhough et al., 1993, Recio-Pinto et al., 1986), motor (Caroni et al., 1994) and sympathetic (Zackenfels et al., 1995, Recio-Pinto et al., 1986) neurons. Schwann cells also require IGF-1 and IGF1R signaling for survival, myelination, cell proliferation and phenotypic remodeling (Syroid et al., 1999, Chattopadhyay and Shubayev, 2009, Russell et al., 2000, Cheng et al., 2000).

Therapeutic options with insulin/IGF-1. In type 1 diabetes, tight glycemic control slows the progression of neuropathy in humans (DCCT, 1993, DCCT, 1995). Insulin injection prevents the progression of DSPN in animal models which could be due to either insulin-mediated neuronal signaling or glucose homeostasis or both (Grote and Wright, 2016, Brussee et al., 2004). In patients with type 1 diabetes who were deficient in C-peptide, a short polypeptide which links proinsulin A-chain to its B-chain, the reduced endoneurial blood supply and nerve dysfunction was improved by C-peptide supplementation (Ekberg and Johansson, 2008). C-peptide therapy in animal models with DSPN has also improved nerve function (Kamiya et al., 2006a).

In type 1 diabetic patients with neuropathy, tight glycemic control with insulin injections confirmed the possible direct role of insulin and insulin-linked glucose homeostasis and suppression of hyperglycemia as key factors in reducing incidence and severity of neuropathy (Martin et al., 2014). However, this is not always the case in type 2 diabetes since there are also other factors such as dyslipidemia and insulin resistance which develop over years of obesity in patients and counteract the effectiveness of glycemic control (Grote et al., 2013a, Callaghan et al., 2012). It is not clear if there is also insulin resistance in CNS or PNS neurons in type 2 diabetes in addition to its occurrence in fat and muscle tissues, although some in vitro studies have demonstrated that neurons fail to respond to insulin or IGF-1 in terms of enhanced axonal outgrowth. For example, neurons exposed to high doses of insulin for a period of time blunted Akt activation when they were treated with exogenous insulin (Singh et al., 2012). Other mediators of the insulin signaling pathway including p70S6K and GSK3β did not respond to insulin after chronic exposure to insulin or in hyperinsulinemic conditions in sensory neurons thus mimicking insulin resistance in muscle and adipocytes (Kim et al., 2011, Cusi et al., 2000). Similarly, Akt stimulation was reduced in DRG neurons from *ob*/*ob* mice that developed DSPN (Grote et al., 2011). In insulin resistance cases in type 2 diabetes, IGF-1 is a good therapeutic option as it binds to a different receptor (IGF-1R) while activating the same signaling pathway as insulin.

1.8.2 Growth factor deficiency

Many growth factors including but not limited to insulin-like growth factors (IGFs), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) have both neurotrophic and angiogenic effects. Deficiency

in any of these growth factors could lead to less neurotrophic support and induction of diabetic neuropathy compromising normal nerve structure and function. NGF was first identified as a polypeptide that induces nerve growth, and the discovery led to a Nobel prize by the Italian developmental biologist Rita Levi-Montalcini and the American biochemist Stanley Cohen in 1986 (Shelton, 2014). Early studies showed a reduction of NGF production and transport in sciatic nerve and superior cervical ganglion in STZ-diabetic rats after three weeks of diabetes and prolonged for 6 months duration of diabetes (Hellweg and Hartung, 1990). There was a reduction in NGF and NT-3 mRNA levels in sensory neuron target tissues such as foot-skin and soleus muscle in STZ-diabetic rats (Fernyhough et al., 1994, Tomlinson et al., 1997). The level of BDNF mRNA was shown to be elevated in DRG and sciatic nerve tissues correlating with increased NGF mRNA in sciatic nerve over 12 weeks of diabetes in STZ-diabetic rats. Intensive insulin treatment reversed the levels of both neurotrophins to normal levels (Fernyhough et al., 1995a). The level of NGF protein was reduced while its mRNA was elevated after 4 weeks of diabetes in DRGs in STZ-diabetic rats indicating activation of compensatory pathways to increase endogenous NGF in DRG tissues (Gao et al., 2017, Nori et al., 2013). The serum level of NGF and BDNF in patients with DSPN and in diabetic animal models was significantly lower than normal (Decroli et al., 2019, Sun et al., 2018, Gao et al., 2017).

The reduction in neurotrophic factors such as NGF and NT-3 can occur in diabetes as a result of impaired retrograde axonal transport. Retrograde transport of NGF and NT-3 was reduced in sciatic nerve in STZ-diabetic rats, this reduction was primarily attributed to reduced target tissue production of these growth factors (Delcroix et al., 1997, Fernyhough et al., 1995b, Fernyhough et al., 1998b, Hellweg et al., 1994). In addition, in the vagal nerve the retrograde axonal transport of NGF and NT-3 was reduced at later stages of diabetes in STZ-diabetic rats

(Lee et al., 2001). Retrograde and anterograde transport of BDNF in sciatic nerve was decreased in galactose-fed and STZ-diabetic rats compared with control rats, and it was independent of receptor-mediated transport (Mizisin et al., 1999). Interestingly, the up-regulation of BDNF gene expression in DRG of STZ-diabetic rats maybe an endogenous repair process to counteract this depletion of transported BDNF.

A wide range of studies have shown neuroprotective and neurotrophic effects of growth factors including NGF, VEGF and BDNF in animal models of diabetic neuropathy (Mata et al., 2006). NGF treatment prevented the deficits in the levels of CGRP and substance P in DRG in STZ-induced diabetic rats (Diemel et al., 1994, Fernyhough et al., 1995b). NGF administration prevented elevation of tailflick threshold and restored the levels of substance P and calcitonin in DRG in STZ-diabetic rats (Apfel et al., 1994, Diemel et al., 1994). Exogenous BDNF treatment improved motor nerve conduction velocity, myelin splitting of motor axons and caliber of the central sensory projections in galactose-fed rats that developed neuropathy (Mizisin et al., 1997). NT-3 administration increased the sensory nerve conduction velocity in STZ-diabetic rats, and NGF administration following nerve crush injury stimulated nerve regeneration in both control and diabetic rats (Tomlinson et al., 1997). Intrathecal delivery of GDNF restored the IENF density and axonal branching in footskin in STZ-diabetic mice. In comparison, NGF only stimulated cutaneous axonal branching in these animals (Christianson et al., 2003a). GDNF but not insulin administration restored loss of nerve fiber terminals of nonpeptidergic unmyelinated sensory neurons stained with the isolectin IB4 in STZ-diabetic mice (Akkina et al., 2001). In STZ-diabetic mice, intrathecal delivery of NGF and insulin reversed mechanical and chemogenic behavioral responses although GDNF delivery only restored behavioral responses to chemogenic stimuli (Christianson et al., 2003b).

IGF-1 mRNA and protein levels were reduced in the nerves and DRGs of alloxan- or STZ-diabetic rats or spontaneously diabetic obese Zucker rats (Li et al., 2001, Aghanoori et al., 2019, Bitar et al., 1997). Treatment with IGF-1 peptide elevated neurite outgrowth of sensory neurons, motor neurons and neuroblastoma cells as well as in vivo endpoints of neuropathy such as thermal sensitivity, corneal nerve profile and IENF in STZ-diabetic rodents (Aghanoori et al., 2019, Fernyhough et al., 1993, Chu et al., 2008, Ishii and Lupien, 2003, Zhuang et al., 1997). In addition, the role of IGFs in myelination and Schwann cell survival, proliferation and mitogenesis has been approved by many studies (Liang et al., 2007, Cai et al., 2011, Lin et al., 2005, Apel et al., 2010). VEGF, a well-known angiogenic factor, increased Schwann cell survival and migration and enhanced neurite outgrowth of DGR neurons other than its main role in improving tissue ischemia (Storkebaum et al., 2004, Verheyen et al., 2013).

The delivery of the neurotrophic growth factors and the design of a proper trial are still challenging in human diabetic patients with DSPN (Mata et al., 2006). Recombinant human NGF (rh) NGF injection significantly improved the sensory component of a standard neurologic examination in 250 patients with DSPN after 6 months (Apfel et al., 1998, Freeman, 1999). Unfortunately, the subsequent phase III clinical trial with rhNGF showed no beneficial effect on diabetic neuropathy and the therapy elevated muscle pain (Apfel et al., 2000). Clinical trials on VEGF had promising results for DSPN patients when initiated, but stopped due to unexpected low efficacy (Ropper et al., 2009).

1.8.3 Dyslipidemia

Dyslipidemia is described as a condition in which the levels of very low-density lipoproteins (VLDL), LDL and triglycerides (TGs) are increased and the level of high-density lipoproteins

(HDLs) is decreased (Mooradian, 2009). The serum level of triglyceride (TG) was increased in type 2 diabetic patients and was contemporaneous with decreased high-density lipoprotein-cholesterol (HDL-C). Under diabetic conditions, low-density lipoproteins (LDLs) were oxidized and glycated and thus had lowered function (Jacobs et al., 2005). In type 2 diabetic rodents that were fed a highly fat diet, accumulation of oxidized lipids and activated lipoxygenases were observed in peripheral nerves before the progression of diabetes (Obrosova et al., 2007). High-fat diet alloxan-induced WBN/Kob rats showed increased plasma levels of TGs and cholesterol compared to high-fat diet nondiabetic and normal diet diabetic rats. They also displayed a reduction in myelin thickness and size in sensory sural nerve as well as a reduction in the size of motor nerve axons (Ozaki et al., 2018).

Dyslipidemia in cultured DRG neurons interfered with mitochondrial trafficking, membrane potential and bioenergetics thus inhibiting proper axonal energy supplementation (Rumora et al., 2018, Rumora et al., 2019). However, a study on 71 type 2 diabetic patients revealed that there was no association between serum levels of TGs and LDLs, and nerve conduction velocity indicating no significant role of dyslipidemia in axonal dysfunction and the pathogenesis of DSPN (Kwai et al., 2016). Therefore, some controversy remains over the role of dyslipidemia in the pathogenesis of DSPN especially in type 1 diabetes and the mechanism through which dyslipidemia contributes to DSPN in type 2 diabetic patients and animal models needs to be further investigated.

1.8.4 Vascular deficiency

Vascular disease and diabetic neuropathy are closely intertwined. Diabetes is a major risk factor for both small and large vessel vascular complications and poses a series of risk factors for

diabetic complications such as peripheral neuropathy, retinopathy and nephropathy. Additionally, blood vessels and axons share cell-cell and molecular signals for terminal arborization, nerve regeneration and navigation (Carmeliet and Tessier-Lavigne, 2005).

Maintaining sufficient nutrients through blood vessels in the peripheral nerves is crucial for fine nerve structure and function. Thus, any deficiencies of the blood supply can confer nerves to pathogenesis leading to neuropathy (Stirban, 2014). Endoneurial microangiopathy has been reported in diabetic patients with neuropathy, but less severe in diabetic patients without neuropathy providing correlative evidence of parallel progression of neuropathy and microvascular complications in persons with diabetes (Khawaja et al., 2000, Stirban, 2014, Malik et al., 1989). The most prominent structural changes of microvasculature in peripheral nerves in human includes thickening of the intraneural vessel wall due to reduplication of capillary basal lamina, endothelial cell hyperplasia and pericyte degeneration despite no histologic changes in capillary density (Khawaja et al., 2000, Powell et al., 1985). These changes might be preceded by platelet activation, erythrocyte aggregation and fibrin deposition causing vessel occlusion and hypoxia which predominantly occur in endoneurium rather than in epineurium or perineurium in human (Dyck et al., 1985, McMillan et al., 1978, O'Malley et al., 1975). STZ-diabetic mice and db/db mice displayed impaired blood flow and vascularization in their sciatic nerve (Himeno et al., 2011). In diabetic rats, nerve conduction velocity slowing occurred following defective vasodilation in epineurial arterioles, which was mediated by reactive oxygen species (ROS) overproduction (Coppey et al., 2003). However, it is still not clear and less likely that vascular pathology and ischemia are the main culprits in the progression of DSPN since nerve degeneration has a symmetrical and length-dependent nature. This is in contrast to the finding that neuronal lesions in DSPN patients resembled non-diabetic cases with

vasculitis (Johnson et al., 1986). On the other hand in a neuroregeneration study, microvascular growth preceded Schwann cell migration and axonal sprouting into the denervated region of experimental intracutaneous axotomy. All these measures of neuroregeneration were more robust in healthy controls vs. diabetic neuropathy patients (Ebenezer et al., 2011).

Despite all the arguments on the structural changes of vasa nervora in DSPN patients and animal models, it is well accepted that DSPN is accompanied by hypoxia and ischemia in the nerve (Dyck et al., 1986). This area needs more investigation to further clarify the microvasculature-related mechanism of nerve pathophysiology in diabetic patients and experimental models. A decrease in angiogenic factors including NGF, IGF and VEGF in DSPN provides evidence for mechanistic microangiopathy in the nerves. This led to a study in which local delivery of VEGF in diabetic rats improved nerve conduction velocity and vasa nervora density (Schratzberger et al., 2001).

1.8.5 Neurovascular deficiency

Neurovascular deficiency refers to those abnormalities that are caused as a result of aberrant interaction between the nerve and blood vessels in the periphery. It is well established that there is a good correlation between pathological changes in vasa nervorum and nerve structure and function such as thermal discrimination and conduction velocity in human (Malik et al., 1994, Tesfaye et al., 2005, Malik et al., 1993). The vascular damage is minimal when nerve pathology is developing in DSPN patients. Hypoxia and loss of sural nerve blood flow exacerbated with progress in neuropathy in diabetic patients (Tesfaye et al., 1993, Ibrahim et al., 1999). One underlying mechanism revealed by epi/perineurial photography was an increase in arterio-venous shunting bypassing endoneurial microcirculation in diabetic patients with

neuropathy (Tesfaye et al., 1993). There was also a denervation in perineurial blood vessels indicating autonomic nerve loss as a result of long term diabetes thus contributing to defective nerve perfusion (Beggs et al., 1992).

Diabetic animal models display similar correlation between pathologic blood vessels and nerve damage as DSPN develops (Himeno et al., 2011, Cameron and Cotter, 2001). Drug intervention by using vasodilator agents such as angiotensin converting enzyme (ACE) inhibitors, endothelin-1 receptor antagonists and alpha1 adrenoceptor blockers on these experimental animals revealed improvements in nerve electrophysiology (sensory and motor nerve conduction velocity) which strongly correlated with corrected endoneurial perfusion (Cameron et al., 2001). Similar correlation between nerve blood flow and thermal sensitivity or tactile allodynia was reported in STZ-diabetic rats treated with ACE inhibitors (Cameron and Cotter, 2007, Cameron and Cotter, 2001). This correlation suggests the interaction between nerve and microvasculature is essential for proper function of both systems. Some of the growth factors such as BDNF and VEGF stimulated angiogenesis and vascular remodeling thus playing a major role as mediators of neurovascular deficiency in the pathology of diabetic neuropathy (Kermani et al., 2005). Although there remains no clear mechanistic link derived from preclinical work between microvascular changes in the nerve and the initial pathophysiological triggers for DSPN, the parallel development of microangiopathy and early nerve dysfunction and associated structural damage suggest a reasonable case for a causal linkage between these processes (Kobayashi and Zochodne, 2018).

1.8.6 Metabolic syndrome

Metabolic syndrome is defined as a syndrome with at least three of the following medical conditions: obesity, high blood sugar and TG, low serum HDL and high blood pressure. The syndrome is associated with high risk of type 2 diabetes and cardiovascular disorders (Kaur, 2014). A body of literature links metabolic syndrome to DSPN risk in type 1 and type 2 diabetes (Callaghan et al., 2016). Patients with metabolic syndrome developed autonomic neuropathy, cardiovagal deficiency, even in the absence of diabetes (Laitinen et al., 2011, Stein et al., 2007). Early autonomic neuropathy in type 2 diabetic patients demonstrated a metabolic deficit (Gottsater et al., 1999).

In a study on high-fat fed mice with prediabetes, exercise normalized neurotrophin levels particularly NGF and its receptor (TrkA) in hind paw skin and hyperalgesia associated with prediabetes and improved epidermal innervation (Groover et al., 2013). In a study on 218 type 2 diabetic patients with/without DSPN symptoms it was revealed that TGs, obesity and metabolic syndrome were independent risk factors for DSPN. Elevated hemoglobin A1C levels correlated with lower motor nerve conduction velocity, and TG and obesity were associated with C fiber loss indicating preferential damage to specific nerves by specific metabolic syndrome components (Smith and Singleton, 2013). In a different study, metabolic syndrome was found to be a risk factor for early DSPN in both type 1 and type 2 diabetic patients (Bonadonna et al., 2006). Patients with type 2 diabetes treated with Steno-2, an agent targeting hyperglycemia, dyslipidemia and hypertension, were less likely to develop autonomic neuropathy or microvascular complications compared to an untreated diabetic group (Gaede et al., 2003). All these data suggest a critical role of metabolic syndrome in the pathogenesis of DSPN in both type 1 and type 2 diabetes.

1.8.7 Hyperglycemia

Hyperglycemia is the most typical abnormality of all kinds of diabetes and is the most important factor which contributes to pathogenesis of DSPN. Hyperglycemia affects, to some extent, all other contributing factors to DSPN mentioned above. It activates a wide variety of other pathways including polyol pathway, nonenzymatic glycation of proteins, glycosylation, protein kinase C activity and oxidative stress to modify a plethora of molecular targets inside the cell eventually leading to nerve pathophysiology in DSPN (Figure 1.2). Excessive load of intracellular glucose because of elevated glucose transport via Glut3 and Glut1 in neurons and glial cells overloads these pathways (Morgello et al., 1995, Wu et al., 2010, Leino et al., 1997).

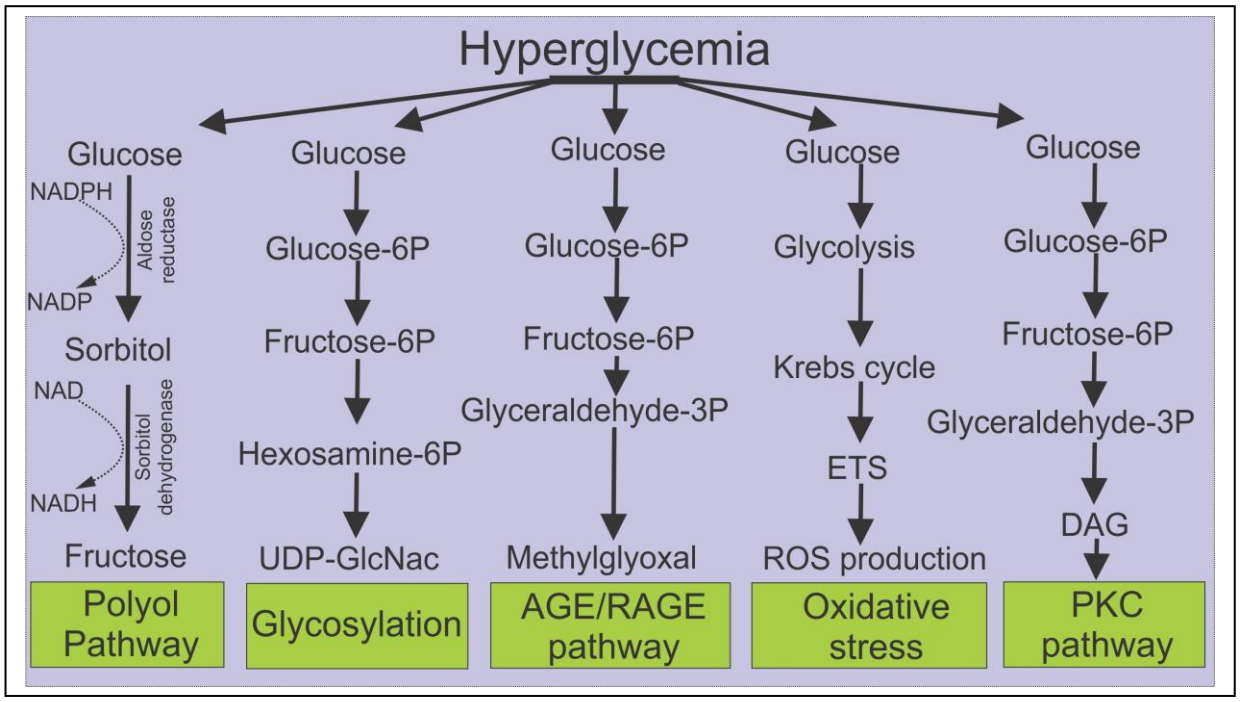

Figure 1.2. Hyperglycemia-driven pathways contributing to DSPN. Hyperglycemia activates a wide variety of other pathways including polyol pathway, nonenzymatic glycation of proteins (AGE/RAGE pathway), glycosylation, protein kinase C activity and oxidative stress. PKC: Protein kinase C, ETS: Electron transport system in mitochondria, DAG: Diacylglycerol, ROS: Reactive oxygen species, RAGE: Receptor for advanced glycation of end products, 6P: 6 phosphate, UDP-GlcNac: UDP-5-diphosphate-N-acetylglucosamine. This figure is original and was prepared by myself.

1.8.7.1 Polyol pathway

Under diabetic conditions excessive intracellular glucose undergoes flux through a catabolic pathway called the polyol pathway in which glucose is firstly converted to sorbitol by aldose reductase (AR). NADPH that is normally utilized to reduce glutathione is oxidized. In the second step, the sorbitol dehydrogenase enzyme catabolizes sorbitol to fructose and reduces one NADH (Yan, 2018). The efflux of myoinositol, required for optimal sodium/potassium (Na/K) ATPase activity and for normal nerve physiology, is the result of osmotic stress caused by elevated cellular sorbitol levels (Oates, 2008). NADPH is depleted in this pathway leading to a defective antioxidant system and generation of ROS and ROS-mediated cellular dysfunction (Oates, 2008). Experimental studies on STZ-diabetic rats demonstrated activation of the polyol pathway and intermediates which were associated with DSPN symptoms (Cameron and Cotter, 1993, Willars et al., 1987). Pre-clinical studies of drugs targeting polyol pathway such as ARIs mitigated nerve conduction velocity slowing and nerve damage in STZ-diabetic rats (Schemmel et al., 2010, Calcutt et al., 1990a). Ablation of AR in STZ-diabetic mice improved preservation of glutathione, nerve structure and function and the overexpression of this enzyme exacerbated the severity of DSPN (Ho et al., 2006, Yagihashi et al., 2001). Despite robust results in animal models of diabetes, ARIs failed to improve DSPN in numerous clinical trials in humans due to appearance of hepatotoxicity and poor drug bioavailability to nerves (Chalk et al., 2007, Grewal et al., 2016). Weak trial design and selection of sub-optimal clinical endpoints also contributed to these failures. In a more recent study, ARIs were used to treat ischemia in kidney injury which was successful implying its possible advantageous impact on microangiopathy in DSPN (Takahashi et al., 2012).

1.8.7.2 Nonenzymatic glycation of proteins

Spontaneous attachment of glucose to mature proteins forms a Shiff base and then Amadori products under hyperglycemic conditions. These intermediates can transform to methylglyoxal, crosslink together and form advanced glycation end-products (AGEs) (Lukic et al., 2008, Thornalley, 2002). AGE deposits were present in the nerves of diabetic patients and animal models (Sugimoto et al., 1997, Sugimoto et al., 2008). Methylglyoxal and AGE were both associated with hyperalgesia in diabetic patients (Bierhaus et al., 2012). AGEs can bind to their receptors, the receptor for AGE (RAGE), and drive vasoconstriction, inflammation and neurotrophic factor deficiency possibly through activation of nuclear factor κB (NF-κB) (Lukic et al., 2008). In endoneurial endothelial cells, NF-κB activation led to swelling and vacuolar disruption in AGE-treated Wistar rats (Lukic et al., 2008, Nishizawa et al., 2010). Glycated tubulin disturbed the axonal flow of this protein leading to axonal degeneration (McLean et al., 1992, Ryle et al., 1997). In an animal study, rats developed microangiopathy, reduced Na/K ATPase activity, nerve conduction slowing and axonal loss as seen in human diabetes when treated with exogenous AGE (Nishizawa et al., 2010). Aminoguanidine, an anti-glycation agent, prevented the progression of neuropathy and improved nerve blood flow in diabetic rodents (Kihara et al., 1991). Diabetic RAGE-deficient mice displayed significantly higher nerve conduction velocity and myelinated fiber densities after an acute sciatic nerve crush when compared to control mice (Juranek et al., 2013, Wada and Yagihashi, 2005). Finally, benfotiamine, with AGE clearing properties, was efficacious especially on pain scores in patients with diabetic neuropathy (Haupt et al., 2005, Stracke et al., 1996).

1.8.7.3 Glycosylation

Excessive fructose-6-phosphate as an intermediate in glycolysis, due to excess glucose influx, undergoes a series of reactions in which UDP-5-diphosphate-N-acetylglucosamine (UDP-GlcNac) is formed (Du et al., 2000). This product has an affinity for the cell membrane and transcription factors, and modifies protein function by binding to serine/threonine residues and causing glycosylation (Du et al., 2000). This modification may contribute to pathogenesis of diabetes complications such as diabetic retinopathy, diabetic neuropathy and cardiovascular diseases (Peterson and Hart, 2016, Semba et al., 2014). UDP-GlcNac was highly toxic and activated a death signal *in vitro* in motor neurons (Lim et al., 2010a). For example, it can bind to T-type calcium channels, modify their function and affect neuronal excitability and contribute to painful diabetic neuropathy (Todorovic, 2015). The detailed role of UDP-GlcNac in the pathogenesis of DSPN is still unclear and needs to be explored.

1.8.7.4 Protein kinase C activity

Another intermediate in glycolysis which accumulates when there is excess intracellular glucose is diacylglycerol (DAG). DAG is a substrate for protein kinase C isoform beta (PKC-β) and activates this enzyme (Geraldes and King, 2010). Over-activated PKC-β triggered metabolic imbalances including insulin resistance, Na/K ATPase dysfunction, altered transforming growth factor-β (TGF-β) and VEGF gene expression, and nerve dysfunction in STZ-diabetic rats (Geraldes and King, 2010, Way et al., 2001).

Pre-clinical therapeutic studies on diabetic rodents were promising as nerve physiology and structure improvement was observed in STZ-diabetic rats when treated with a PKC inhibitor (Cameron and Cotter, 2002, Cotter et al., 2002). In similar studies, a PKC-β specific inhibitor

had beneficial effects on nerve blood flow and nerve function, such as nerve conduction velocity (Nakamura et al., 1999). Contradictory results were also observed in STZ-diabetic mice, the main PKC isoform (PKC-α), which also requires DAG for activity, in the sciatic nerve showed lower activity and expression, and the main PKC isoform (PKC-β) in the vessels showed higher expression and activity (Yamagishi et al., 2003). As a consequence, PKC-β specific inhibitors were not successful in clinical trials as they could not improve IENF density and nerve conduction velocity and other neuropathy indices in DSPN subjects other than an increase in skin blood flow (Casellini et al., 2007).

1.8.7.5 Oxidative stress

ROS molecules are required for normal cell signaling and function, and the balance in their production are controlled by antioxidant agents like thioredoxin (Trx), glutathione and vitamin E and enzymes like superoxide dismutase (SOD) inside the cell (Vincent et al., 2011, Halliwell, 1995). When the ROS production exceeds the amount of available antioxidants, it causes oxidative stress which is harmful to cells by oxidation of proteins, membrane lipids and DNA, nitrosylation, ATP synthase inhibition in mitochondria and many more disturbing reactions (Schieber and Chandel, 2014). Poly (ADP)-ribose polymerase (PARP) activated under oxidative stress to repair DNA, inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) leading to an accumulation of glycolysis substrates in the cytosol (Obrosova et al., 2005).

NADPH oxidase is the main source of ROS production and a major player in stress-related cell signaling (Kuroda et al., 2010, Van Heerebeek et al., 2002). Other than that, respiratory complexes I and III also produce ROS in cells through electron leakage to oxygen and water molecules at the mitochondrial inner membrane (Vincent et al., 2011, Sztanek et al., 2016).

Normally, the electron transport system (ETS) Complexes receive electrons from the electron donors NADH and $FADH_2$ produced by the Krebs cycle. There is electron leakage as part of this ATP generating process that under normal conditions contributes to less than 5% of total cellular ROS (Vincent et al., 2011, Sztanek et al., 2016). It was hypothesized that different pathogenic mechanisms described above originated from hyperglycemia-induced oxidative stress particularly superoxide overproduction by mitochondrial ETS (Du et al., 2000, Nishikawa et al., 2000). Brownlee et al hypothesized that hyperglycemia increases glucose flux through glycolysis and Krebs cycle thus subsequently saturating the proximal axis of the ETS in mitochondria which thus generates excess superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2) which then drive up ROS levels (Halliwell, 1995, Sztanek et al., 2016). However, supporting evidence for this hypothesis stemmed mainly from endothelial cell culture studies and did not reflect hyperglycemia-driven pathways in DRG neurons (Akude et al., 2011, Fernyhough, 2015). It remains likely that the main cellular source of ROS in neurons under conditions of hyperglycemia is the polyol pathway (Akude et al., 2011).

Accumulating evidence has shown that oxidative stress induces of peripheral nerve loss and dysfunction in diabetic animal models (Rabbani and Thornalley, 2015, Thornalley, 2002, Baynes and Thorpe, 1999). Treatment of STZ-diabetic rats with alpha-lipoic acid, a potent antioxidant, protected against microangiopathy, nerve conduction velocity slowing and nerve loss (Stevens et al., 2000). PARP-knock out mice made type 1 diabetic with STZ showed no nerve conduction velocity impairment or abnormal nerve blood vessels (Obrosova et al., 2004). A combination of ALA and coenzyme Q10 therapy in STZ-diabetic rats improved motor nerve conduction velocity, increased the levels of ATP and glutathione, and decreased oxidative stress via lowering the level of ROS in the nerves (Sadeghiyan Galeshkalami et al., 2019).

In a randomized double-blind study in human type 2 diabetic patients with DSPN, ubiquinone, a ROS scavenger, improved nerve conduction velocity compared to placebo (Hernandez-Ojeda et al., 2012). Alpha-lipoic acid has alleviated symptoms of neuropathy in human diabetic patients (Ziegler et al., 2016, Ziegler et al., 1995, Ziegler and Gries, 1997, Papanas and Ziegler, 2014). Treatment with the biogenic antioxidant R(+)-thioctic acid in 12 patients with DSPN corrected motor and sensory nerve conduction slowing and improved their general wellness, sensation and pain (Mrakic-Sposta et al., 2018). There will be more clinical data available very soon on this category of drugs as no or less side effects have been reported.

1.9 Key molecular and cellular players contributing to DSPN

There are other contributing factors to pathogenesis of DSPN and hence good targets for therapy in DSPN. Here, we review some of these components including mitochondrial function, AMPK activity and insulin/insulin-like growth factor-1 signaling in diabetes and DSPN which are also targets in our present study.

1.9.1 Mitochondria

The mitochondrion is a two-layer organelle in eukaryotic cells that efficiently generates high levels of ATP inside a cell and thus it is also called the powerhouse of the cell. It is speculated that α-proteobacterium was at one time engulfed into a primary eukaryotic cell thus forming an organelle that evolved to its modern form that is specialized in a series of processes such as energy supplementation, cell growth and death, differentiation, calcium buffering, cell cycle and signaling inside the modern eukaryotic cell (Figure 1.3) (Lane and Martin, 2010, Gabaldon and Huynen, 2004). Each human mitochondrion is typically 0.75-3 micron in diameter and each cell contains 0 (red blood cells)-2000 (liver cells) mitochondria (Chan, 2006, Wiesner

et al., 1992, das Neves et al., 2010). Mitochondria have one to ten copies of a 16.5kb-circular DNA (mtDNA) which encodes 37 genes for 22 mitochondrial tRNA, 2 mitochondrial rRNA and 13 subunits of the electron transport system complexes I, III, IV and V (Chan, 2006, Wiesner et al., 1992). All other proteins including more than 70 subunits of ETS required for normal mitochondrial function are encoded by the nuclear DNA and imported into mitochondria. This unique organelle has its own replication, transcription and translation machinery independent of the cytosolic machinery (Friedman and Nunnari, 2014).

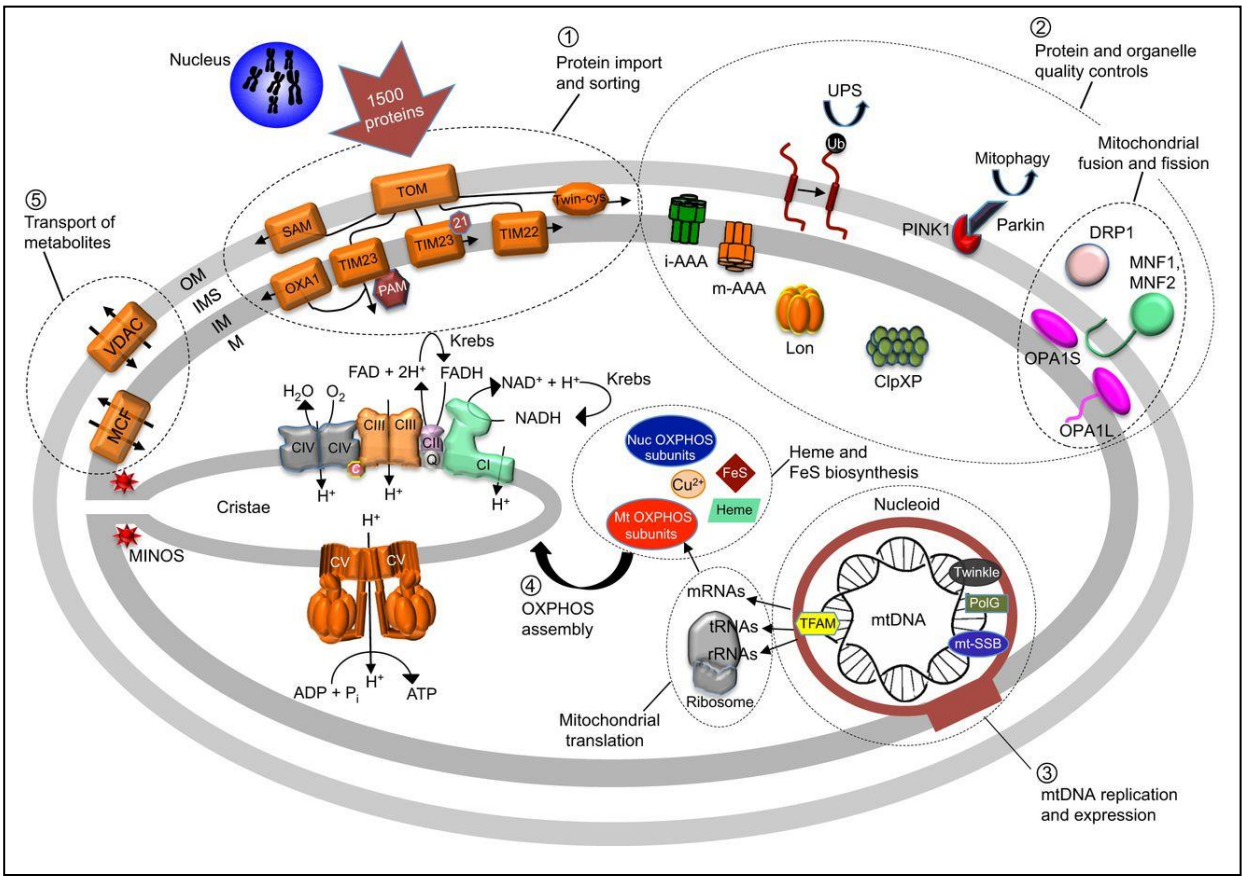

Figure 1.3. Overview of mitochondrial processes and components. This is a schematic cross-section of a human mitochondrion, showing a number of components involved in mitochondrial function. Translocase of the outer membrane (TOM); translocase of the inner membrane (TIM); sorting and assembly machinery (SAM; also known as TOB); intermembrane space (IMS); mitochondrial targeting sequence (MTS), matrix (M); inner membrane (IM); Twin-cys (the so-called mitochondrial disulfide relay system); mitochondrial carrier family (MCF); MCF with no cleavable MTS (OXA1); ubiquitin proteasome system (UPS); mitofusin-1 (MNF1); mitofusin-2 (MNF2); optic atrophy 1 (OPA1); dynein-related protein 1 (DRP1); mitochondrial transcription factor A (TFAM); DNA polymerase gamma (PolG); mitochondrial inner membrane organizing system (MINOS). Proteases and chaperones present in the IM (i-AAA, m-AAA) or the matrix (Lon, ClpXP) and proteins involved in mtDNA maintenance (PolG, Twinkle, mt-SSB). ["Used

with permission": Directly copied from "Jean-Paul Lasserre et al. Dis. Model. Mech. 2015;8:509-526 Published by The Company of Biologists Ltd" with permission under Attribution 4.0 International license (CC BY 4.0: https://creativecommons.org/licenses/by/4.0/) for the use of figure in thesis/dissertation, Lasserre, J. P., A. Dautant, R. S. Aiyar, R. Kucharczyk, A. Glatigny, D. Tribouillard-Tanvier, J. Rytka, M. Blondel, N. Skoczen, P. Reynier, L. Pitayu, A. Rotig, A. Delahodde, L. M. Steinmetz, G. Dujardin, V. Procaccio and J. P. di Rago (2015). "Yeast as a system for modeling mitochondrial disease mechanisms and discovering therapies." Dis Model Mech 8(6): 509-526.] (**Lasserre et al., 2015**).

1.9.1.1 Structure and function

In mammals, the mitochondrion consists of the outer mitochondria membrane, inner mitochondrial membrane, cristae structure formed by inner membrane folding, intermembrane space and the matrix (Friedman and Nunnari, 2014). The ETS in the mitochondria which produces energy is situated in the inner mitochondrial membrane. Special machinery is recruited to generate ATP and supply to all other parts of a cell for various cellular processes (Spinelli and Haigis, 2018). Briefly, pyruvate generated from glucose in the glycolysis process and shuttled through mitochondrial membranes undergoes a series of reactions in a catabolic process called Krebs cycle in the mitochondrial matrix and a large quantity of energy packages in the form of ATP, NADH and $FADH_2$ are produced (Spinelli and Haigis, 2018, Walsh et al., 2017). Then, the ETS system comprised of five complexes I, II, III, IV and V and located in the inner mitochondrial membrane converts the energy stored in NADH and $FADH_2$ into ATP via electron transfer and oxidative phosphorylation (OxPhos) (Walsh et al., 2017). In this process, electron transfer to complex IV and then to oxygen is normally coupled with pumping protons (H^+) from the mitochondrial matrix into the intermembrane space. A strong electrochemical gradient is established across the inner mitochondrial membrane by which protons in the intermembrane space return to the matrix through complex V (ATP synthase) and ATP is generated from ADP (Spinelli and Haigis, 2018) (Watt et al., 2010).

Mitochondrial biogenesis is transcriptionally regulated through the action of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) family of proteins that respond to energy status of the cell mediated by AMP-activated protein kinase (AMPK) and silent mating type information regulation 2 homolog 1 (SIRT1) enzymes via sensing of the AMP/ATP and NAD+/NADH ratios, respectively (Jager et al., 2007, Jeninga et al., 2010). PGC-1 accomplishes

the regulation of mitochondrial function by interacting with transcription factors known as nuclear respiratory factors 1 (NRF-1) and 2 (NRF-2) (Scarpulla et al., 2012). Mitochondrial replication and transcription is regulated by DNA polymerase γ and mitochondrial transcription factor A (TFAM) (Bogenhagen, 2012). Mitochondrial division resembles bacterial division, and its fission and fusion are mediated by dynamin-related proteins (DRPs) and mitofusin 1 (MFN1), mitofusin 2 (MFN2) and dynamin-like 120 kDa protein called OPA1, respectively (Labrousse et al., 1999, Meeusen et al., 2004). When daughter mitochondria are made from parental mitochondria, they are distributed and trafficked to different regions of the cell via Miro GTPases (Rhot1 and Rhot2), trafficking kinesin-binding protein 1 (TRAK1) and 2 (TRAK2), and other adaptor proteins on microtubules or actin filaments (Fransson et al., 2006).

The key features of mitochondria are illustrated in figure 1.3 (Figure 1.3) (Lasserre et al., 2015). In brief, import and sorting of proteins of nuclear origin: the translocase of the outer membrane (TOM) complex mediates translocation of proteins across or into the outer membrane (OM); sorting and assembly machinery (SAM; also known as TOB) facilitates protein insertion from the intermembrane space (IMS) into the OM; TIM23 takes in proteins with a cleavable mitochondrial targeting sequence (MTS), directing them either into the matrix (M) (when associated to protein import machinery (PAM)) or the inner membrane (IM) (when associated to Tim21). Twin-cys (the so-called mitochondrial disulfide relay system) mediates, in a redox-dependent manner, the delivery into the IMS of proteins containing specific cysteine motifs; TIM22, together with small soluble proteins in the IMS (called Tim), delivers into the IM the proteins of the so-called mitochondrial carrier family (MCF) that lack a cleavable MTS; OXA1 helps the insertion of proteins from the matrix into the IM. Mitochondrial quality control is a process in which misfolded and damaged mitochondrial proteins and organelles are eliminated

by proteases and chaperones present in the IM (i-AAA, m-AAA) or the matrix (Lon, ClpXP) by the cytosolic ubiquitin proteasome system (UPS), and by the PINK1 and Parkin proteins at the surface of mitochondria. Fusion (which is mediated by MNF1, MNF2, OPA1L and OPA1S) and fission (mediated by DRP1) of mitochondria contribute also to mitochondrial quality surveillance. Mitochondrial DNA (mtDNA) maintenance and expression: mtDNA is packaged into structures called nucleoids that contain proteins involved in mtDNA maintenance (PolG, Twinkle, mt-SSB), RNA synthesis (TFAM), and the processing of RNAs into messenger (mRNA), transfer (tRNA) and ribosomal (rRNA) RNAs, which are then used to translate the mtDNA-encoded proteins on mitochondrial ribosomes. OXPHOS assembly: the nDNA-encoded subunits of the OXPHOS system (Nuc OXPHOS subunits; CI-V) assemble with their partner subunits of mitochondrial origin (Mt OXPHOS subunits; all except CII, which is entirely encoded by nDNA) together with their redox prosthetic groups (heme and FeS, which are in part synthetized in the mitochondria, and Cu2+). CI-IV together with ubiquinone (Q) and cytochrome c (c) transfer electrons to oxygen from reduced cofactors (NADH, FADH) produced by the Krebs cycle, which is coupled to the pumping of protons out of the matrix. The protons are transported back into the matrix by CV, which is coupled to ATP synthesis from ADP and inorganic phosphate (Pi) (discussed earlier). Transport of metabolites: systems in the OM (VDAC; also known as porin) and IM [MCF (mitochondrial carrier family)] enable the transport of small solutes and ions into and outside the organelle. Parts of the IM protrude into the matrix, forming the cristae, at the basis of which narrow tubular structures termed ‘cristae junctions’ are maintained by proteins of the mitochondrial inner membrane organizing system (MINOS) complex (Lasserre et al., 2015).

Mutation in the corresponding genes, epigenetic modification, environmental signals, or any transcriptional/translational modification of these essential proteins are associated with aberrant mitochondrial phenotype resulting in many mitochondrial or mitochondria-driven disorders (Chakrabarty et al., 2018). Mutations in mtDNA gives rise to various diseases including MELAS syndrome, Kearns-Sayer syndrome, Leber's hereditary optic neuropathy, Pearson syndrome, myoclonic epilepsy with ragged red fibers (MERRF) and progressive external ophthalmoplegia (Zeviani and Di Donato, 2004). Mutations in nuclear DNA-encoded mitochondrial proteins results in mitochondrial dysfunction and diseases such as hereditary spastic paraplegia, Friedreich's ataxia and Wilson's disease (Chinnery and Schon, 2003). Mitochondrial dysfunction has been reported to be a pathogenetic factor in a wide variety of disorders including autism, epilepsy, Alzheimer's disease (Lim et al., 2010b), cardiomyopathies, stroke, Parkinson's disease (Sherer et al., 2002) and diabetic complications (Schapira, 2006, Pieczenik and Neustadt, 2007).

1.9.1.2 Mitochondrial abnormalities in DSPN

Mitochondria in the Schwann cells in the sural nerve of human diabetic patients were enlarged along with effacement of cristae, however, axonal mitochondria appeared normal (Kalichman et al., 1998). Accumulated glycogen granules in the intermembrane space in the axons and reduced complex IV expression in epidermal nerves has been reported in human diabetes with sensory neuropathy (Kalichman et al., 1998, Casanova-Molla et al., 2012). In persons with diabetic neuropathy, mitochondria accumulated in axonal swellings of IENF (Figure 1.4) (Lauria et al., 2003, Ebenezer et al., 2007). These mitochondrial ultrastructural changes have been observed in diabetic felines (Mizisin et al., 2007). However, type 1 diabetic rodent models only displayed aberrant mitochondrial phenotype in Schwann cells but not axons

(Kamiya et al., 2006b). In STZ-diabetic mice, accumulation of small mitochondria in autonomic ganglia has been demonstrated (Schmidt et al., 2003). Mitochondrial ultrastructure in sympathetic ganglia from NOD mice or Ins2Akita mice or persons with diabetic neuropathy showed increased number of small mitochondria as a result of increased fission (Schmidt et al., 2009, Schmidt et al., 2008, Schroer et al., 1992). Similarly, greater number of mitochondria with smaller sizes was observed in DRG neurons and axons from db/db mice (Vincent et al., 2010).

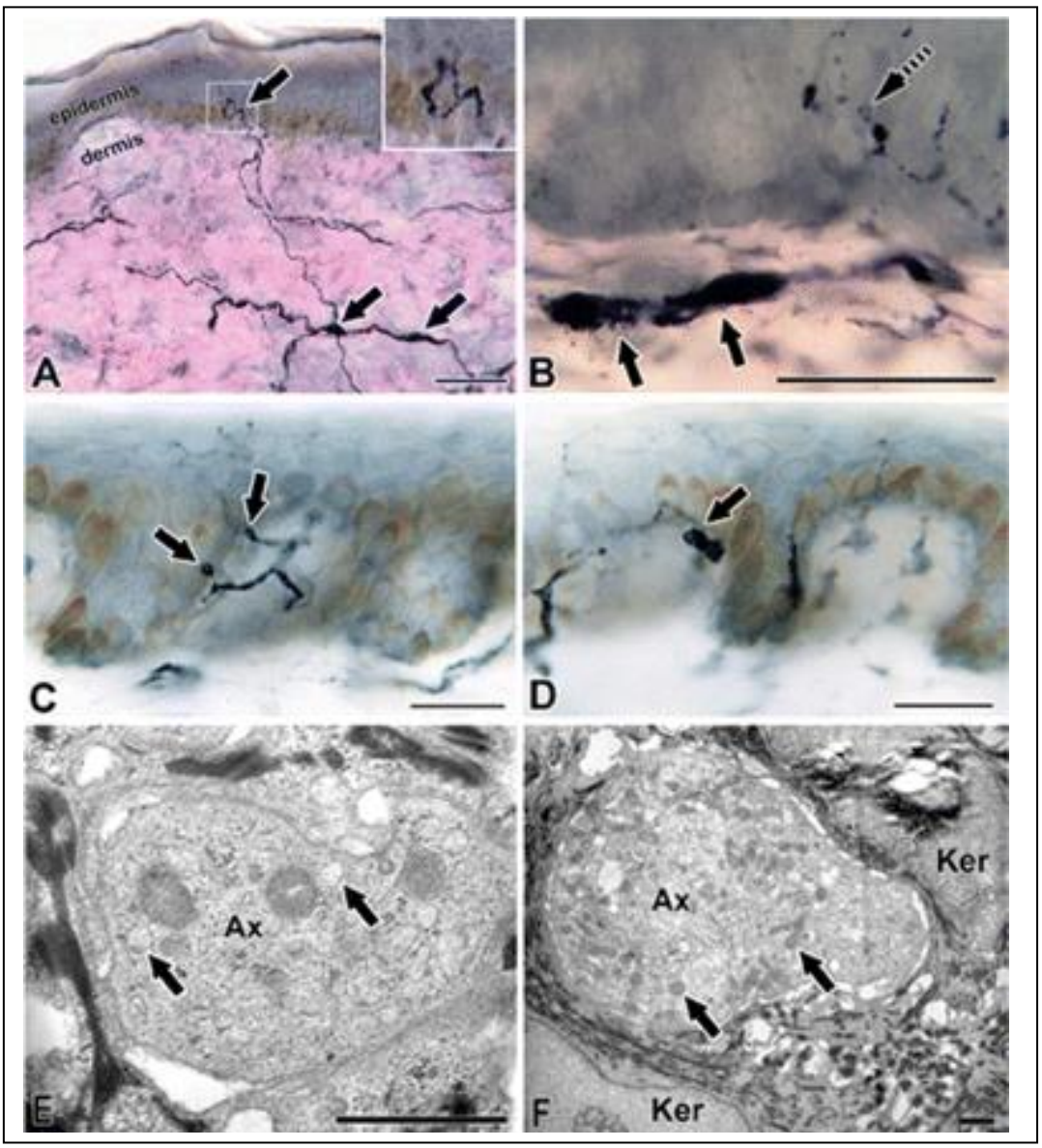

Figure 1.4. Light and electron microscopic findings of cutaneous nerves in chronic neuropathy patients. (A) Skin with the epidermal and dermal nerve fibers showing axonal swellings (arrows). Scale bar = 50 µm (Inset: epidermal axonal swelling close to the basement membrane). (B) Skin showing a fusiform axonal swelling in the dermis (arrows) epidermal axonal swellings and a degenerating nerve fibre with attenuated segments (broken arrow). Scale bar = 25 µm. (C) Skin with epidermal axonal swellings. The arrows identify swellings with

pallor of PGP in the centre, probably reflecting sites of organelle accumulation. Scale bar = 25 µm. (D) Skin with axonal swellings at the dermo–epidermal junction with vacuolar alteration (arrow). Scale bar = 25 µm. (E) Globular axonal swelling (ax) containing accumulations of particulate organelles (arrows). Scale bar = 1 µm. (F) Large axonal swelling (ax) between the keratinocytes (ker). The swelling contains accumulation of mitochondria (arrows), vesicular organelles and neurofilaments. Scale bar = 1 µm. ["Used with permission": Copyright permission note: This is a License Agreement between MohamadReza Aghanoori ("You") and Oxford University Press ("Oxford University Press") provided by Oxford University Press and Copyright Clearance Center for the use of figure/text in dissertation/thesis. License number: 4551520509534, Ebenezer, G. J., J. C. McArthur, D. Thomas, B. Murinson, P. Hauer, M. Polydefkis and J. W. Griffin (2007). "Denervation of skin in neuropathies: the sequence of axonal and Schwann cell changes in skin biopsies." Brain 130(Pt 10): 2703-2714] **(Ebenezer et al., 2007)**.

Mitochondrial complex activities were reduced in skeletal muscle in persons with type 2 diabetes (Mogensen et al., 2007) and in the kidney and heart in STZ-diabetic rodents (Dugan et al., 2013, Yang et al., 2009). Our lab and others have shown a mitochondrial depolarization and reduced complex activities in sensory neurons in type 1 and type 2 diabetic rat and mouse models (Aghanoori et al., 2019, Akude et al., 2011, Chowdhury et al., 2010, Ma et al., 2014, Urban et al., 2012). Mitochondrial oxygen consumption rate in isolated DRGs from STZ-diabetic rats revealed a significant reduction compared to controls (Chowdhury et al., 2010). A reduction of citrate synthase (in Krebs cycle) and AMPK activity, and PGC-1α was also associated with mitochondrial dysfunction in DRGs isolated from diabetic rodents compared to controls (Chowdhury et al., 2010, Roy Chowdhury et al., 2012) indicating a possible crucial role for AMPK signaling pathway in regulating mitochondrial function in diabetes.

1.9.2 AMP-activated protein kinase (AMPK)

1.9.2.1 Structure and function

AMPK is an enzyme with kinase activity that senses the energy status of a cell and inhibits anabolic pathways and promotes catabolic pathway in response to low intracellular ATP levels. It is a 3-subunit protein composed of one (catalytic) α subunit (α1 or α2), one β subunit (β1 or β2) and one γ subunit (γ1 or γ2 or γ3) that are diversely combined across varying tissues (Figure 1.5) (Ross et al., 2016). The γ subunit is comprised of four cystathione β-synthase repeats (CBSs) spanning its two Bateman domains. AMP, ADP and ATP bind to two of these repeats competitively while one site is non-functional and one site is constantly occupied by AMP. Thus, depending on the energy status of the cell and binding sites occupied by AMP, ADP or ATP, it is the AMP/ATP that primarily determines the AMPK basal activity (Xiao et al., 2011, Chen et al., 2012).

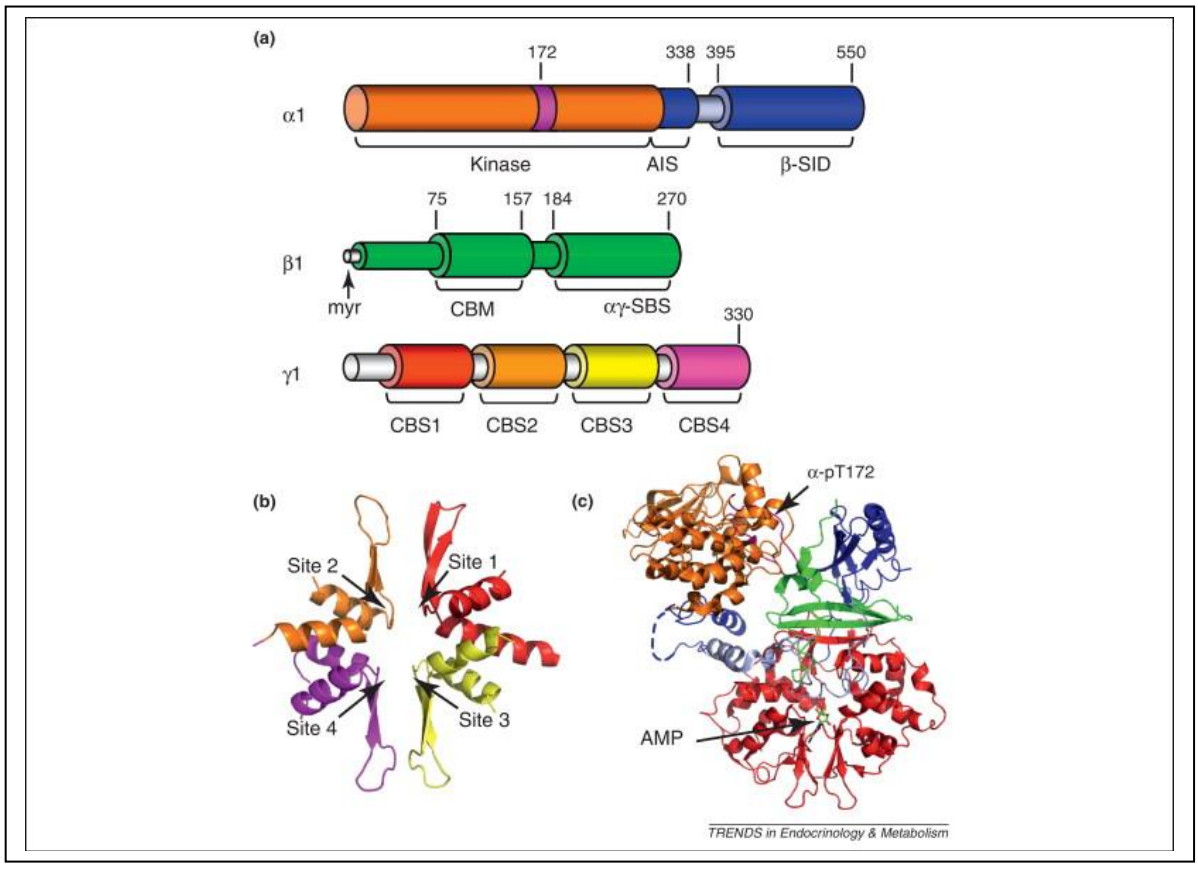

Figure 1.5. AMPK domains and structure. (a) Domain organization of AMPK subunits. Residue numbering refers to human α1, β1 and γ1 isoforms. The α subunit consists of an N-terminal kinase domain, an autoinhibitory sequence (AIS) and a β-subunit interacting domain (β-SID). The β subunit is N-terminally myristoylated (myr) and contains a mid-molecule carbohydrate-binding module (CBM) and C-terminal αγ subunit-binding sequence (SBS). The γ subunit contains four cystathione β-synthase (CBS) domains, paired (1+2 and 3+4) to form two Bateman modules. (b) Tetrad organization of CBS domains in the γ-subunit, colored as in (a), showing locations of nucleotide binding sites (black arrows). (c) Structure of the mammalian AMPK regulatory core and kinase domain [PDB 2Y94: rat α1 (7–299)/(331–469)/(524–548), human β1 (198–272) (green), rat γ1 (23–326) (red)]; α-subunit regions are colored as in (a).

AMP bound at γ site 3 is evident. [“Used with permission”: Copyright permission note: This is a License Agreement between MohamadReza Aghanoori ("You") and Elsevier ("Elsevier") provided by Elsevier and Copyright Clearance Center for the use of figure/text in dissertation/thesis. License number: 4551480066045, Oakhill, J. S., J. W. Scott and B. E. Kemp (2012). "AMPK functions as an adenylate charge-regulated protein kinase." Trends Endocrinol Metab **23**(3): 125-132.] **(Oakhill et al., 2012)**.

Two upstream kinases liver-kinase-B-1 (LKB1: which senses low energy inside the cell) and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ: which is regulated by calmodulin and Ca^{2+} ions) phosphorylate AMPK at Thr172 site on the kinase domain of the catalytic subunit (Figure 1.6). Phosphorylation of AMPK on its Thr172 site is required for its full activation which reaches 100-fold activity (Hawley et al., 2005, Woods et al., 2003). The α subunit has other domains including an auto-inhibitory domain (AID) and C terminus domain (CTD) which has binding sites for βγ. The β subunit also has a CTD which has binding sites for αβ subunits, a myristoylation-containing domain and carbohydrate-binding module (CBM) which has a binding site for glycogen (Calabrese et al., 2014).

When activated, AMPK regulates a wide variety of pathways including glucose and lipid metabolism, protein metabolism, autophagy and mitophagy (Figure 1.7). AMPK phosphorylates and activates TBC domain family, member 1(TBC1D1) and thioredoxin-interacting protein (TXNIP) to regulate glucose uptake via GLUT4 and GLUT1. It phosphorylates 6-phosphofructo-2-kinase (PFKFB3) to regulate glycolysis and inhibits storage of glycogen by modifying glycogen synthase (GS) (Hardie, 2013). AMPK plays an important role in lipid metabolism by phosphorylating and inhibiting its substrates acetyl-coA carboxylase (ACC) 1 and ACC2 leading to activation of carnitine palmitoyltransferase 1 (CPT1) which transfers fatty acids to mitochondria for oxidation (Kim et al., 2016).

AMPK inhibits protein synthesis via blockade of mammalian target of rapamycin complex 1 (mTORC1). An antagonistic effect of AMPK on eukaryotic elongation factor 2 kinase (eEF2K) against mTORC1 and S6K1 has also been reported. Insulin can activate the Akt signaling pathway thus counteracting AMPK activity which leads to activation of mTORC1 and S6K1 enzymes and protein synthesis (Inoki et al., 2003, Leprivier et al., 2013). Autophagy is a

process in which macromolecules, organelles and proteins are targeted to lysosomes for degradation thus saving energy. AMPK implements its role in mitophagy/autophagy by phosphorylating and activating unc-51-like autophagy-activating kinase 1 (ULK1), a critical initiating factor in autophagic/mitophagic cascade, and Forkhead box O3 (FOXO3) (Greer et al., 2007, Egan et al., 2011). AMPK can also control cell growth and proliferation, manage redox status, regulate circadian rhythm and perform a variety of other regulations inside the cell. Therefore, any malfunction of AMPK can predispose an organism to various disorders (Novikova et al., 2015). For example, hyperglycemia is known to downregulate AMPK in different tissues including liver, skeletal muscle and DRGs in type 1 and type 2 diabetes (Kraegen et al., 2006, Lee et al., 2007, Chowdhury et al., 2011). AMPK activation during exercise has shown promising results in type 2 diabetic patients in terms of blood glucose reduction and general health (Musi et al., 2001). Constitutively activated AMPK using pharmacological activators during ischemia contributed to protection against ischemic injury and restoration of metabolism (Russell et al., 2004). AMPK activators such as AICAR and metformin have shown beneficial effects in the treatment of heart failure, cardiac hypertrophy, obesity and diabetes (Li et al., 2007, Russell et al., 2004, Owen et al., 2000). Some activators of AMPK can also decrease the incidence of cancers in diabetic patients and therefore, metformin is now in clinical trials for breast cancer treatment (Evans et al., 2005).

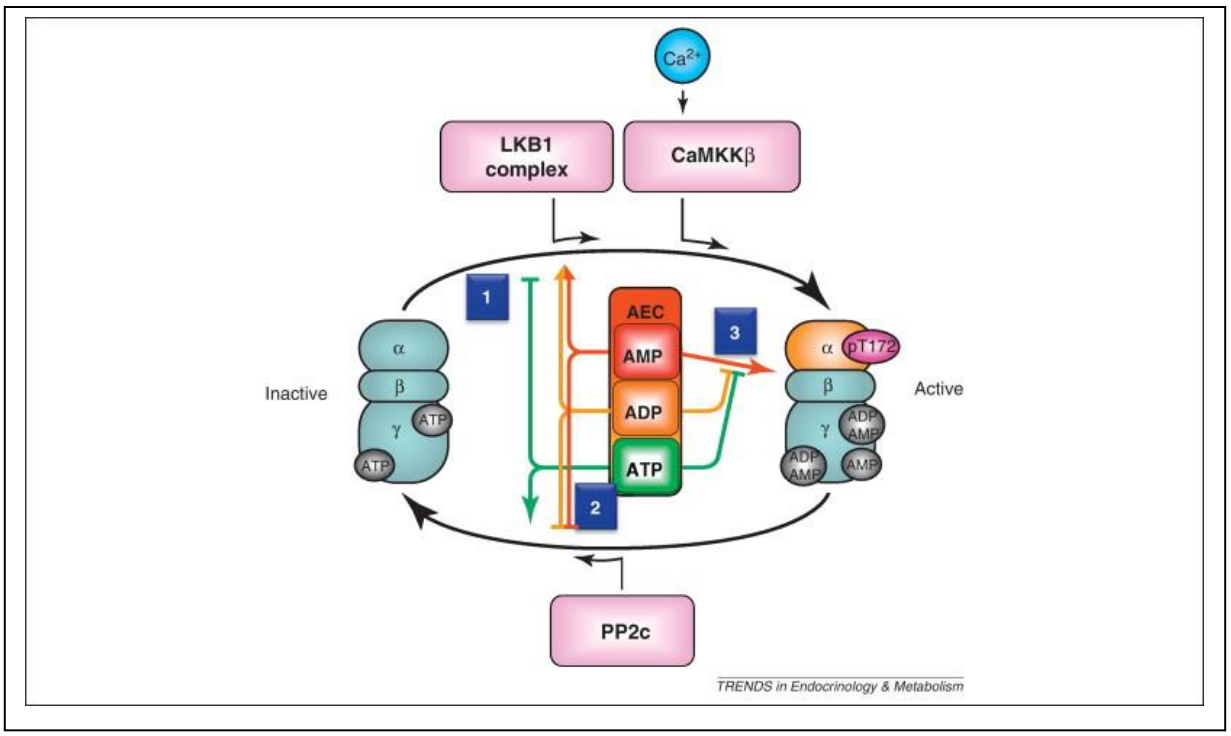

Figure 1.6. AMPK regulation by adenylate energy charge (AEC). Rises in intracellular ADP/AMP trigger (1) α-Thr172 phosphorylation and (2) simultaneous inhibition of phosphatase-mediated α-pThr172 dephosphorylation. (3) AMP allosterically activates phosphorylated AMPK directly, although it is doubtful that this occurs in the cell due to antagonism by ADP and ATP. ATP acts antagonistically on all three mechanisms. [“Used with permission”: Copyright permission note: This is a License Agreement between MohamadReza Aghanoori ("You") and Elsevier ("Elsevier") provided by Elsevier and Copyright Clearance Center for the use of figure/text in dissertation/thesis. License number: 4551480066045, Oakhill, J. S., J. W. Scott and B. E. Kemp (2012). "AMPK functions as an adenylate charge-regulated protein kinase." Trends Endocrinol Metab **23**(3): 125-132.] **(Oakhill et al., 2012)**.

1.9.2.2 AMPK abnormalities in DSPN

In STZ-diabetic rats with neuropathy AMPK was suppressed in DRG tissue, skeletal muscles, heart and liver, and it was correlated with mitochondrial dysfunction (Chowdhury et al., 2011, Guo et al., 2007, Mei et al., 2018). Resveratrol, an AMPK activator (Dasgupta and Milbrandt, 2007), improved bioenergetics profile and reversed thermal hypoalgesia, IENF density profile and mean axonal caliber in myelinated fibers in STZ-diabetic rats (Roy Chowdhury et al., 2012). Metformin is currently used as an effective treatment for type 2 diabetic patients. Metformin, although a non-specific AMPK activator, was found to activate AMPK in sciatic nerve and modulate mechanical sensitivity, and cold allodynia in a rat model of painful diabetic neuropathy (Ma et al., 2015). It also significantly increased nerve conduction velocity and reduced the pro-inflammatory cytokines in DRG tissue in a rat model of diabetic neuropathy (Hasanvand et al., 2016). STZ-diabetic rats treated with A769662, an AMPK activator (Goransson et al., 2007), exhibited improved thermal sensitivity, motor and sensory nerve conduction velocities and nerve blood flow. These features were associated with increased AMPK T172 phosphorylation, mitochondrial biogenesis and polarization, PGC-1α stimulation and normalized mitochondrial ROS production in cultured Neuro2a (N2A) cells (Yerra et al., 2017). DRG neurons exposed to high glucose suppressed pAMPK together with a reduction in SIRT2 expression and the levels of Complexes II/III and respiratory capacity (Schartner et al., 2018).

Other regulators of mitochondrial biogenesis and function that operate in parallel to, or downstream of AMPK, were found to play a role in diabetic neuropathy. For instance, the levels of PGC-1α and NRF-1 proteins and mitochondrial DNA were reduced in diabetic mice, and PGC-1α ablation in diabetic mice caused further mitochondrial degeneration and decreased

TFAM and NRF1 protein levels in DRG tissue together with severe neuropathy in animals (Choi et al., 2014). Overexpression of TFAM, a protein involved in mitochondrial DNA replication and transcription, improved nerve conduction velocity, intra-epidermal nerve fiber density and mitochondrial DNA content in STZ-diabetic mice (Chandrasekaran et al., 2015). Similar improvement in terms of mitochondrial biogenesis and neuropathy was achieved in STZ-diabetic rats when treated with metabotropic glutamate receptor (mGluR) agonists (Chandrasekaran et al., 2017). In almost all cases, a clear role of mitochondrial dysfunction and its impaired biogenesis contributing to the development of diabetic neuropathy has been identified.

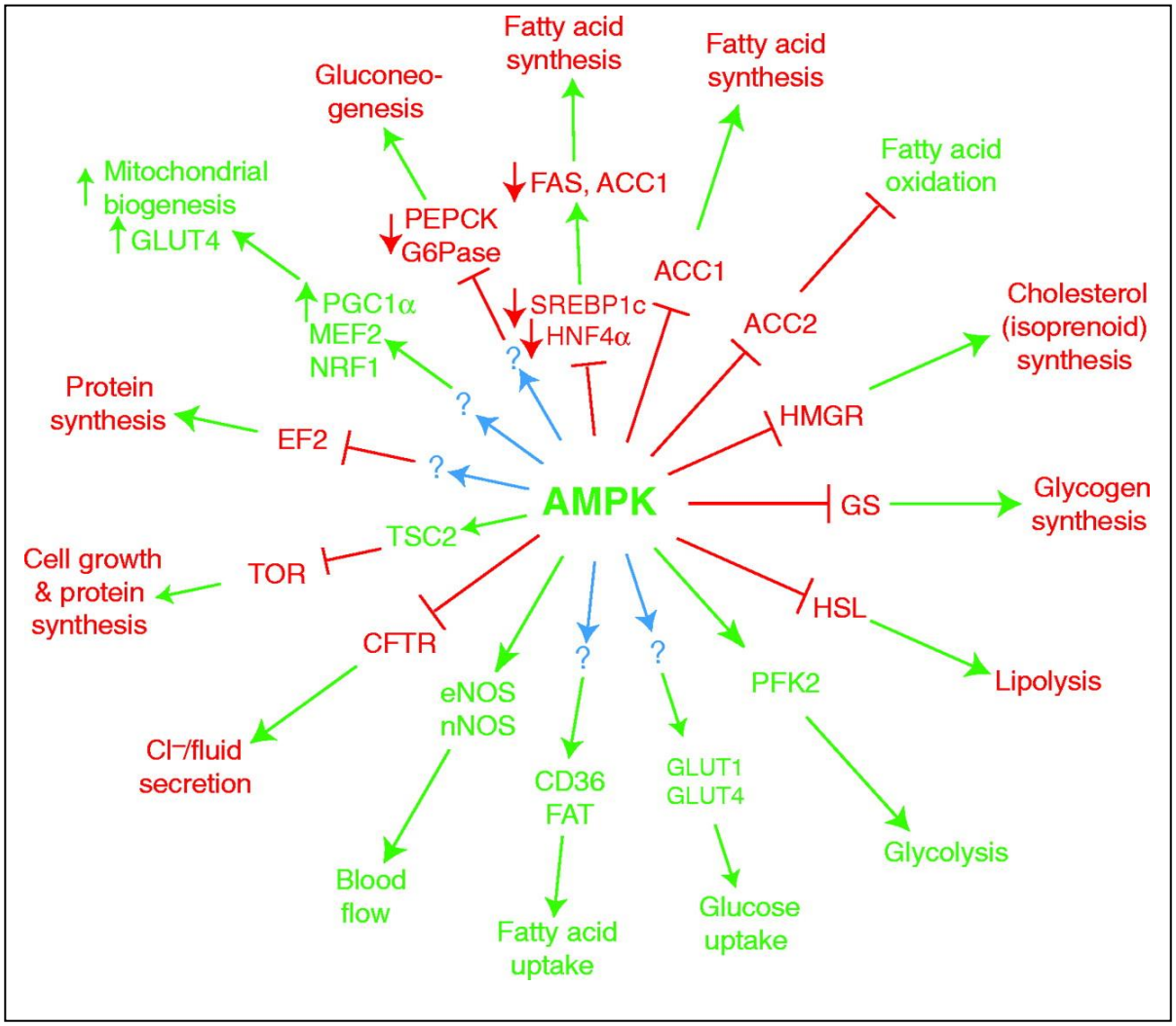

Figure 1.7. Targets for AMPK. Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression. It is not established which proteins are involved and how they implement their role downstream to blue arrows. Abbreviations: ACC1/ACC2, 1 (α) and 2 (β) isoforms of acetyl-CoA

carboxylase; CD36/FAT, CD36/fatty acid translocase; CFTR, cystic fibrosis transmembrane regulator; EF2, elongation factor-2; eNOS/nNOS. endothelial/neuronal isoforms of nitric oxide synthase; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; GLUT1/4, glucose transporters; GS, glycogen synthase; HMGR, 3-hydroxy-3-methyl-CoA reductase; HSL, hormone-sensitive lipase; MEF2, myocyte-specific enhancer factor-2; NRF1, nuclear respiratory factor-1; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator-1α; TOR, mammalian target of rapamycin. [“Used with permission”: Copyright permission note: This is a License Agreement between MohamadReza Aghanoori ("You") and The Company of Biologists Ltd ("The Company of Biologists Ltd") provided by Copyright Clearance Center ("CCC") for the use of figure/text in dissertation/thesis. License number: 4562540357095, Hardie, D. G. (2004). "The AMP-activated protein kinase pathway--new players upstream and downstream." J Cell Sci **117**(Pt 23): 5479-5487.] **(Hardie, 2004)**.

1.9.3 Insulin and insulin-like growth factor 1

1.9.3.1 Insulin structure, expression and function

Humans have one single gene (*INS*) located on chromosome 11 encoding insulin (there are two in rodents, *ins1* and *ins2*), which is transcriptionally regulated by transcription factors MafA, PDX1 and NeuroD1 (Artner I., 2008). Insulin is expressed in β-cells of the pancreas in response to glucose transported to these cells via GLUT1 (GLUT2 in rodents) glucose transporters (McCulloch et al., 2011). Translated preproinsulin transits to and is cleaved by peptidase in the endoplasmic reticulum where proinsulin is properly folded and stabilized, then transferred to the Golgi for further processing such as C-peptide cleavage from the A and B domains, and then insulin is stored in secretary granules (Hutton, 1994).

After glucose transits to β-cells, it is phosphorylated by glucokinase, and feeds the glycolysis and mitochondrial Krebs cycle for ATP generation. Excess intracellular ATP inhibits ATP-sensitive K (K_{ATP}) channels, triggering membrane depolarization and further Ca^{2+} and Na^{+} channel openings leading to increased intracellular Ca^{2+} which is coordinately synchronized across β-cells (Rorsman and Braun, 2013, Tarasov et al., 2013). Intracellular Ca^{2+} causes actin remodeling and exocytic SNARE protein recruitment, and subsequently plasma membrane and insulin granule fusion and oscillated insulin release into the peripheral vasculature for delivery to the liver. First-pass and second-pass insulin clearance occurs in the liver via insulin-insulin receptor (IR) complex internalization and endosomal lysis in hepatocytes to control insulin amount in the circulation (Gaisano, 2017, Jung et al., 2018).

Insulin in peripheral tissues binds to its receptor to activate the insulin receptor substrates IRS1 and IRS2 and thus driving two canonical pathways: PI3K/Akt signaling pathway and

mitogen-activated protein kinases, also called ERK, extracellular signal-regulated kinase (MAPK/ERK) pathway (Tokarz et al., 2018). Insulin controls glucose uptake via GLUT4 and cellular metabolism in muscle and fat tissues (Jaldin-Fincati et al., 2017) through these main pathways. Insulin regulates a wide variety of processes including nitrite oxide (NO)-activated vasodilation in the blood vessels (Vicent et al., 2003), appetite, memory, cognition and neurotrophic support in the brain (Schwartz et al., 2000, Lee et al., 2016). Insulin is cleared from the blood in no more than 30 min via the kidney clearance system. Mechanistic defects in any of the insulin production, secretion and action processes is associated with a wide range of abnormalities and disorders especially insulin resistance in type 2 diabetes (Tokarz et al., 2018).

1.9.3.2 IGF-1 structure, expression and function

IGF-1 and IGF-2 have similar structure and function, but here we focus on IGF-1 specifically as it is the core part of the current study. *IGF-1* gene consists of 6 exons and 5 introns in human (chromosome 12) and rodents differentially spliced to produce six different protein precursors from different transcript variants (Rotwein, 2017). The mature IGF-1 protein that is secreted from the liver in an endocrine manner and from peripheral tissues in an autocrine/paracrine manner has B, C, A and D domains made of 70 amino acids (Figure 1.8) (Rotwein, 2017, Filus and Zdrojewicz, 2015).

Studies have identified two promoters in the *IGF-1* gene and specific binding sites for transcription factors and regulators. Growth hormone (GH) is the most important activator of IGF-1 especially in the liver which fulfills its role through stimulating Stat5b that binds to enhancer elements on the *IGF-1* gene (Rosenfeld and Hwa, 2009). Several other transcription factors including hepatocyte nuclear factors (HNF)-1, HNF-3, CCAAT/enhancer-binding protein

(C/EBP) α, β and δ have binding sites on the *IGF-1* gene and regulate its transcription (Nolten et al., 1994, Nolten et al., 1996, Nolten et al., 1995, Umayahara et al., 1999). IGF-1 is expressed and mainly secreted from the liver as a hormone into the circulation and regulates a wide range of pathways and processes in our body. Almost all of the IGF-1 produced and circulated is bound to six IGF-1 binding proteins (IGFBPs) that have an activating or inhibitory effect on IGF-1 function in target tissues. As we age the level of IGF-1 in serum is reduced (Allard and Duan, 2018).

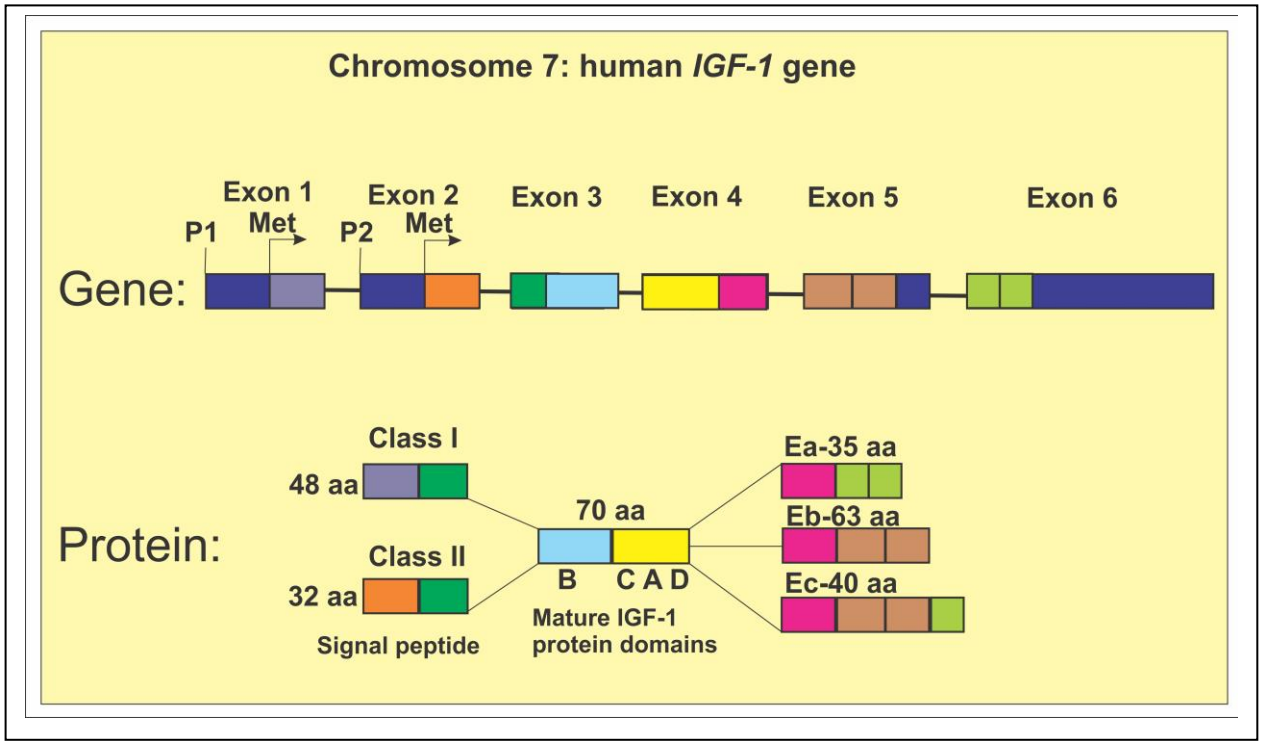

Figure 1.8. IGF-1 gene and protein structure. IGF-1 has 6 exons and 5 introns. They give rise to different transcript variants from which six different proteins are produced. There is only one mature IGF-1 expressed which has 70 aa. This figure is original and was prepared by myself.

At the target tissue, IGF-1 binds to the IGF-1 receptor (IGF1R: a tyrosine kinase receptor) and signals through IRS1 to activate two main pathways PI3K/Akt and MAPK/ERK which are essential for protein synthesis, cell survival and growth. Any deficiency or incompetency of IGF-1 function in our body produces a distinctive set of disorders (Filus and Zdrojewicz, 2015). For example, GH receptor or IGF-1 gene mutations can cause growth failure such as Dwarfism, and overproduction of GH and IGF-1 cause Acromegaly syndrome (Giustina et al., 2014, Ren et al., 2016). IGF-1 affects the aging process and improves neuropathy but its higher levels in adults is associated with cancer (Lewis et al., 1993, Arnaldez and Helman, 2012, Fletcher et al., 2009). IGF-1 is currently being tested in clinical trials for Rett syndrome and ALS but failed to improve memory and cognition in clinical trials for Alzheimer's disease (Lewis et al., 1993, Vaught et al., 1996, Nagano et al., 2005, Pini et al., 2016).

1.10 Therapeutic approaches for DSPN

1.10.1 Glycemic control

To date, no FDA-approved drug has been introduced for diabetic neuropathy. All clinical trials for the treatment of DSPN have failed possibly due to poor trial design, incorrect reasoning of pathogenesis, severity of neuropathy, method choice of DSPN assessment and many more possible confounders (Malik, 2014). One of the first clinical trials which was based on glycemic control was conducted in Stockholm on 48 patients. Patients treated with intensive or standard insulin as a tool to control blood glucose, showed suboptimal prevention of nerve conduction slowing although no major effect on thermal and vibration threshold (Reichard et al., 1993). In 1995, the Diabetes Control and Complications Trial (DCCT) achieved more than 30% reduction of neuropathy after 5 years in 1400 type 1 diabetic patients with intensive therapy (DCCT, 1993,

DCCT, 1995). A meta-analysis of eight studies on 34000 patients with type 2 diabetes did not find any prevention of clinical neuropathy after tight glycemic control (Callaghan et al., 2012).

1.10.2 Oxidative stress control

Several studies have reported that many hyperglycemic-induced pathways lead to overproduction of ROS and NOS since oxidative stress markers such as peroxynitrite and superoxide are increased and antioxidant defense moieties are reduced in patients with DSPN (Ziegler et al., 2004). Therefore, ARIs, protein kinase C inhibitors, α-lipoic acid as antioxidant agents, methylfolate and γ-linoleic acid, benfotiamine as AGE inhibitor and a plethora of other agents that reduce oxidative stress have been used and are still under investigation for treatment of DSPN (Ziegler et al., 2004). There is still a disagreement on which compound or pathway has antioxidant properties, and whether the antioxidant agents have a direct effect on general health in human is not established (Hollman et al., 2011).

Polyol pathway depletes NADPH, a ROS scavenger in cells, which contributes to oxidative stress in addition to aberrant glucose metabolism. Polyol pathway inhibitors, especially ARIs, found their way into many early clinical trials after minimal success in glycemic control trials. ARIs showed an improvement of nerve conduction velocity, sural nerve regeneration and myelinated fiber density in a variety of studies (Greene et al., 1999, Dyck et al., 1988); nevertheless, all follow-up clinical trials failed (Boulton et al., 2004). Although Epalrestat as an ARI has been licensed and has limited use in India and Japan (Goto et al., 1995), 32 clinical trials with ARIs involving 4970 diabetic subjects failed in treatment of DSPN (Chalk et al., 2007). Many animal studies utilizing antioxidants have been successful in controlling oxidative stress and improvement of neuropathy indices. For example, a combination of α-lipoic acid and

coenzyme Q10 therapy in STZ-diabetic rats suppressed oxidative stress, increased the reduced form of glutathione (GSH) and was associated with improved motor nerve conduction velocity and DSPN symptoms (Sadeghiyan Galeshkalami et al., 2019). Further, meta-analysis of four human clinical trials with daily administration of α-lipoic acid reported a significant improvement in the symptoms of DSPN after 3 weeks. However, in one multicenter randomized trial, α-lipoic acid failed to improve quantitative sensory testing (QST) score, neurophysiology and neuropathy scores in diabetic patients after 4 years (Dyck et al., 2007).

1.10.3 Growth factor replacement

The pathogenesis of growth factor deficiency in DSPN, and therapeutic approaches and studies using growth factor replacement has been discussed earlier. Here, we review two most common neurotrophic factors, insulin and IGF-1, that share the same signaling pathway (insulin signaling pathway). Insulin and IGF-1 as therapies for DSPN are discussed in detail.

1.10.3.1 Insulin as therapy for DSPN

There is insulin impairment in diabetes that contributes to pathogenesis of DSPN in patients and animal models of diabetes. Topical insulin to the eye of STZ-diabetic mice prevented depletion of sub-basal nerve plexus in the cornea (Chen et al., 2013). Insulin implants in WBN/Kob rats delayed tibial nerve conduction slowing, axonal atrophy, endoneurial fibrosis and myelin distension (Ozaki et al., 2013). Axonal elongation and caliber developed much slower in type 1 diabetic BB/Wor-rats than type 2 diabetic BB/Z-rat with hyperinsulinemia after crush injury. In parallel, the level of neurocytoskeleton proteins and neurotrophic factors were less normal in type 1 diabetic rats indicating insulin deficiency rather than hyperglycemia accounting for the progress of DSPN (Pierson et al., 2003). Consistently, providing insulin

systemically or local injection to peripheral nerves prevented deficits in sensory and motor nerve conduction velocities and mitochondrial dysfunction in STZ-diabetic rats independent of correction of hyperglycemia (Huang et al., 2003, Singhal et al., 1997, Brussee et al., 2004). Low doses of direct insulin by intrathecal delivery, insufficient to correct hyperglycemia, improved sensory and motor nerve conduction velocities (Brussee et al., 2004). Insulin implants (releasing minimal insulin that is not enough to change hyperglycemia) prevented neuropathy indices in rats without affecting hyperglycemia indicating a unique role for insulin as a neuroprotective factor in diabetic rodents (Aghanoori et al., 2017).

Insulin replacement by direct insulin and/or C-peptide replacement and/or pancreas transplantation have had beneficial effects on diabetic neuropathy in human. Insulin treatment of type 2 diabetic patients did not prevent neuropathy compared to non-treated diabetic group (Tovi et al., 1998). In type 2 diabetic subjects, local insulin application recovers nerve function in carpal tunnel syndrome (Ozkul et al., 2001). However, there is no clinical trial specifically designed for local insulin treatment to assess the efficacy of this protein in the treatment of neuropathy.

1.10.3.2 IGF-1 as therapy for DSPN

The level of IGF-1 in the serum of diabetic patients with neuropathy is significantly lower than patients without neuropathy indicating a role for IGF-1 in the progress of neuropathy (Guo et al., 1999). Early studies on STZ-diabetic rats treated with IGFs (IGF-1 and IGF-2) subcutaneously showed the reversal of sensory nerve regeneration within 2 weeks despite unabated hyperglycemia and weight (Zhuang et al., 1996). Intrathecal delivery of IGF-1 prevented and reversed sensory and motor nerve conduction velocity and sural nerve atrophy in

STZ-diabetic rats (Brussee et al., 2004). AAV-bearing mouse IGF-1 targeting the liver of STZ-diabetic mouse prevented hypo- and hyperalgesia, sensory nerve demyelination, and muscle atrophy (Chu et al., 2008). IGFBP5, an IGF-1 binding and inhibiting protein, was increased in nerve biopsies from diabetic patient with DSPN. Mice deficient in IGFBP5 or motor neuron-specific IGF1R developed motor and sensory neuropathy associated with axonal degeneration (Rauskolb et al., 2017).

Providing IGF-1 to the peripheral nerves during nerve injury improved sciatic nerve regeneration in age-matched and STZ-diabetic rats (Ishii and Lupien, 1995, Ekstrom et al., 1989, Sjoberg and Kanje, 1989). IGF-1 also prevented/reversed hyperalgesia, loss of IENF density, sural nerve axonal degeneration, and nerve conduction slowing in STZ-diabetic rats (Toth et al., 2006, Brussee et al., 2004). Intrathecal delivery of an adenovirus-bearing IGF-1 overexpressing construct improved myelination, and motor and sensory nerve conduction velocities in mouse models of diabetic neuropathy (Homs et al., 2014, Chu et al., 2008). The therapeutic potential of IGF-1 is evident in animal models of diabetes; however, the cellular mechanisms by which IGF promotes neuroprotection in diabetes is not clear. And, no clinical trial data is available for IGF-1 treatment of diabetic neuropathy.

1.10.4 AMP-activated protein kinase (AMPK) and mitochondrial function activators/modulators

Under hyperglycemic conditions it has been proposed that nutrient excess triggers this down-regulation of AMPK leading to mitochondrial dysfunction and eventually axonal degeneration (Fernyhough, 2015). As the activity of AMPK decreases over the course of diabetes and neuropathy, and many therapeutic agents act through AMPK to improve

neuropathy, it is logical to consider the AMPK pathway as a therapeutic target in treatment of DSPN. In a recent study on STZ-diabetic rats, metformin improved nerve conduction velocity together with a reduction in pro-inflammatory cytokines (Hasanvand et al., 2016). Resveratrol, an AMPK activator (Dasgupta and Milbrandt, 2007), increased AMPK phosphorylation along with increased mitochondrial function and Complex activities, and reversed thermal hypoalgesia and foot skin intraepidermal nerve loss in STZ-diabetic rats (Roy Chowdhury et al., 2012). Chinese herbal medicine is reported to activate AMPK and improve mitochondrial function was proposed to protect against neuropathy in diabetic patients (Zhang and Liang, 2019). Gentiopicroside improved sensory and motor nerve conduction velocity, enhanced nerve blood flow and ameliorated hyperalgesia by modulating the PPAR-gamma/AMPK signaling pathway (Lu et al., 2018). Donepezil also showed an improvement of hyperalgesia in STZ-diabetic mice through activation of AMPK signaling pathway (Atef et al., 2019).

As mitochondria can contribute to oxidative stress, any free-radical scavengers can optimize mitochondrial function to improve indices of neuropathy. On the other hand, AMPK signaling pathway is linked to mitochondrial biogenesis and function, thus, drugs modulating AMPK can also affect mitochondrial function. It is also noted above that mitochondria are partially dysfunctional in diabetic neuropathy. However, to date, no direct modulators/activators of mitochondrial function have been investigated, although there are potential mitochondrial sites suitable for therapeutic targeting (Leinninger et al., 2006b).

Rationale, hypotheses and aims

Each part is covered by one chapter of the current thesis. Specific aims of part 1 are addressed in chapter 2, specific aims of part 2 are addressed in chapter 3 and specific aims of part 3 are addressed in chapter 4.

Rationale part 1: Neuropathy, the most common complication of diabetes, is characterized by distal dying-back of nerve fibers combined with impaired axon regeneration (Vinik et al., 2016, Zochodne, 2016a). Oxidative stress, defective insulin signaling, neurotrophic factor deficiency, dyslipidemia and aberrant neurovascular interactions have all been proposed as contributors to pathogenesis of diabetic neuropathy (Calcutt et al., 2008, Davidson et al., 2010, Vincent et al., 2009, Yagihashi, 2016, Zochodne, 2016a). Other than an improvement in indices of diabetic neuropathy by tight glycemic control in persons with type 1 diabetes (Nathan et al., 1993), there are no promising therapies for diabetic or other peripheral neuropathies, many of which display some degree of mitochondrial dysfunction (Bennett et al., 2014, Cashman and Hoke, 2015).

In human tissues derived from persons with diabetes there is down-regulation of the AMPK/PGC-1 α pathway (Mootha et al., 2003, Patti et al., 2003). In animal models of DSPN, the levels of expression and activity of AMPK and PGC-1α are also significantly depressed in the DRG. Under hyperglycemic conditions it has been proposed that nutrient excess triggers this down-regulation of AMPK leading to mitochondrial dysfunction and eventually axonal degeneration (Fernyhough, 2015). Neurons are under direct regulatory control by insulin/IGF-1 and impaired insulin/IGF-1 signaling in diabetes provides a parallel pathogenic mechanism to hyperglycemia.

Hypothesis 1: We hypothesized that exogenous insulin could optimize AMPK activity and mitochondrial function to promote axonal repair in DRG neurons in type 1 diabetes.

Specific aim 1: To investigate the effect of insulin on AMPK activity and mitochondrial function in cultured DRGs from control and STZ-diabetic rats.

Specific aim 2: To investigate the effect of minimal insulin release (insulin implant) on nerve repair and mitochondrial phenotype in STZ-diabetic rats.

The two aims of this part are addressed in chapter 2.

Rationale part 2: IGF-1 functioning as a neurotrophic factor has been a therapeutic target in many disorders including Alzheimer's disease, Fragile X syndrome, Rett syndrome, ALS and Parkinson's disease (Costales and Kolevzon, 2016). Suppressed IGF-1 expression and subsequent depression of its signaling pathway have also been proposed to contribute to neurodegeneration in diabetes (Ishii, 1995, Rauskolb et al., 2017, Zochodne, 2016b) since the level of systemic or background IGF-1 is markedly diminished in humans and animal models of type 1 and type 2 diabetes (Zhuang et al., 1997, Palta et al., 2014, Ishii et al., 1994, Ekstrom et al., 1989).

Hypothesis 2: We hypothesized that exogenous IGF-1 could optimize AMPK activity and mitochondrial function to promote axonal repair in DRG neurons in type 1 diabetes.

Specific aim 1: To investigate the effect of IGF-1 on AMPK activity and mitochondrial function in cultured DRGs from control and STZ-diabetic rats.

Specific aim 2: To scrutinize the mechanism through which IGF-1 regulates mitochondrial function in DRG neurons derived from rats.

Specific aim 3: To investigate the effect of IGF-1 therapy on AMPK and mitochondrial function, nerve metabolism and nerve repair in STZ-diabetic rats.

The three aims of this part are addressed in chapter 3.

Hypothesis 3: We also hypothesized that impaired autocrine/paracrine IGF-1 in DRGs was a contributing factor to progressive neurodegeneration and impaired nerve regeneration in DSPN.

Specific aim 1: To investigate the main source of endogenous IGF-1 in DRG and nerve tissues.

Specific aim 2: To scrutinize the mechanism by which endogenous IGF-1 is suppressed in DRGs.

Specific aim 3: To explore regulatory proteins involved in endogenous IGF-1 gene expression in sensory neurons.

The three aims of this part are addressed in chapter 4.

Chapter 2: Insulin Prevents Aberrant Mitochondrial Phenotype in Sensory Neurons of Type 1 Diabetic Rats

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Abstract

Diabetic neuropathy affects approximately 50% of diabetic patients. Down-regulation of mitochondrial gene expression and function has been reported in both human tissues and in dorsal root ganglia (DRG) from animal models of type 1 and type 2 diabetes. We hypothesized that loss of direct insulin signaling in diabetes contributes to loss of mitochondrial function in DRG neurons and to development of neuropathy. Sensory neurons obtained from age-matched adult control or streptozotocin (STZ)-induced type 1 diabetic rats were cultured with or without insulin before determining mitochondrial respiration and expression of mitochondrial respiratory chain and insulin signaling-linked proteins. For *in vivo* studies age-matched control rats and diabetic rats with or without trace insulin supplementation were maintained for 5 months before DRG were analyzed for respiratory chain gene expression and cytochrome *c* oxidase activity. Insulin (10nM) significantly ($P<0.5$) increased phosphorylation of Akt and P70S6K by 4-fold and neurite outgrowth by 2-fold in DRG cultures derived from adult control rats. Insulin also augmented the levels of selective mitochondrial respiratory chain proteins and mitochondrial bioenergetics parameters in DRG cultures from control and diabetic rats, with spare respiratory capacity increased by up to 3-fold ($P<0.05$). Insulin-treated diabetic animals exhibited improved thermal sensitivity in the hind paw and had increased dermal nerve density compared to untreated diabetic rats, despite no effect on blood glucose levels. In DRG of diabetic rats there was suppressed expression of mitochondrial respiratory chain proteins and cytochrome *c* oxidase activity that was corrected by insulin therapy. Insulin elevates mitochondrial respiratory chain protein expression and function in sensory neurons and this is associated with enhanced neurite outgrowth and protection against indices of neuropathy.

Key words: axon regeneration; bioenergetics; diabetic neuropathy; dorsal root ganglia; mitochondrial function; neurotrophin

Introduction

Neuropathy, the most common complication of diabetes, is characterized by distal dying-back of nerve fibers combined with impaired axon regeneration (Vinik et al., 2016, Zochodne, 2016a). Oxidative stress, defective insulin signaling, neurotrophic factor deficiency, dyslipidemia and aberrant neurovascular interactions have all been proposed as contributors to pathogenesis of diabetic neuropathy (Calcutt et al., 2008, Davidson et al., 2010, Vincent et al., 2009, Yagihashi, 2016, Zochodne, 2016a). Other than an improvement in indices of diabetic neuropathy by tight glycemic control in persons with type 1 diabetes (Nathan et al., 1993), there are no promising therapies for diabetic or other peripheral neuropathies, many of which display some degree of mitochondrial dysfunction (Bennett et al., 2014, Cashman and Hoke, 2015).

The high energy consumption of neurons requires fine control of mitochondrial function (Chowdhury et al., 2013, Fernyhough, 2015) and the growth cone motility required to maintain fields of innervation consumes 50% of ATP supplies in neurons due to high rates of actin treadmilling (Bernstein and Bamburg, 2003). Unmyelinated axons are more energetically demanding than myelinated axons, consuming 2.5-10-fold more energy per action potential (Wang et al., 2008). There is mounting evidence that diabetes suppresses mitochondrial function in dorsal root ganglia (DRG) (Chowdhury et al., 2010, Roy Chowdhury et al., 2012, Urban et al., 2012, Ma et al., 2014, Freeman et al., 2016, Sas et al., 2016). We have previously proposed that hyperglycemia-induced down-regulation of the AMP-activated protein kinase (AMPK)/ peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α) signaling axis can result in axon degeneration and failure to regenerate (Roy Chowdhury et al., 2012, Chowdhury et al., 2013, Fernyhough, 2015, Calcutt et al., 2017). However, there is also a growing appreciation that hyperglycemia is not the sole initiating factor in the pathogenesis of diabetic neuropathy. A

number of authors have presented data indicating that loss of direct insulin signaling contributes to diabetic neuropathy and retinopathy (Zochodne, 2016a, Ishii, 1995, Reiter and Gardner, 2003). For example, providing systemic insulin at low levels or injecting insulin adjacent to the sciatic nerve can prevent deficits in sensory and motor nerve conduction velocity (NCV) in streptozotocin (STZ)-induced diabetic rats independent of correction of hyperglycemia (Huang et al., 2003, Singhal et al., 1997, Brussee et al., 2004). Local injection of insulin to the skin, or topical application to the cornea also enhances sensory nerve fiber density in diabetic rodents (Chen et al., 2013, Guo et al., 2011). In humans, the local application of insulin can enhance nerve recovery in carpal tunnel syndrome in patients with type 2 diabetes (Ozkul et al., 2001).

The potential for direct neurotrophic and neuroprotective actions of insulin is supported by reports that neurons express the appropriate proteins to facilitate responses to insulin exposure. Insulin receptors (IRs) and receptor substrate scaffolds (IRS1, IRS2) are expressed by sensory neurons and activate signal transduction pathways that modulate neurite outgrowth and axonal plasticity (Fernyhough et al., 1993, Huang et al., 2005, Singh et al., 2012, Grote et al., 2013b). In neurons, the insulin receptor pathway activates several messengers that include the important survival kinase, phosphatidylinositide 3-kinase (PI3-K), that is directly associated with, and activated by, IRS-1 and induces Akt activation (Grote et al., 2013b, Huang et al., 2005, Kim et al., 2011). P70S6K is a serine/threonine kinase that acts downstream of the PI-3K/Akt pathway to regulate survival and growth of neurons. Upon phosphorylation on the T389 site, P70S6K is activated and triggers protein synthesis via activation of S6 ribosomal protein (Chung et al., 1994). Neurons are therefore under direct regulatory control by insulin and impaired insulin signaling in diabetes provides a parallel pathogenic mechanism to hyperglycemia.

In the present study, we tested the hypothesis that exogenous insulin could correct mitochondrial dysfunction in adult rat sensory neurons under hyperglycemic conditions using both *in vitro* and *in vivo* models.

Materials and methods

Animals

As mentioned in chapter 1, we used type 1 diabetic rats because of low cost of maintenance and ease of use while reflecting most of neuropathy indices seen in human. To be consistent throughout our study, we focused on one gender (male) in the present study. Male Sprague-Dawley rats (275–325 g) were used as a model of type 1 diabetes after delivery of a single intraperitoneal injection of 90 mg/kg STZ (Sigma, St Louis, MO, USA). Insulin implants (Linplant, Linshin Canada Inc., Canada) were injected subcutaneously into the nape of the neck of a subgroup of STZ-induced diabetic rats after approximately 4 weeks of diabetes and at monthly intervals thereafter. Fasting blood glucose concentration was monitored weekly using the AlphaTRAK glucometer (Abbott) to ensure that insulin therapy did not alter hyperglycemia. At the end of 5 months, blood glucose, glycated haemoglobin (HbA1C) and body weight were recorded before tissue collection. Animal procedures were approved by the University of Manitoba Animal Care Committee.

Hind paw thermal sensitivity test in adult rats

Hind paw thermal response latencies were measured using a Hargreaves apparatus (UARD, La Jolla, CA, USA) as previously described *(Jolivalt et al., 2016).* Briefly, rats were placed in plexiglass cubicles on top of the thermal testing system. The heat source was placed below the middle of one of the hind paws and latencies of the paw withdrawal to the heat source were

automatically measured. Response latency of each paw was measured three times at 5 min intervals.

Adult DRG sensory neuron culture

DRGs were isolated and dissociated using previously described methods (Calcutt et al., 2017). Neuron-enriched cells were cultured in Hams F12 media supplemented with Bottenstein's N2 without insulin (0.1 mg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite 0.1 mg/ml BSA; all additives were from Sigma, St Louis, MO, USA; culture medium was from Caisson labs, USA). DRG neurons from control rats were cultured in the presence of 5 mM D-glucose and DRG neurons derived from STZ-induced diabetic rats with 25 mM D-glucose and zero insulin. Porcine insulin powder (Sigma, St Louis, MO, USA) dissolved in PH=2 and different doses (10 or 100 nM) were used as treatments. No neurotrophins were added to any DRG cultures. In this culture system there is approximately 5% cell loss over a 24 hr period.

Quantitative Western blotting for insulin signaling and mitochondrial proteins

Rat DRG neurons were harvested from culture or isolated intact from adult rats and then homogenized in ice-cold lysis buffer containing: 0.1 M Pipes, 5 mM $MgCl_2$, 5 mM EGTA, 0.5% Triton X-100, 20% glycerol, 10 mM NaF, 1 mM PMSF, and protease inhibitor cocktail. Proteins were assayed using DC protein assay (BioRad; Hercules, CA, USA). The samples (2-5µg total protein/lane) were resolved and separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA) and immunoblotted with specific antibodies against pP70S6K T389 (1:1000, Cell Signaling

Technology), pAkt S473 (1:1000, Santa Cruz, TX, USA), Total OXPHOS (1:2000, MitoSciences, Abcam, USA), Porin (1:1000, Abcam), β-actin (1:1000, Santa Cruz Biotechnology) and total-ERK (1:1000, Santa Cruz Biotechnology). Of note, total protein bands were captured by chemiluminescent imaging of the blot after gel activation (TGX Stain-Free™ FastCast Acrylamide Solutions, Bio-Rad, CA, USA) in addition to use of T-ERK and porin levels for target protein normalization. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (H+L) or donkey anti-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, PA, USA. The blots were incubated in ECL Advance (GE Healthcare) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad). All raw data signals for each antibody were normalized to T-ERK (in vitro work) or total protein (in vivo work) from the same blot. Please note that electron transport chain protein bands in Figures 2.2, 2.4 and 2.7, and Figures 2.9 and 2.10. supplemental Figure were obtained from one single blot with different exposure times.

Real-Time PCR of DRG samples

RNA was extracted from previously frozen tissue samples using TRIzol® Reagent (Invitrogen). RNA samples underwent DNase treatment and reverse transcription by using the iScript™ gDNA Clear cDNA Synthesis Kit and iQ cycler system (Bio-Rad, CA, USA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed by using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA). The ΔΔCt method was used to quantify gene expression. The expression of porin gene was used for normalization. Primer sequences for gene expression analysis are listed as follows: *SDHB*-Forward: 5′-ATCTGCAATCCATCGAGGACC-3′, *SDHB*-Reverse: 5′-AGCGAT AAGCCTGCATGAGAA-3′, *MT-CO1*-Forward: 5′-TCCAGATGCTTACACCACATGA-3′, *MT-CO1*-Reverse: 5′-

AGTTGAGGAGTAGGAAATTGAGAGT-3′, *VDAC1* (porin) -Forward: 5′-GCTTTTCGGCCAAAGTGAACA-3′, *VDAC1* (porin) -Reverse: 5′-CGCATTGACGTTCTTGCCAT-3′.

Mitochondrial respiration in cultured neurons

To measure the basal level of mitochondrial oxygen consumption, the ATP-linked oxygen consumption (proton leak), the maximal respiration, the spare respiratory capacity and the non-mitochondrial oxygen consumption, an XF24 Analyzer (Seahorse Biosciences, Billerica, MA, USA) was used (Brand and Nicholls, 2011). In short, DRG culture medium was changed 1hr before the assay to unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 1mM pyruvate, and 5 mM D-glucose. For diabetic rat DRG cultures, 25 mM D-glucose was used. Four mitochondrial complex inhibitors including oligomycin (1 µM), FCCP (1 µM) and rotenone (1 µM) + antimycin A (1 µM) were injected sequentially through ports in the Seahorse Flux Pak cartridges. Oligomycin acts as an irreversible ATP synthase inhibitor, FCCP as an uncoupler, rotenone as Complex I inhibitor, and antimycin A as an inhibitor of Complex III of the mitochondrial electron transport system. The XF24 machine which creates a transient 7µl chamber in specialized 24-well microplates, allowed real time recording of live cell mitochondrial oxygen consumption rate (OCR). After OCR measurement, cells were fixed with 4% paraformaldehyde and stained for β-tubulin III which specifically labels sensory neurons. Plates were then placed into a Cellomics Arrayscan-VTI HCS Reader (Thermo Scientific, Pittsburgh, PA, USA) equipped with Cellomics Arrayscan-VTI software to facilitate a full neuronal count of each well. OCR measures from each well were normalized to neuronal count for that specific well. Data are presented as OCR per 5000 cells.

Immunocytochemistry for β-tubulin III for neurite outgrowth in DRG cultures

DRG neurons were removed from culture and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature then permeabilized with 0.3% Triton X-100 in PBS for 5 min. Cells were incubated with blocking buffer for 1 h and with neuron-specific -tubulin III primary antibody (1:1000; from Sigma Aldrich) overnight. Following three washes with PBS, cells were incubated with fluorescein isothiocynate-conjugated secondary antibody (1:1000, Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Slides were mounted using mounting medium with DAPI and imaged by a Carl Zeiss Axioscope-2 upright fluorescence microscope equipped with AxioVision3 software. The fluorescent signal was collected as total pixel area for neurites and was measured by ImageJ software and normalized to number of cell bodies to calculate total neurite outgrowth per neuron.

Nerve density in footpads

The plantar dermis and epidermis of the hind paw were removed and placed in 4% paraformaldehyde. Tissue was processed to paraffin blocks, cut as 6µm sections, immunostained using an antibody to PGP 9.5 (1:1000, Biogenesis Ltd. Poole, UK) and the number of immunoreactive intra-epidermal fiber s(IENF) and sub-epidermal nerve profiles (SNP) per unit length quantified under blinded conditions by light microscopy (Jolivalt et al., 2016).

Enzymatic activity of respiratory Complex IV in DRG

Enzymatic activity of cytochrome *c* oxidase (a subunit of Complex IV of the mitochondrial electron transport system) was measured by a temperature controlled Ultrospec 2100 UV–visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech). Briefly, 0.02% lauryl maltoside was mixed with 10 µg DRG homogenates and incubated for 1 min before

addition of 40 µM reduced cytochrome *c* and 50 mM KPi to the mixture. The resulting absorbance decrease of reduced cytochrome *c* at 550 nm was monitored for 2min (Roy Chowdhury et al., 2012).

Statistical analysis

Data were analyzed using two-tailed Student's t-tests or one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, as appropriate and indicated (GraphPad Prism 4, GraphPad Software). A P value < 0.05 was considered to be significant.

Results

Insulin augments pAkt (short-term), pP70S6K and neurite outgrowth (long-term) in cultured DRG neurons from normal adult rats

Sensory neurons derived from a normal adult rat were maintained *in vitro* and exposed to insulin at the physiologically relevant concentration of 10 nM. Akt was activated within 15 min of exposure to insulin while P70S6K exhibited enhanced phosphorylation on residue T389 within 2h (Figure 2.1A, B). To confirm that insulin was acting as a neurotrophic factor for adult sensory neurons, DRG-derived neurons were treated with insulin (1, 10 and 100 nM) for 24h. At doses of 10 nM and 100 nM, but not 1 nM, insulin significantly ($P<0.001$ and $P<0.0001$, respectively) enhanced total neurite outgrowth (Figure 1.1C, D).

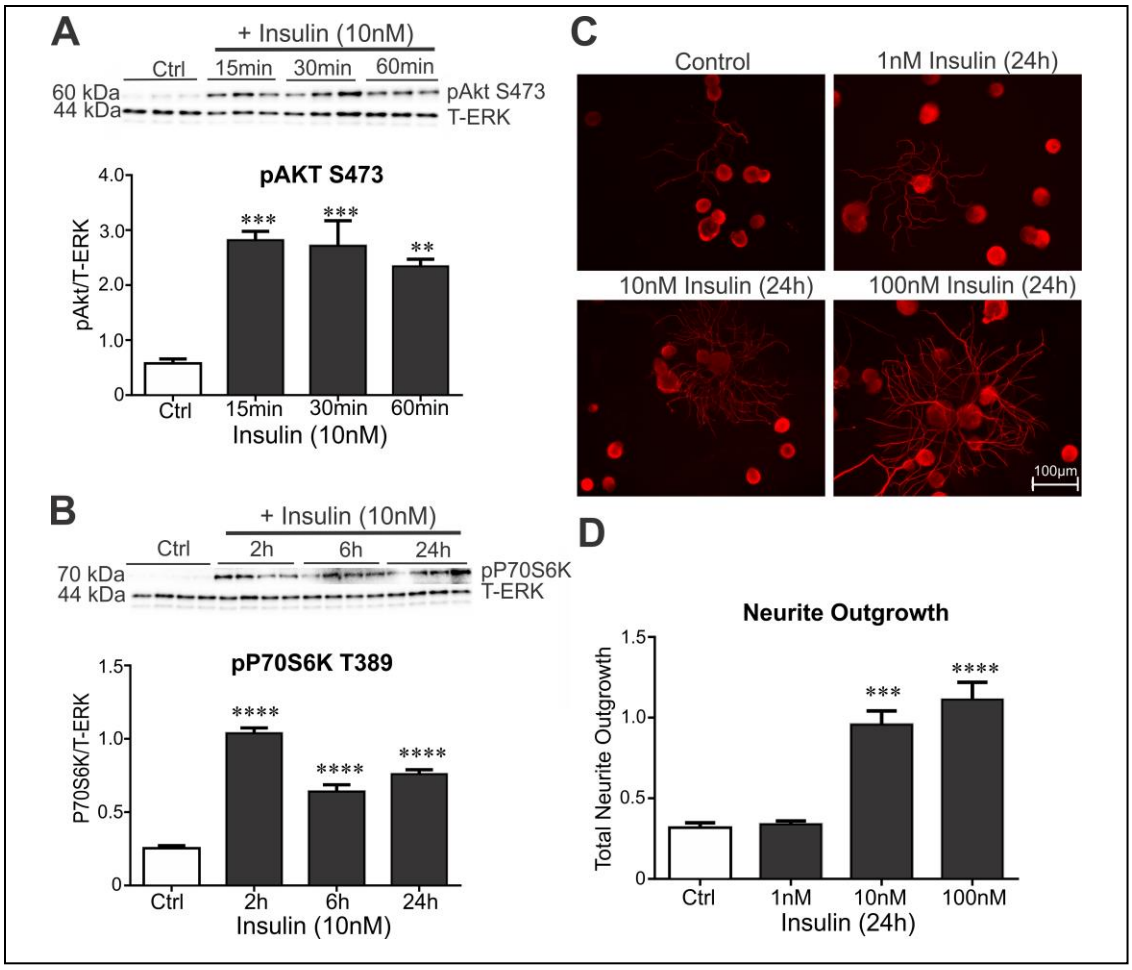

Figure 2.1. Insulin treatment increases the expression of pAkt, pP70S6K and elevates neurite outgrowth in DRG neurons. DRG neurons derived from adult control rats were grown in the absence of neurotrophic factors and under defined conditions then treated with/without 10 nM insulin for (A) 15-60min or (B) 2-24h. Lysates were collected and subjected to Western blotting. In (C,D) DRG neuron cultures from adult control rats were treated with/without 1 nM, 10 nM and 100 nM insulin for 24h then fixed and immunostained for β-tubulin III. In (A,B) Western blot data were normalized to T-ERK. In (D) total neurite outgrowth data is presented relative to neuron number. Data are mean ± SEM of N=3-4 replicates; **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

Insulin up-regulates mitochondrial gene expression and function in cultured neurons derived from normal adult rats

Mitochondrial respiratory complex proteins, including the NDUFB8 subunit of Complex I and ATP5A subunit of Complex V, were significantly ($P<0.05$ and 0.01 respectively) up-regulated in cultures of DRG neurons derived from control rats 24h after treatment with insulin (Figure 2.2). Subunits of Complex II and IV were also raised 2-fold at 24h when compared to the untreated control group but this enhancement was not statistically significant. Insulin treatment elevated OCR (Figure 2.3A) and bioenergetic parameters such as basal respiration, maximal respiration and spare respiratory capacity were significantly ($P<0.05$) augmented 2h after insulin delivery (Figure 2.3B).

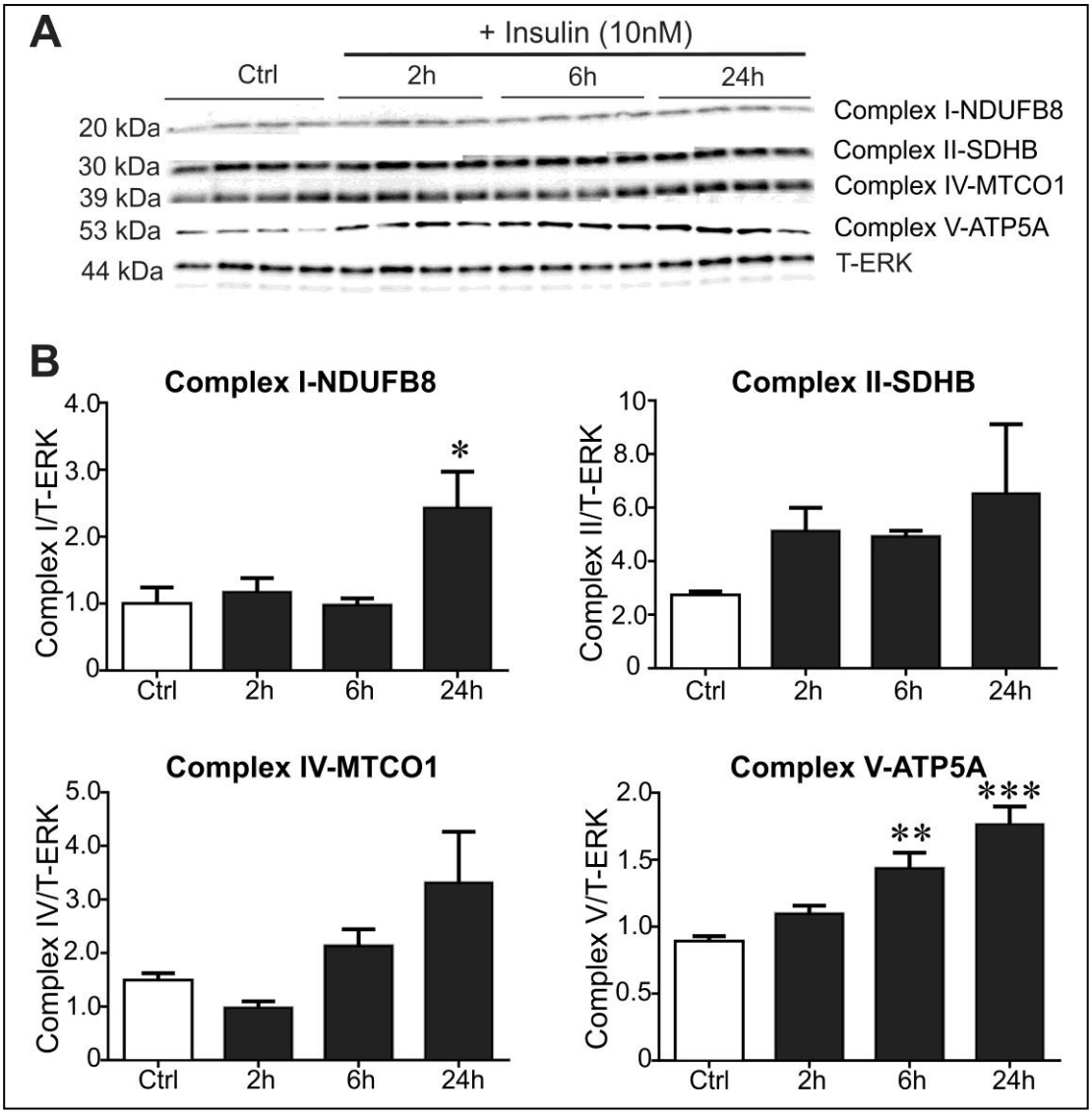

Figure 2.2. Insulin treatment increases the expression of electron transport chain proteins. DRG neurons derived from adult control rats were treated with/without 10 nM insulin for 2-24 h and lysates subjected to Western blotting. Specific proteins from each respiratory Complex were quantified and expressed relative to T-ERK. Complex III-UQCRC2 subunit protein was not detectable. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

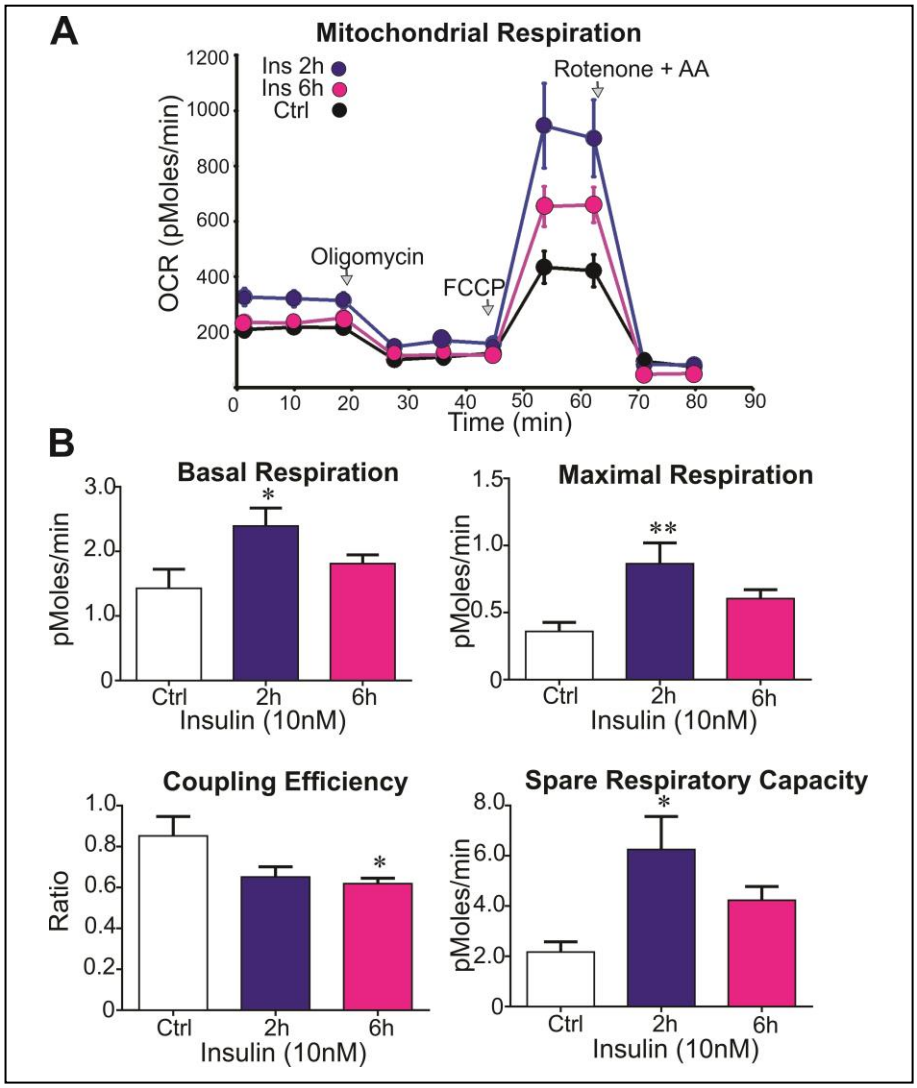

Figure 2.3. Insulin enhances mitochondrial respiration in cultured adult sensory neurons. DRG neurons derived from adult control rats were maintained overnight and then were treated with/without 10 nM insulin for 2 and 6 h. The culture plate was then inserted into the Seahorse XF24 Analyzer and oligomycin, FCCP and rotenone+AA (antimycin A) added sequentially. Data were normalized to cell number per well derived from immunocytochemical counting using the Seahorse software prior to statistical analysis. Data are presented as OCR in pmoles/min per 5000 cells. Data are mean ± SEM of N=5 replicates; *= $p<0.05$ or **= $p<0.01$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

Insulin enhances mitochondrial gene expression and function in cultured neurons derived from a diabetic rat

To investigate if diabetes impedes insulin driven induction of mitochondrial gene expression and mitochondrial function, we analyzed expression of mitochondrial proteins in DRG neurons derived from STZ-induced diabetic rats cultured in the presence of 25mM D-glucose. Mitochondrial gene expression of subunits of Complexes II, IV and V was up-regulated within 24h of exposure to 10 nM insulin (Figure 2.4). This stimulatory effect of insulin was also observed in a complementary experiment where DRGs were derived from age-matched control and diabetic rats at the same time, cultured, and the diabetic culture was treated with/without insulin for 24h (Figure 2.9.Supplemental Figure). The same dose of insulin enhanced respiration in cultured sensory neurons derived from diabetic rats (Figure 2.5A) with both basal and maximal respiration significantly ($P<0.001$ and 0.01 respectively) increased at 6h, but not 2h (Figure 2.5B), indicating a delayed response compared to cells from control rats (Figure 2.3B).

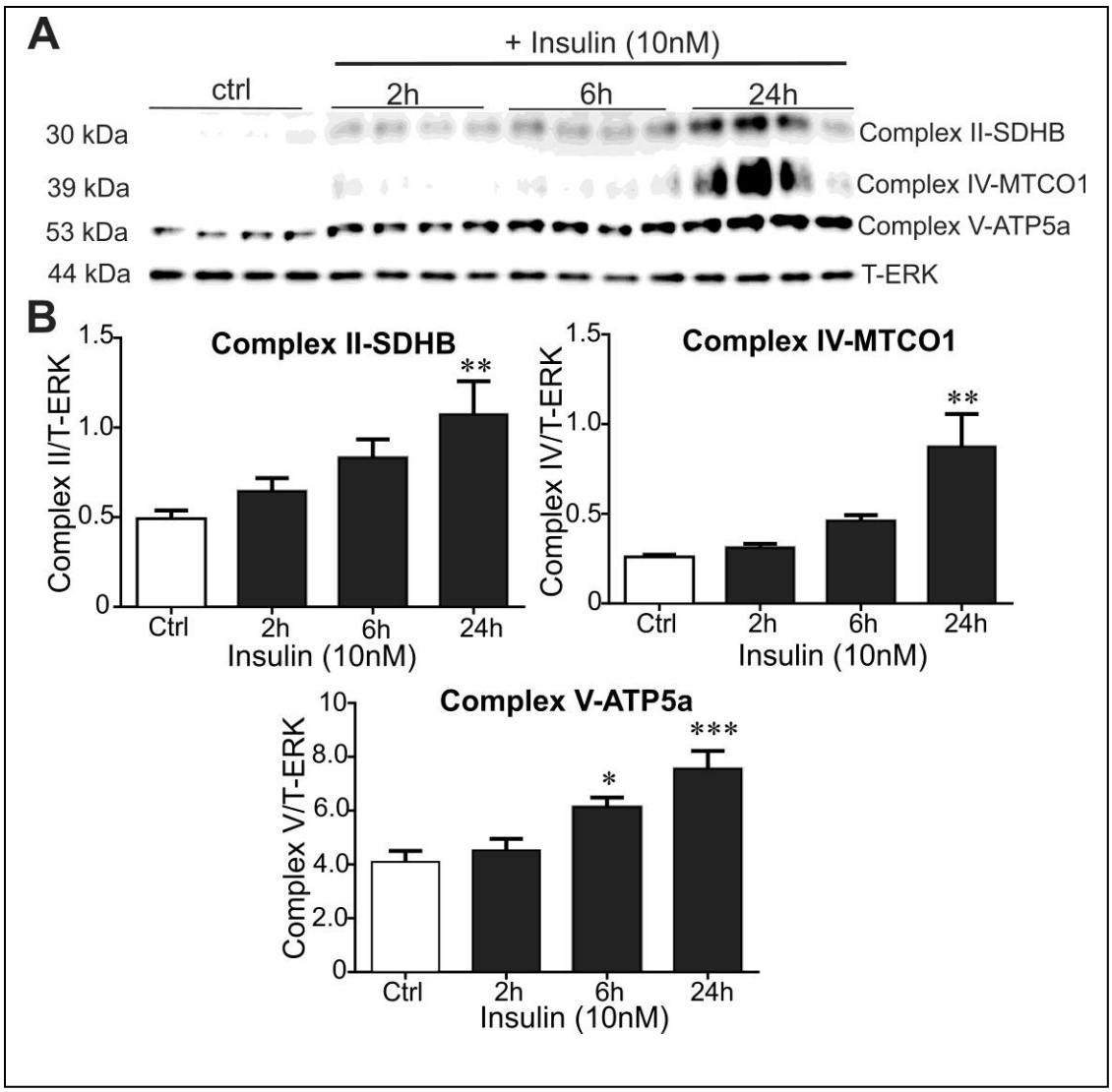

Figure 2.4. Insulin treatment increases the expression of electron transport chain proteins in DRG cultures from a diabetic rat. DRG neurons derived from adult STZ-diabetic rats were cultured in the presence of 25mM glucose, and treated with/without 10 nM insulin for 2-24h. Complex I and III subunit proteins were not detectable. Protein band intensity was normalized to T-ERK. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

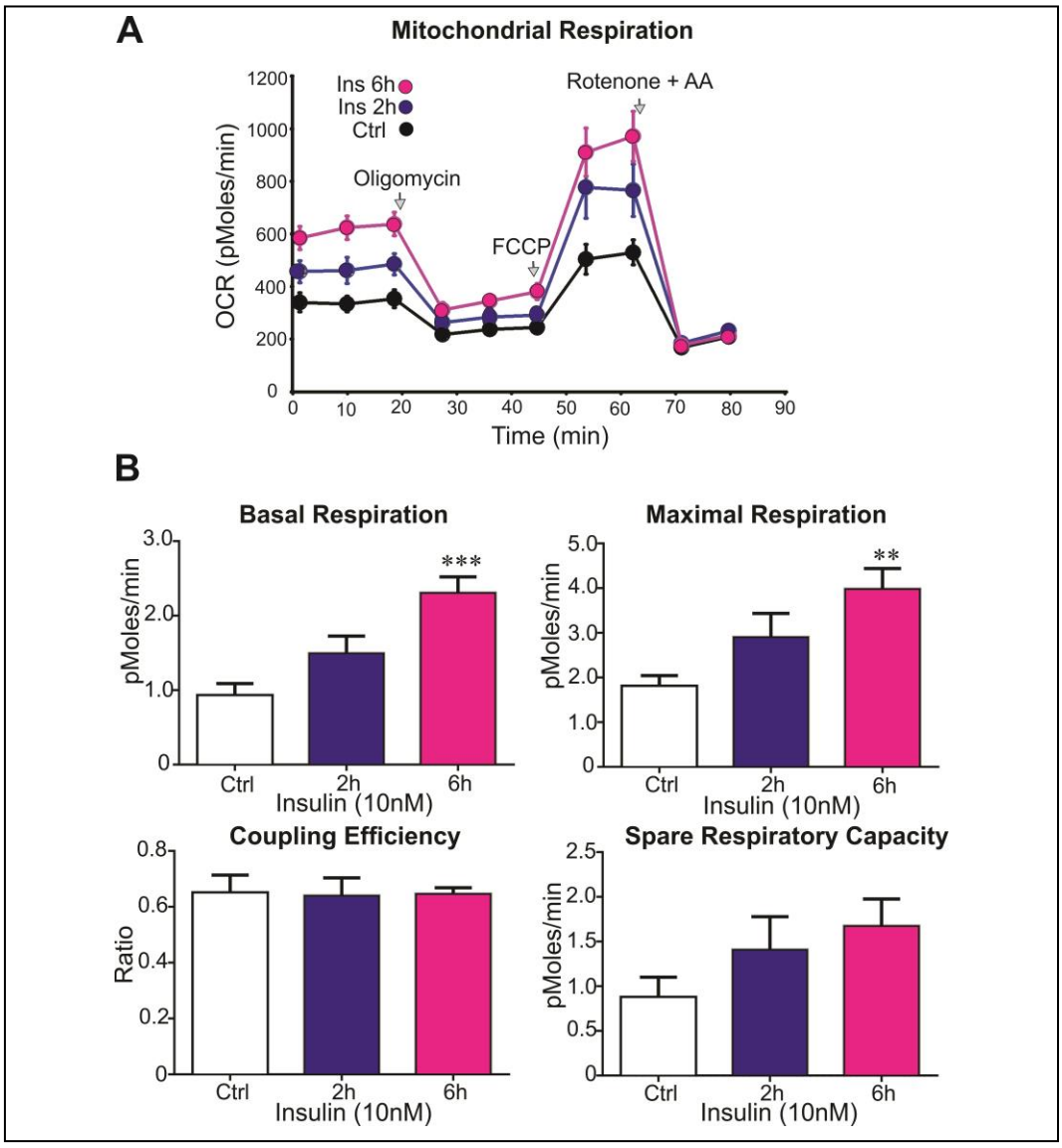

Figure 2.5. Insulin enhances mitochondrial respiration in cultured DRG neurons from a diabetic rat. DRG neurons derived from adult STZ-diabetic rats were cultured in the presence of 25mM glucose, and treated with/without 10 nM insulin for 2 and 6 h. Data were normalized to cell number using Seahorse software prior to statistical analysis. Data are presented as OCR in pmole/min per 5000 cells. Data are mean ± SEM of N=5 replicates; **= $p<0.01$ or ***= $p<0.001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

Insulin therapy prevents thermal hypoalgesia and partially corrects dermal nerve loss in diabetic rats

Type 1 diabetic rats showed significant hyperglycemia, elevated HbA1c and reduced weight gain after 5 months when compared with their age-matched controls (Table 2.1). Insulin treatment of diabetic rats significantly improved body weight ($P<0.001$ vs untreated diabetic) but did not alter terminal blood glucose or HbA1c levels (Table 2.1). STZ-diabetic rats exhibited marked thermal hypoalgesia that was prevented by insulin (Figure 2.6A). The footpads from untreated diabetic rats exhibited significant ($P<0.01$) loss of IENF and SNP profiles (Figure 2.6B, C). Insulin partially attenuated nerve loss, most notably in the SNP, where values did not differ significantly from age-matched control animals.

Table 2.1. Insulin-treated diabetic rats exhibited no change in their blood glucose and HbA1c compared to untreated diabetic rats. Animals were maintained for 5 months. Blood glucose, HbA1C and body weight were recorded at tissue collection. Data are mean ± SEM of N=6; ¶= $p<0.001$ vs. control and *= $p<0.0001$ vs. other groups by one-way ANOVA with Tukey's *post-hoc* test.

Group	**N**	**Body weight (g)**	**Blood glucose (mmol/L)**	**HbA1c (%)**
Control	**6**	725.6 ± 21.0*	8.1 ± 0.6	5.1 ± 0.1
Diabetic	**6**	395.2 ± 22.0	38.9 ± 2.1*	11.3 ± 0.7*
Insulin-implanted diabetic	**6**	589.6 ± 14.3*¶	35.5 ± 1.7*	12.7 ± 0.3*

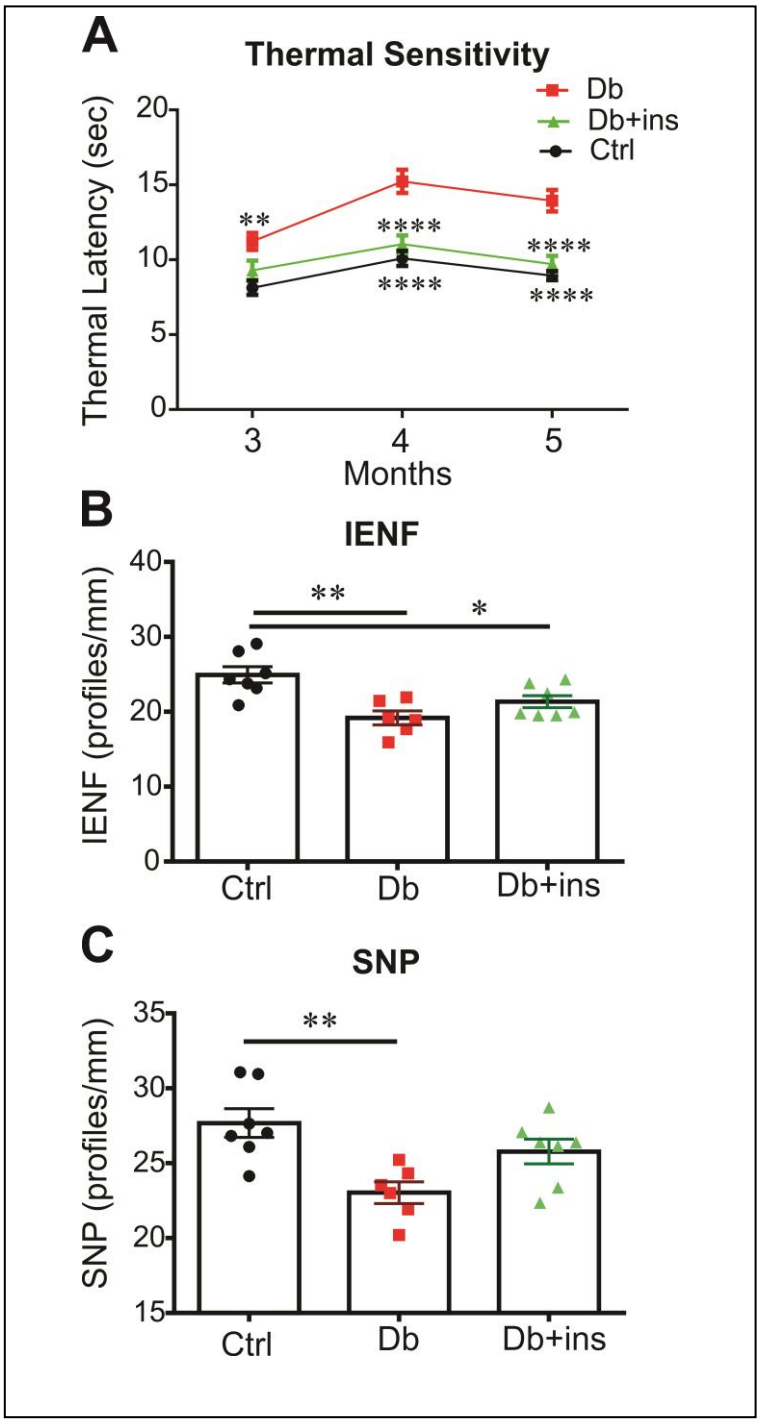

Figure 2.6. Insulin implants prevented thermal hypoalgesia and sub-epidermal neural plexus (SNP) loss in diabetic rats. Three groups of animals**:** control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were tested for their thermal response latency (A). **= $p<0.01$ vs. control and ****= $p<0.0001$ vs. diabetic; analyzed by two-way ANOVA with Tukey's *post-hoc* test. IENF (B) and SNP (C) profiles in plantar hind paw skin (from the three groups of animals which were maintained for 5 months) were also measured; *= $p<0.05$ or **=$p<0.01$ or ****= $p<0.0001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test. Data are mean ± SEM of N=6-7 animals.

Decreased mitochondrial respiratory protein expression and activity in DRG of diabetic animals is corrected by insulin

DRG derived from age-matched control, diabetic, and insulin-implanted diabetic rats were homogenized and subjected to mRNA and protein expression analysis. Western blotting showed a significant decreased expression in subunits of mitochondrial Complexes II, IV and V proteins in DRG of diabetic rats compared with age matched controls (Figure 2.7A, B). In insulin-implanted animals, levels of Complex IV and V proteins in DRG were raised compared with untreated diabetic and were not significantly different from age-matched control - except for Complex II protein where the increase was less dramatic (Figure 2.7A, B). Similar findings were seen when the data was normalized to the mitochondrial protein, porin (Figure 2.10.Supplemental Figure). Insulin also significantly up-regulated mRNA for the MTCO1 subunit of Complex IV compared to both control ($P<0.001$) and diabetic ($P<0.01$) rats (Figure 2.8A, Figure 2.11.Supplemental Figure). Complex II-SDHB mRNA levels were not different between groups. Enzymatic activity of cytochrome *c* oxidase was significantly ($P<0.001$) reduced in the DRG of diabetic animals relative to controls and this decrease was partially prevented by insulin treatment (Figure 2.8B and Figure 2.12.Supplemental Figure).

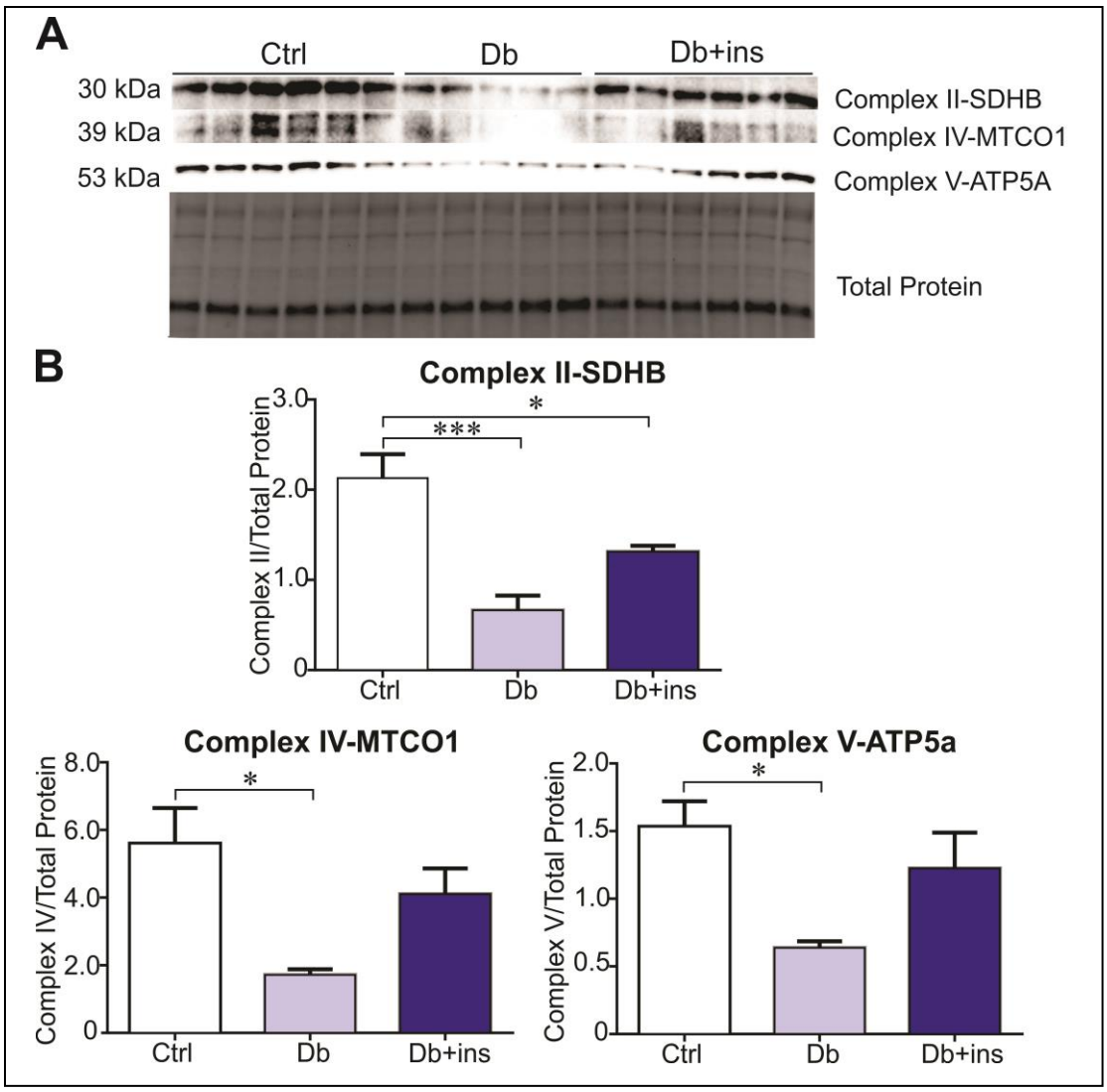

Figure 2.7. Insulin corrects deficits in mitochondrial protein expression in DRG of diabetic animals. DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were isolated and subjected to Western blotting. Complex I and III subunit proteins were not detectable. Western blot band intensity of these proteins was normalized to total protein bands of the same blot. Data are mean ± SEM of N=5-6 animals; *= $p<0.05$ or ***= $p<0.001$ analyzed by one-way ANOVA with Tukey's *post-hoc* test.

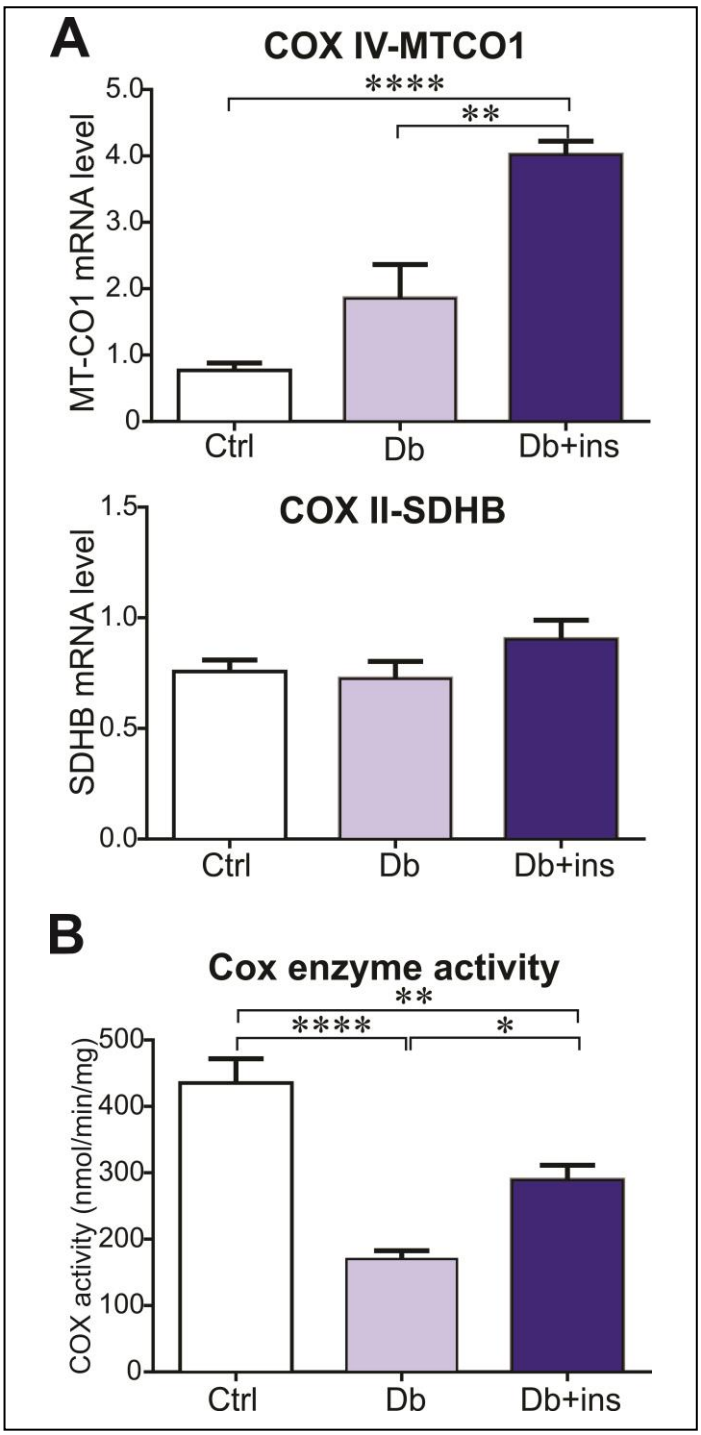

Figure 2.8. Increased MT-CO1 mRNA expression and restored cytochrome c oxidase (Cox) activity in insulin implanted diabetic rats. A) DRG tissues from control (Ctrl), diabetic (Db) and insulin implanted diabetic (Db+ins) rats were used for Real-Time PCR assay. Complex II-SDHB and Complex IV-MT-CO1 mRNA levels were calculated relative to porin mRNA levels using delta dela Ct method. Data are mean ± SEM of N=5-6 animals (duplicate test tubes). B)Tissues were used for cytochrome *c* oxidase enzymatic activity assay. Data are in nmol/mim/mg tissue and are mean ± SEM of N=5 animals. *= $p<0.05$, **= $p<0.01$ and ****= $p<0.0001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

Discussion

Insulin signaling was identified in sensory neurons of adult rat by detection of Akt and P70S6K activation, confirming earlier work (Grote et al., 2013b, Huang et al., 2005). We used a physiologically relevant concentration (10 nM) of insulin in all cell culture experiments to minimize confounding effects deriving from potential cross-occupation of insulin-like growth factor receptors by excess insulin (Recio-Pinto and Ishii, 1988, Benyoucef et al., 2007, Kleinridders, 2016). Other reported consequences of insulin stimulation of sensory neurons include increased transcription and synthesis of cytoskeletal proteins, such as tubulin and neurofilament (Fernyhough et al., 1989, Wang et al., 1992) that are essential for axonal growth, regeneration and structural stability. Demonstration that exogenous insulin also dose-dependently enhanced neurite outgrowth of sensory neurons is consistent with prior studies (Fernyhough et al., 1993, Recio-Pinto et al., 1986) and highlights the direct neurotrophic properties of insulin in sensory neurons.

Studies in liver, muscle and cardiac cells have suggested that insulin can modulate mitochondrial phenotype. For example, *in vitro* studies on the liver of alloxan-induced diabetic rats and de-pancreatized cats revealed depressed mitochondrial oxygen consumption and ATP production that was restored by addition of insulin (Hall et al., 1960). More recently, a 20min exposure of skeletal muscle myotubes to 10 and 100 nM insulin increased respiratory control ratio and coupling efficiency of oxidative phosphorylation (Nisr and Affourtit, 2014). The effects of insulin are also apparent *in vivo*. Acute infusion of insulin in healthy humans resulted in higher ATP production, increased mitochondrial proteins including MT-CO1 and NADH dehydrogenase subunit IV at mRNA and protein levels, and elevated citrate synthase and COX enzymatic activities as measured in biopsies of skeletal muscle cells (Stump et al., 2003).

Conversely, knockout of insulin receptor or PI3-K signaling in mouse myocardial tissue triggered maladaptive responses in mitochondria including reduced expression of genes and proteins related to oxidative phosphorylation (Boudina et al., 2009, O'Neill et al., 2007). We have now extended these studies to adult sensory neurons by demonstrating that a physiologic concentration of insulin raised the expression of mitochondrial electron transport chain proteins and enhanced respiration *in vitro*. Moreover, exogenous insulin had similar effects on sensory neurons derived from diabetic rats and maintained under hyperglycemic conditions, although the time course of OCR enhancement was delayed. This delay could perhaps reflect the inhibitory effect of hyperglycemia, as we have previously shown that diabetes, via hyperglycemia mediated nutrient excess, down-regulates expression of mitochondrial proteins and respiration (Chowdhury et al., 2011). Insulin appears to circumvent the effects of hyperglycemia without lowering glucose levels *per se*.

It is well established that insulin triggers transcription and translation within cells via both the PI3-K/Akt and MAPK/ERK pathways (Cheng et al., 2010). AMPK is a sensor of energy status that augments mitochondrial function and adaptability (Hardie et al., 2012). Insulin signaling via Akt down-regulates this activity in cardiac tissue (Gamble and Lopaschuk, 1997), in part, through phosphorylation on the ST loop of AMPKα at Ser 485/491 (in rats) (Hawley et al., 2014, Valentine et al., 2014). Insulin interaction with the AMPK pathway is multifaceted (Towler and Hardie, 2007) and adipocytes maintained under conditions of ongoing fatty acid metabolism, insulin can activate AMPK over a period of 40 min via elevation in the AMP/ATP ratio (Hebbachi and Saggerson, 2012, Liu et al., 2010a). In hippocampal neurons, compounds that activate the insulin receptor also mobilize the AMPK-SIRT signaling axis with associated enhancement of mitochondrial function (Barhwal et al., 2015). Insulin can also boost

intracellular Ca^{2+} ion concentration (Contreras-Ferrat et al., 2014) which could trigger activation of an upstream activator of AMPK, namely Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Hawley et al., 2005, Hurley et al., 2005, Woods et al., 2005). Thus, insulin signaling and coordinated activation of AMPK could augment mitochondrial function in sensory neurons. Insulin signaling via the Akt/IKK/NF-□B (Saleh et al., 2013b, Mauro et al., 2011, Salminen and Kaarniranta, 2010) and the PI3-K pathway (by-passing Akt involvement) (O'Neill et al., 2007) could also regulate mitochondrial function. The putative involvement of all these pathways requires further study.

We have previously reported that by 22 weeks of type 1 diabetes, rat sensory neurons showed impaired mitochondrial respiration, reduced mitochondrial Complexes I and IV enzymatic activities and reductions in expression of selective mitochondrial respiratory chain proteins compared with controls (Chowdhury et al., 2010). Insulin therapy in a subgroup of STZ-diabetic rats for the final 4 weeks improved mitochondrial respiration but also that corrected hyperglycemia so that it was not feasible to determine whether the effect was due to insulin per se or secondary to glycemic control. Our present findings show that protein expression of subunits of mitochondrial Complexes II, IV and V and activity of Complex IV were recovered in insulin-implanted diabetic animals without correcting the hyperglycemic state. This supports actions of insulin that are not mediated by glycemic control.

Down-regulation of specific mitochondrial OXPHOS proteins and Complex activities in diabetic conditions has been demonstrated by other investigators. Skeletal muscle biopsies from type 2 diabetic patients showed a significant reduction in mRNA levels of multiple subunits of Complexes I, II, III, IV and V proteins (Patti et al., 2003). Protein levels of OXPHOS subunits (I, II, III, and IV) were significantly repressed in cardiac mitochondria of $Ins2^{+/-}$Akita mice

(C57BL/6) compared to control mice (Bugger et al., 2009). In kidney cortex in mouse models of type 1 and type 2 diabetes components of Complexes I-IV were suppressed as well as activity of Complex IV (Dugan et al., 2013, Sas et al., 2016). Reduced expression of NADH dehydrogenase Fe-S protein 3 (Complex I-NDUFS3) and MT-CO1 (Complex IV) together with the enzymatic activities of NADH-cytochrome c reductase (Complex I) and cytochrome c oxidase (Complex IV) in STZ-diabetic rodent DRGs vs. control has been reported in our previous studies (Akude et al., 2011, Chowdhury et al., 2010, Roy Chowdhury et al., 2012). In the current study we had significant down-regulation of Complex IV subunits and activity. It can be inferred from all these studies that mitochondrial OXPHOS protein expression, especially within Complex IV and its activity, is depressed under diabetic conditions. Finally, to illustrate the complexity of interactions in diabetes in the nervous system proteomic studies of distal sciatic nerve of type 1 diabetic rats reveal elevations in specific components of the mitochondrial electron transport chain (Freeman et al., 2016).

Studies with a variety of neurotrophic growth factors reveal that mitochondrial function is under the control of an array of signal transduction pathways. For example, in adult sensory neurons IL-1β, IL-17A and ciliary neurotrophic factor all enhance neurite outgrowth in hand with augmentation of mitochondrial function (Habash et al., 2015, Saleh et al., 2013a, Saleh et al., 2013b). These growth factors utilize multiple signal transduction pathways, include NF-kB and JAK/STAT, to directly stimulate mitochondrial function. NT3 most likely signals through Akt to modulate mitochondrial polarization in sensory neurons from control or STZ-diabetic rats (Huang et al., 2005). Insulin and IGF-1 share very similar transduction pathways, PI3K/Akt and MAPK, which could lead to protein synthesis and transcription initiation, respectively. IGF-1 possibly by activating these two main transduction pathways promotes growth and impedes

mitochondrial aberration and cell death mediated by caspase activation in SH-SY5Y cell cultures exposed to high glucose concentration (Leinninger et al., 2004). For the first time insulin can be added to this group of neurotrophic proteins. Insulin has previously been demonstrated to modulate mitochondrial membrane potential (Huang et al., 2003, Huang et al., 2005) and the current study reveals that insulin has the potential to control mitochondrial function via up-regulation of mitochondrial electron transport system protein expression and Complex activity. It is possible that insulin is optimizing responses to neurotrophic factors. For example, insulin was shown to up-regulate high affinity NGF binding in the human neuroblastoma SH-SY5Y neuronal cell line (Recio-Pinto et al., 1984). All experiments in the present were performed in the absence of exogenous neurotrophic factors (no exogenous growth factors were added to the culture) so this eventuality seems less likely. However, we cannot exclude the possibility that the cultured neurons may be secreting neurotrophins such as BDNF (Tonra et al., 1998, Acheson et al., 1995) whose function may be augmented by insulin via enhanced $p75^{NTR}$ and/or trkB interaction. Further studies will dissect the potential for direct vs indirect actions of insulin on mitochondrial function.

Loss of background levels of systemic insulin and insulin-like growth factors has long been suspected of contributing to the development of diabetic neuropathy (Ishii, 1995) and recent studies have reported that insulin can activate several signal transduction pathways to enhance nerve repair in diabetes (Grote et al., 2013b, Zochodne, 2016a). The present study supports this concept and demonstrates that insulin modulates mitochondrial function and thus may contribute to nerve repair. As all in vitro studies were performed with 10 nM insulin, we are confident that signal transduction via the insulin receptor accounts for the positive effects on mitochondrial function. Interpretation of the in vivo data is not so straightforward. Given that insulin can cross-

occupy IGF type 1 receptors at concentrations above 30 nM (Recio-Pinto and Ishii, 1988) it cannot yet be discounted that some of the in vivo efficacy of insulin is a consequence of stimulation of IGF receptor signaling. Ongoing studies are addressing this issue (manuscript in preparation). Future work will attempt to identify the signal transduction pathway activated by IGF-1 to modulate mitochondrial function, for example Akt vs AMPK. It will be interesting to see if this pathway is shared with NT-3, cytokines or IGF-1 and a major goal will be to characterize key components of the mitochondrial respiratory chain, e.g. proteins such as MTCO1 within Complex IV, and sites of regulation that may be commonly modulated by these pathways.

Acknowledgements

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Author contributions:

MRA performed the majority of work including primary neuron cell culture, Western blotting, real-Time PCR and immunocytochemistry. DRS and NAC maintained, treated, and performed behavioral, electrophysiological and biochemical assays on groups of rats. MRA, SKR and MGS performed Seahorse analysis of mitochondrial function and cell counting. MRA and SKR carried out the mitochondrial enzymatic activities. MRA, NAC and DRS analyzed data. DRS and MRA designed experiments. MRA contributed to write the first draft of paper. PF designed experiments, analyzed data and wrote and edited the paper.

Supplementary Materials:

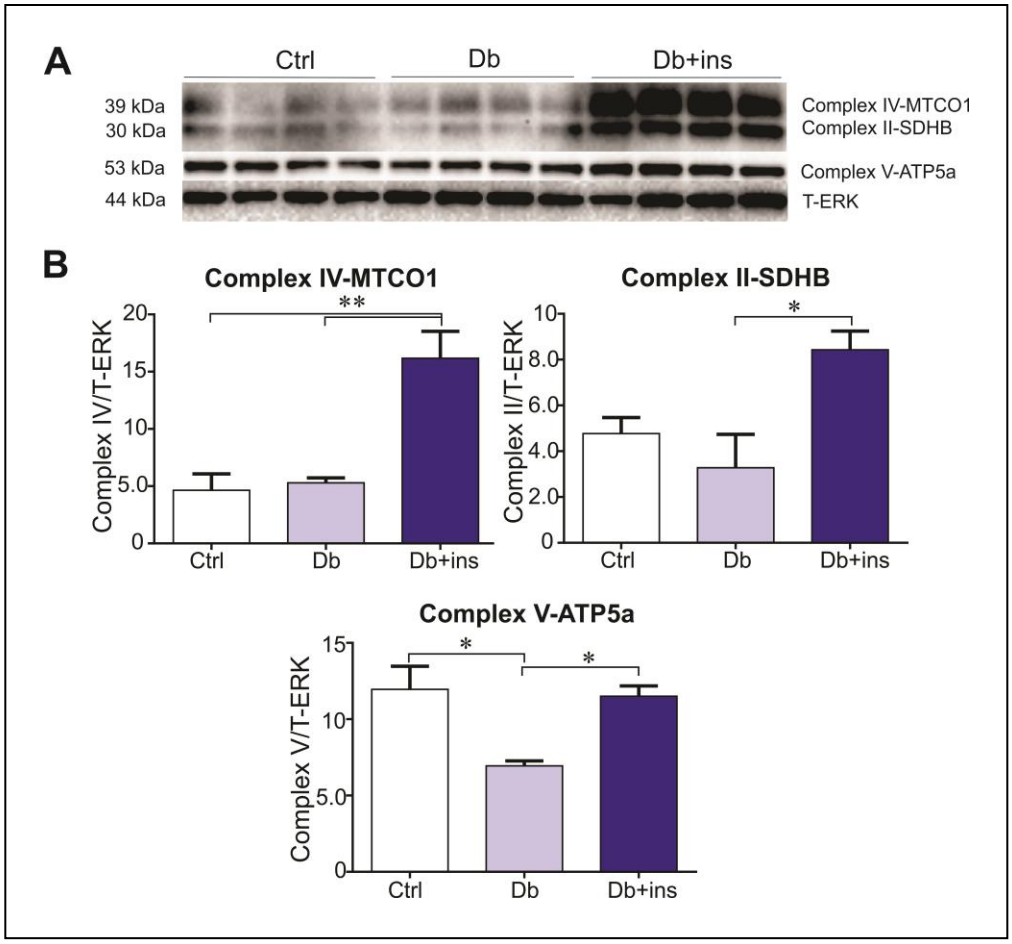

Figure 2.9. Supplemental Figure. Insulin treatment of DRG cultures from diabetic rats elevates mitochondrial electron transport chain proteins above baseline controls levels. DRG neurons derived from age-matched control (Ctrl) and STZ-diabetic rats (Db) were cultured in the presence of 5mM and 25mM glucose, respectively. DRG neurons from STZ-diabetic were treated with/without 10 nM insulin for 24h. Complex I and III subunit proteins were not detectable. Protein band intensity was normalized to T-ERK. This figure illustrates that insulin elevates the expression of subunits of Complexes II, IV and V compared to non-treated diabetic and the control group. There was no significant difference in protein levels for subunits of

Complexes II (SDHB) and IV (MT-CO1) in untreated diabetic vs. control group. This was different than observed in *in vivo* data where there were deficits in diabetic samples. This may be due to an axotomy effect on primary adult DRG neurons under culture conditions. In response to axonal loss the diabetic cultures may over-produce components of electron transport chain to drive neurite outgrowth. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

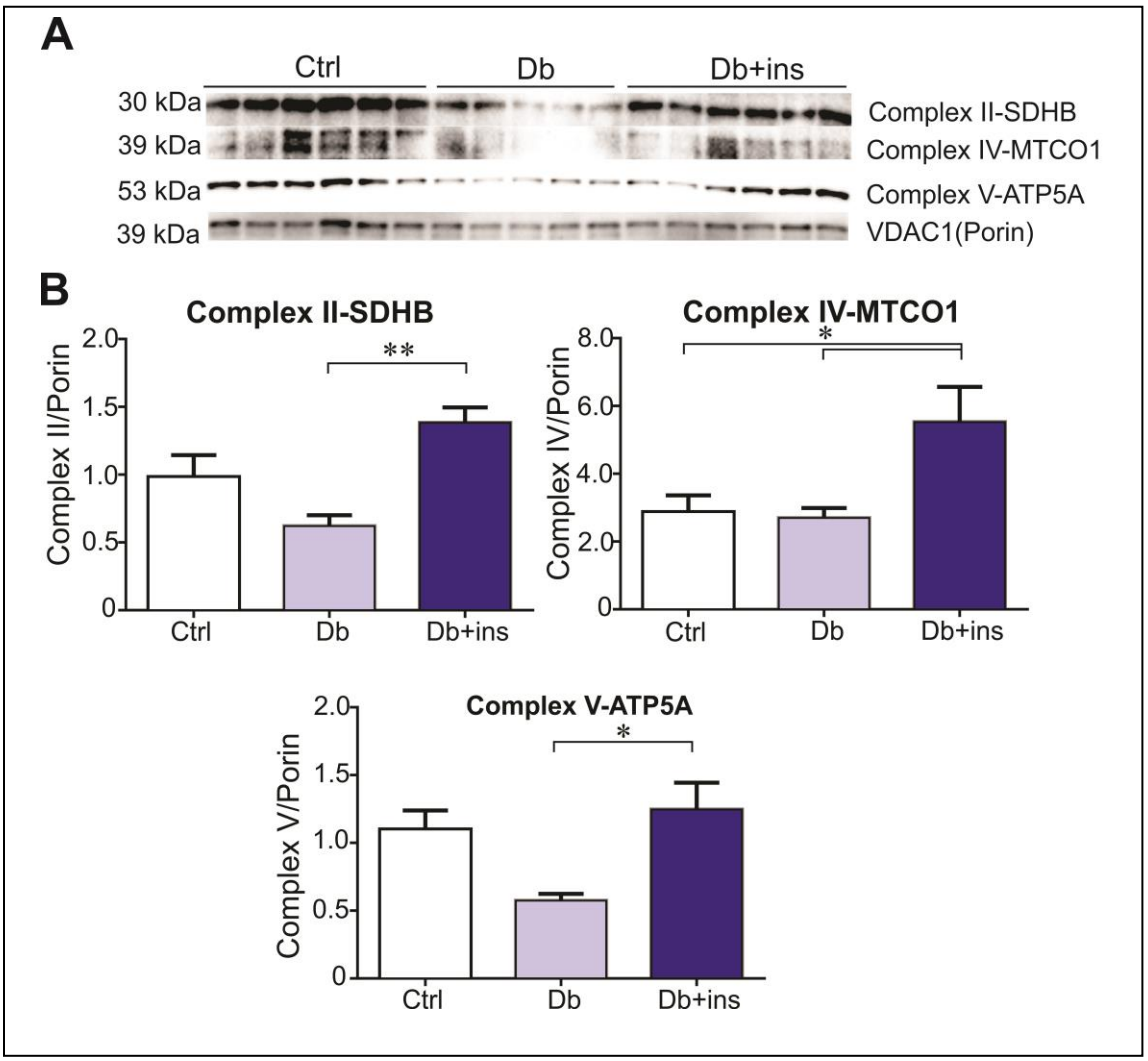

Figure 2.10. Supplemental Figure. Correction of mitochondrial protein expression in DRG of insulin-implanted diabetic animals (normalized to porin). DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were isolated and subjected to Western blotting. Complex I and III subunit proteins were not detectable. Western blot band intensity of proteins was normalized to porin bands on the same blot. Data are mean ± SEM of N=5-6 animals; *= $p<0.05$ or **= $p<0.01$ analyzed by one-way ANOVA with Tukey's *post-hoc* test.

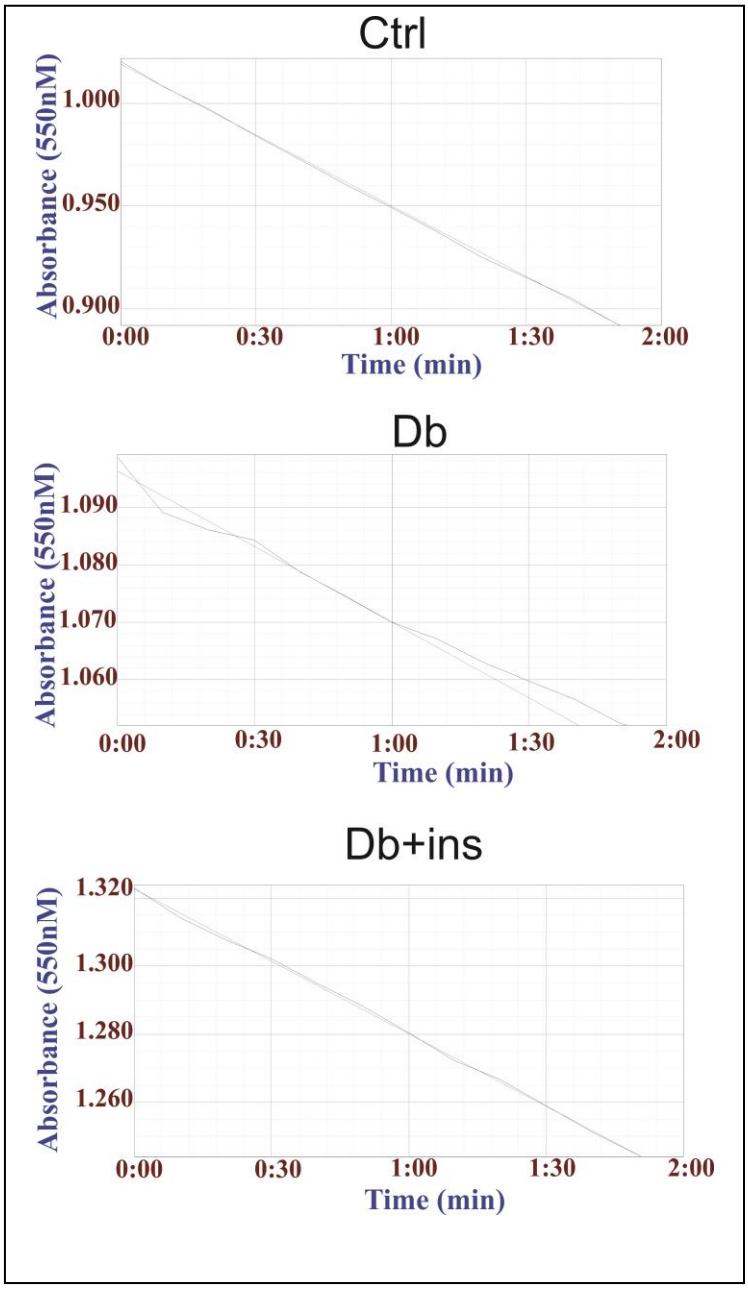

Figure 2.11. Supplemental Figure. RT-PCR dissociation curves for control samples in Figure 2.8A. DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were derived and used for Real-Time PCR assay. Complex II-SDHB, Complex IV-MT-CO1 and porin amplified PCR products were dissociated by gradual denaturation starting at 55°C to 95°C and recorded. The dissociation curves from just the control animals are shown. The different colored curves are each derived from one sample.

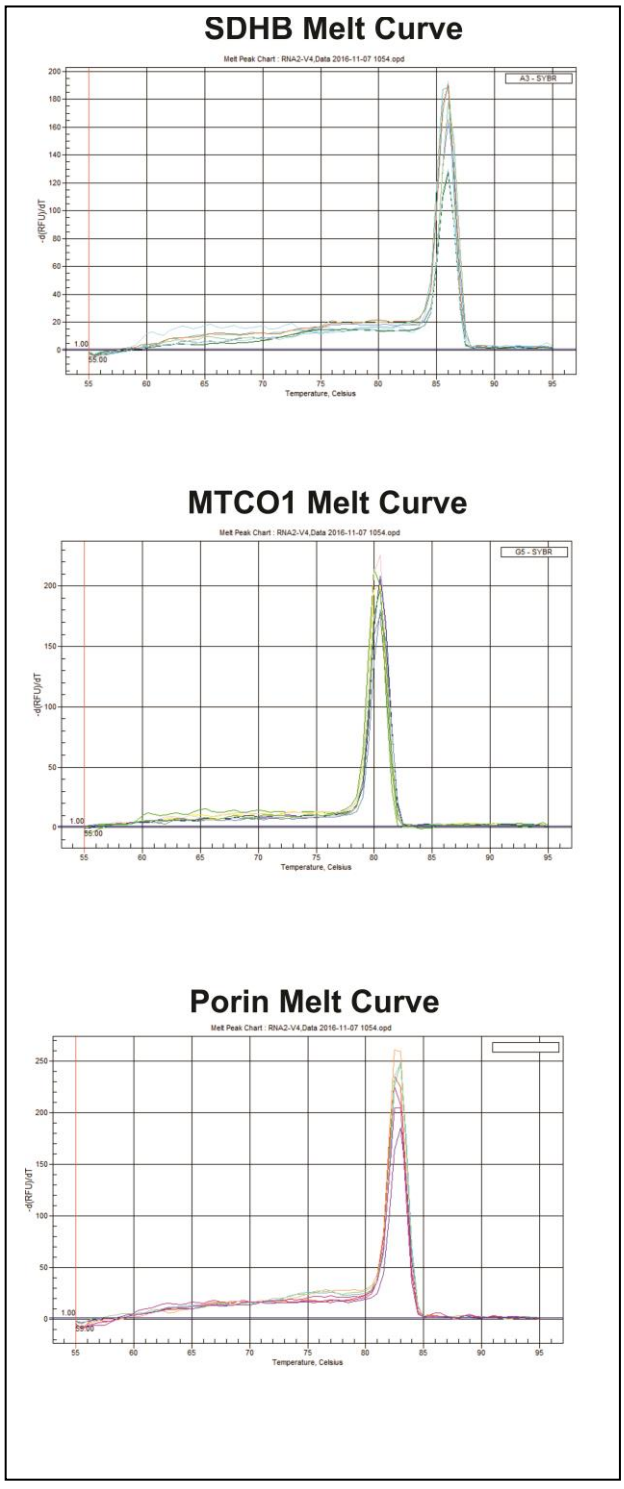

Figure 2.12. Supplemental Figure. Complex activity absorbance curves for Figure 2.8B. DRG tissues from control (Ctrl), diabetic (Db) and insulin-implanted diabetic (Db+ins) rats were isolated and used for cytochrome c oxidase enzymatic activity assay. The rate of cytochrome c oxidation in 10 µg DRG homogenates was measured by its absorbance decrease at 550 nm for 2 min.

Transition Statement One

Aims 1 and 2 of the part 1 (chapter 2) of this thesis were to determine the effect of insulin as a therapy on mitochondrial function and nerve degeneration in sensory neurons in diabetes. Firstly, we showed that insulin signaling exists in sensory neurons of the adult rat by detection of Akt and P70S6K activation. Consistent with previous studies, insulin enhanced neurite outgrowth in cultured DRG neurons from rats. We also demonstrated that a physiologically relevant concentration (10 nM) of insulin raised the expression of mitochondrial electron transport system proteins and enhanced respiration in DRG tissue from both control and diabetic rats. Insulin implants in STZ-diabetic rats improved thermal hypoalgesia and IENF profile. Insulin therapy in a subgroup of STZ-diabetic rats also recovered the protein expression of subunits of mitochondrial Complexes II, IV and V and activity of Complex IV. This part of the study supports this concept and demonstrates that insulin modulates mitochondrial function and thus may contribute to nerve repair. However, the mechanism through which insulin implements its role as a neuroprotective and neurotrophic factor was not determined. For this purpose, we studied its analog, IGF-1, to investigate the mechanism through which mitochondrial function and nerve repair are regulated by these neurotrophic factors. Sensory neurons respond to IGF-1 via IGF-1 receptors they express. IGF-1 is more potent than insulin and can be a therapeutic option in type 1 and 2 diabetes where there is insulin resistance. In three aims of part 2 (chapter 3) of this thesis, IGF-1 effect on AMPK activity, mitochondrial function and nerve structure and function is investigated in cultured sensory neurons and in animal model of diabetic neuropathy.

Chapter 3: Insulin-like growth factor-1 activates AMPK to augment mitochondrial function and correct neuronal metabolism in sensory neurons in type 1 diabetes

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Abstract

Objective: Diabetic sensorimotor polyneuropathy (DSPN) affects approximately half of diabetic patients leading to significant morbidity. There is impaired neurotrophic growth factor signaling, AMP-activated protein kinase (AMPK) activity and mitochondrial function in dorsal root ganglia (DRG) of animal models of type 1 and type 2 diabetes. We hypothesized that sub-optimal insulin-like growth factor 1 (IGF-1) signaling in diabetes drives loss of AMPK activity and mitochondrial function, both contributing to development of DSPN. **Methods:** Age-matched control Sprague-Dawley rats and streptozotocin (STZ)-induced type 1 diabetic rats with/without IGF-1 therapy were used for *in vivo* studies. For *in vitro* studies, DRG neurons from control and STZ-diabetic rats were cultured and treated with/without IGF-1 in the presence or absence of inhibitors or siRNAs. **Results:** Dysregulation of mRNAs for IGF-1, AMPKα2, ATP5a1 (subunit of ATPase) and PGC-1β occurred in DRG of diabetic vs. control rats. IGF-1 up-regulated mRNA levels of these genes in cultured DRGs from control or diabetic rats. IGF-1 treatment of DRG cultures significantly ($P<0.05$) increased phosphorylation of Akt, P70S6K, AMPK and acetyl-CoA carboxylase (ACC). Mitochondrial gene expression and oxygen consumption rate (spare respiratory capacity), ATP production, mtDNA/nDNA ratio and neurite outgrowth were augmented ($P<0.05$). AMPK inhibitor, Compound C, or AMPKα1-specific siRNA suppressed IGF-1 elevation of mitochondrial function, mtDNA and neurite outgrowth. Diabetic rats treated with IGF-1 exhibited reversal of thermal hypoalgesia and in a separate study reversed the deficit in corneal nerve profiles. In diabetic rats, IGF-1 elevated the levels of AMPK and P70S6K phosphorylation, raised Complex IV-MTCO1 and Complex V-ATP5a protein expression, and restored the enzyme activities of Complex IV and I in the DRG. IGF-1 prevented TCA metabolite build-up in nerve. **Conclusions:** In DRG neuron cultures IGF-1 signals via AMPK to

elevate mitochondrial function and drive axonal outgrowth. We propose that this signaling axis mediates IGF-1-dependent protection from distal dying-back of fibers in diabetic neuropathy.

Key words: IGF-1; AMPK; axon regeneration; diabetic neuropathy; oxygen consumption rate

Introduction

Diabetic sensorimotor polyneuropathy (DSPN) frequently presents with a stocking and glove distribution that is proposed to reflect the dying back of the longest peripheral nerve fibers. Incidence can range from 10% to 90% in diabetic patients, depending on the criteria and methods used to define neuropathy (Vinik et al., 2016). In humans and animal models of type 1 and type 2 diabetes, the level of available insulin-like growth factor-1 (IGF-1) in serum is substantially decreased, primarily a consequence of suppressed expression in the liver (Zhuang et al., 1997, Palta et al., 2014, Ishii et al., 1994, Ekstrom et al., 1989). Thus, impaired neurotrophic support by insulin signaling and insulin-like growth factors (IGF-1 and IGF-2) have been proposed to contribute to neurodegeneration in diabetes (Ishii, 1995, Rauskolb et al., 2017, Zochodne, 2016b). In addition to a critical role for IGF-1 during nervous system development and early postnatal growth (Zackenfels et al., 1995), IGF-1 promotes neurite outgrowth in sensory (Fernyhough et al., 1993, Recio-Pinto et al., 1986), motor (Caroni et al., 1994) and sympathetic (Zackenfels et al., 1995, Recio-Pinto et al., 1986) neurons. Further, Schwann cells also require IGF-1 and IGF type 1 receptor signaling for survival, motility, cell proliferation and phenotypic remodeling and myelination (Syroid et al., 1999, Chattopadhyay and Shubayev, 2009, Russell et al., 2000, Cheng et al., 2000).

Following peripheral nerve injury, the local or systemic delivery of IGF-1 improves the rate of sciatic nerve regeneration in age matched control or streptozotocin (STZ)-induced type 1 diabetic rats (Ishii and Lupien, 1995, Ekstrom et al., 1989, Sjoberg and Kanje, 1989). Hyperalgesia was also prevented/reversed in STZ-induced diabetic rats treated with IGF-1 (Zhuang et al., 1996). Lumbar intrathecal injection of IGF-1 reversed indices of neuropathy including the deficit in intra-epidermal nerve fiber (IENF) density, sural nerve axonal

degeneration, and reduced sensory and motor nerve conduction velocities in STZ-induced type 1 diabetic rats (Toth et al., 2006, Brussee et al., 2004). Adenovirus-mediated IGF-1 expression via an intrathecal route or through the liver improved nerve regeneration, myelination, and motor and sensory nerve conduction velocities in mouse models of diabetic neuropathy (Homs et al., 2014, Chu et al., 2008). Finally, IGF binding protein 5, an endogenous inhibitor of IGF-1 action, is up-regulated in sural nerve biopsies from persons with diabetic neuropathy, and its over-expression in transgenic mice induced motor and sensory neuropathy (Simon et al., 2015). Thus, there is extensive evidence of the therapeutic potential of IGF-1 in animal models of diabetes. However, less is known about the cellular mechanisms by which IGF promotes neuroprotection in diabetes.

IGF type 1 receptor mobilizes two widely known pathways, the Akt/phosphoinositide-3 kinase (PI-3K) and the mitogen-activated protein (MAP) kinase pathways, mediated by insulin receptor substrate (IRS) 1 and IRS 2 phosphorylation following ligand binding (Le Roith and Zick, 2001, Cheng et al., 2010). The Akt/PI-3K pathway activates the mammalian target of rapamycin (mTOR) pathway which directs protein synthesis and cell growth via downstream effectors, P70S6K and 4E-binding protein 1(4E-BP1) (Cheng et al., 2010). The MAPK pathway incorporates activation of extracellular signal-regulated kinase (ERK)-1/2 and target transcription factors such as Elk-1 to regulate cell survival (Le Roith and Zick, 2001). IGF-1 also activates AMP-activated protein kinase (AMPK) during osteoblast differentiation (Xi et al., 2016). The AMPK-α subunit is phosphorylated and activated by IGF-1 in an ataxia telangiectasia mutated (ATM)-dependent manner in a human pancreatic cancer cell line (Suzuki et al., 2004a). Alternatively, IGF-1 suppressed AMPK activity in vascular smooth muscle cells mediated through Akt1 which phosphorylated an inhibitory site on AMPK at S485 (Ning et al., 2011).

Any or all of these pathways could be pertinent to the neuroprotective actions of IGF-1 against diabetic neuropathy.

It may be particularly pertinent that IGF-1 activates AMPK as the AMPK/peroxisome proliferator-activated receptor γ co-activator 1-α (AMPK/PGC-1α) energy sensing pathway augments mitochondrial function in a range of cell types (Canto and Auwerx, 2009). A well-characterized upstream activator of AMPK is Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKK β) (Green et al., 2011). A small range of studies have demonstrated that IGF-1 can regulate cellular metabolism and bioenergetics in neurons and astrocytes and protect against Huntington's disease (Logan et al., 2018, Naia et al., 2016, Gazit et al., 2016, Ribeiro et al., 2014). In human tissues derived from persons with diabetes there is down-regulation of the AMPK/PGC-1 α pathway (Mootha et al., 2003, Patti et al., 2003). In animal models of DSPN, the levels of expression and activity of AMPK and PGC-1α are also significantly depressed in the dorsal root ganglia (DRG). Under hyperglycemic conditions it has been proposed that nutrient stress triggers this down-regulation of AMPK (Fernyhough, 2015). However, the mechanistic interactions between IGF-1, AMPK and mitochondrial function are poorly defined and the contribution of impaired IGF-1 signaling and associated pathways to the pathogenesis of DSPN remain to be characterized. We therefore investigated whether exogenous IGF-1 could optimize AMPK activity and mitochondrial function to promote axonal repair in DRG neurons in type 1 diabetes. We used STZ-induced type 1 diabetic rats/mice which represent the most commonly utilized animal models to study diabetic neuropathy and exhibit many clinically relevant features.

Materials and methods

Induction of type 1 diabetes

As mentioned in chapter 1, we used type 1 diabetic rats because of low cost of maintenance and ease of use while reflecting most of neuropathy indices seen in human. To be consistent throughout our study, we focused on one gender (male) in the present study. Male Sprague-Dawley rats obtained in-house were maintained 2 per cage on Sani-Chips bedding (P.J. Murphey, Montville, NJ, USA) in a Canadian Council of Animal Care (CCAC)-accredited vivarium under a 12hr light:dark cycle with free access to diet (5001, LabDiet with fat content of not less than 4.5%, MO, USA) and municipal water. Rats were obtained from our facility at a weight of 201-225g. A randomly selected cohort of rats (275-325g) were made diabetic (non-fasting blood glucose > 19 mmol/l) by a single 90mg/kg i.p. injection of STZ (Sigma, St. Louis, MO, USA). A randomly selected cohort (N=12) of 3-month diabetic rats received thrice weekly subcutaneous injections of 20 µg IGF-1 (recombinant human, Preprotech Inc., Rocky Hill, NJ, USA) per rat (at home cage) between 9-11AM as previously described (Lupien et al., 2003) for three months. Fasting blood glucose concentration was monitored half-way through the injection period and at study end using an AlphaTRAK glucometer (Abbott Laboratories, Illinois, USA) to ensure that IGF-1 injection did not affect hyperglycemia. At the end of 24 weeks, blood glucose, glycated hemoglobin (HbA1c Multi-test system, HealthCheck Systems, Brooklyn, NY, USA) and body weight were recorded before tissue collection (Table 1). No rats died during the study period and at study end all STZ-injected rats remained hyperglycemic (non-fasting blood glucose > 19 mmol/l). Animal procedures were approved by the University of Manitoba Animal Care Committee and followed CCAC rules.

Hind paw thermal sensitivity test in adult rats

Hind paw thermal response latencies were measured using a Hargreaves apparatus (UARD, La Jolla, CA, USA) as previously described *(Jolivalt et al., 2016).* Briefly, between 9-3PM rats were placed in plexiglass cubicles on top of the thermal testing system. The heat source was placed below the middle of one of the hind paws and latencies of the paw withdrawal to the heat source were automatically measured. Response latency of each paw was measured three times at 5 min intervals and the mean values were determined.

Intraepidermal nerve fiber density in hind paw footpads

The plantar dermis and epidermis of the hind paw were removed and placed in 4% paraformaldehyde. Tissue was coded, processed to paraffin blocks, cut as 6µm sections, immunostained using an antibody to PGP 9.5 (1:1000, Biogenesis Ltd. Poole, UK) and the number of immunoreactive intra-epidermal nerve fibers (IENF) and sub-epidermal nerve profiles (SNP) per unit length quantified under light microscopy (Jolivalt et al., 2016).

Corneal nerve density

Adult (20-30g) female Swiss Webster mice (Envigo, CA, USA) were maintained 3-4 per cage on TEK-Fresh bedding (7099, Envigo) in an AALAC-accredited vivarium under a 12hr light:dark cycle with free access to diet (5001, LabDiet, MO, USA) and municipal water. A randomly-selected cohort of mice was made diabetic (non-fasting blood glucose >15 mmol/l) by injection of STZ (75 mg/kg i.p. on 2 consecutive days) following overnight fast. This cohort was maintained untreated for 8 weeks, along with the remaining age- and sex-matched control mice. After 8 weeks of diabetes, the cohort was divided into 2 groups of mice, one of which (N=10) received daily delivery of IGF-1 to one eye by eye-drop (50µl of 25ng/ml solution in 0.9%

saline) while the other diabetic group (N=9) and the control group (N=9) received saline vehicle alone. Treatment was given between 8-10am each day to manually restrained unanaesthetized animals in their home cage and continued for 4 weeks. No mice died during the study period and at study end all STZ-injected mice remained hyperglycemic (non-fasting blood glucose >15 mmol/l). Corneal nerves of the sub-basal nerve plexus were imaged in isofluorane-anesthetized mice at 3 time points: before onset of diabetes, at week 8 of diabetes, and after 4 weeks of treatment using a Heidelberg Retina Tomograph 3 with Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany). Images were collected from randomly selected animals by an investigator unaware of the treatment groups and the image stack from each animal was coded. Nerve occupancy of 5 consecutive images (2 μm intervals) of the cornea between the superficial corneal epithelium and the stroma was calculated using an 8 × 8 grid superimposed on randomized and coded images (Chen et al., 2013), as described in detail elsewhere (Jolivalt et al., 2016). These studies were carried out using protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Adult DRG sensory neuron culture

DRGs were isolated from adult male Sprague-Dawley (300-350g) rats and dissociated using previously described methods (Calcutt et al., 2017). Neurons were cultured in no-glucose Hams F12 media supplemented with Bottenstein's N2 without insulin (0.1 mg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite 0.1 mg/ml BSA; all additives were from Sigma, St Louis, MO, USA; culture medium was from Caisson labs, USA). DRG neurons from control rats were cultured in the presence of 5 mM D-glucose and DRG neurons derived from STZ-induced diabetic rats with 25 mM D-glucose. No neurotrophins or insulin was added to any DRG cultures. The following pharmacological inhibitors were used: Compound C, a

selective and reversible AMPK inhibitor (Abcam, Cambridge, MA, USA), MK-2206, a highly selective pan-Akt inhibitor (Santa Cruz Biotechnology, Texas, USA), U0126, a selective non-competitive inhibitor of MAP kinase kinase (Abcam, Cambridge, MA, USA), and STO-609, a selective CaMKKβ inhibitor (Santa Cruz Biotechnology, Texas, USA).

AMPK isoform-specific knockdown

DRG neurons were dissociated and subjected to AMPK isoform-specific knockdown according to the instruction manual of the Amaxa® Rat Neuron Nucleofector Kit (Lonza Inc., Basel, Switzerland). Briefly, the neuron pellet was resuspended at room temperature in 100 µl of Nucleofector® solution with 200 nM siRNA specific to AMPKα1, 5'-CGAGUUGACUGGACAUAAATT-3' (siRNA ID:194424, Thermo Scientific, Pittsburgh, PA, USA), or AMPKα2, 5'-GGUUGACAAUCGGAGCUAUTT-3' (siRNA ID:s134962, Thermo Scientific, Pittsburgh, PA, USA), or a scrambled siRNA (Cat #:4390843 , Thermo Scientific, Pittsburgh, PA, USA) as a negative control. The suspension was transferred into a certified cuvette and Nucleofector® program O-003 on Amaxa Nucleofector machine (Lonza Inc., Basel, Switzerland) was used to electroporate/transfect the cells with the corresponding siRNA. Neurons were then plated for further experimentation.

Luciferase-based ATP assay in DRG culture

To measure ATP production by DRG neurons, Luminescent ATP detection assay kit (ab113849: Abcam, Cambridge, MA, USA) was used. In brief, cultured DRG neurons and an ATP standard dilution series were treated with detergent supplied in the kit and shaken for 5min. A solution containing D-Luciferin and firefly luciferase were added to the reaction mix and shaken on a plate stirrer for 5min. After 10min adaptation to the dark, luminescence from luciferase activity

was measured and recorded using the Glomax-multi detection system (Promega, Wisconsin, USA). A standard curve was plotted and luminescent units from each sample were interpolated in order to calculate the absolute ATP concentration per mg of total protein lysate.

Quantitative Western blotting

Rat DRG neurons were harvested from culture or isolated intact from adult rats and then homogenized in ice-cold RIPA buffer containing: 25mM Tris pH=8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and protease phosphatase inhibitors. Proteins (2-20 μg total protein/lane) were resolved and separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently transferred to a nitrocellulose membrane (Bio-Rad, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA) and immunoblotted with specific antibodies against pP70S6K T389 (1:1000, Cell Signaling Technology, Massachussetts, USA), pAkt S473 (1:1000, Santa Cruz Biotechnology, Texas, USA), total Akt (1:1000, Abcam, Cambridge, MA, USA), pAMPK T172 (1:1000, Cell Signaling Technology, Massachussetts, USA), total AMPK (1:800, Santa Cruz Biotechnology, Texas, USA), total OXPHOS (1:2000, MitoSciences, Abcam, Cambridge, MA, USA), PGC-1α (1:1000, Abcam), pACC S79 (1:1000, Cell Signaling Technology, Massachussetts, USA) and total ERK (1:1000, Santa Cruz Biotechnology, Texas, USA). Of note, total protein bands were captured by chemiluminescent imaging of the blot after gel activation (TGX Stain-Free™ FastCast Acrylamide Solutions, Bio-Rad, CA, USA) in addition to the use of T-ERK levels for target protein normalization (to adjust for loading). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, PA, USA. The blots were incubated in ECL Advance (GE Healthcare) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad, CA, USA).

Real-time PCR array

RNA was extracted from cultured neurons or previously frozen tissue samples using TRIzol® Reagent (Invitrogen, California, USA). Complementary DNA (cDNA) was synthesized from RNA samples by using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA) or Bright Green Master mix (Abmgood Co., Richmond, Canada) compatible with the iQ5 Cycler machine (Bio-Rad, CA, USA). The ΔΔCt method was used to quantify gene expression. The mRNA level of GAPDH and B2m were used for normalization.

Mitochondrial DNA/Nuclear DNA (mtDNA/nDNA) ratio

Total DNA was extracted from DRGs by using a modified salting-out DNA extraction method and subjected to QRT-PCR using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA). To calculate mtDNA/nDNA ratio, we designed primers specific to D-loop regions on rat mitochondrial DNA, and to ApoB and B2m genes on the rat nuclear DNA. PCR product size and specificity were validated using melt curve and 4% agarose gel (Figure 3.10A and B.Supplemental Figure).

Mitochondrial respiration in cultured neurons

An XF24 analyzer (Seahorse Biosciences, Billerica, MA, USA) was used to measure the basal level of mitochondrial oxygen consumption rate (OCR), the maximal respiration, the spare respiratory capacity and the coupling efficiency. In short, DRG culture medium was changed 1h before the assay to unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 1 mM sodium pyruvate, and 5 mM D-glucose. For diabetic rat DRG cultures,

25 mM D-glucose was used. Four mitochondrial complex inhibitors including oligomycin (1 µM), FCCP (1 µM) and rotenone (1 µM) + antimycin A (1 µM) were injected sequentially through ports in the Seahorse Flux Pak cartridges. Oligomycin acts as an irreversible ATP synthase inhibitor, FCCP as an uncoupler, rotenone as Complex I inhibitor, and antimycin A as an inhibitor of Complex III of the mitochondrial electron transport system. After OCR measurement, cells were subjected to protein assay (DC protein assay, BioRad, USA) for normalization purpose and, in some cases, Western blotting. OCR measures from each well were normalized to total protein levels and are presented as pmoles/min/mg protein.

Neurite outgrowth in DRG cultures

DRG neurons were cultured on glass coverslips. Then, they were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature then permeabilized with 0.3% Triton X-100 in PBS for 5 min. Neurons were incubated with 5% BSA in PBS for 1 h and with neuron-specific β-tubulin III antibody (1:1000; from Sigma, St Louis, MO, USA) overnight. Following three washes with PBS, cells were incubated with Cy3-conjugated secondary antibody (1:1000, Jackson ImmunoResearch Laboratories Inc., PA, USA) for 1 h at room temperature. Coverslips were mounted on slides using VECTASHIELD antifade mounting medium with DAPI (Vectorlabs, inc. CA, USA) and imaged using a Carl Zeiss Axioscope-2 upright fluorescence microscope equipped with AxioVision3 software. The fluorescent signal was collected as total pixel area for neurites and was measured by the high throughput NeurphologyJ plugin in ImageJ software after image enhancement. Total pixel area was normalized to number of cell bodies to calculate total neurite outgrowth per neuron.

Mitochondrial isolation and enzymatic activity of respiratory Complexes I and IV in DRG

DRG tissues were homogenized in mitochondrial isolation buffer (MIB) consisting of 70 mM sucrose, 210 mM mannitol, 5.0 mM HEPES PH 7.2, 1.0 mM EGTA, and 0.5 % (w/v) fatty acid free BSA using a Dounce homogenizer. Supernatant from a double centrifugation of the tissue at 800 g for 15 min was centrifuged twice at 8000 g for 10 min and the pellet subjected to enzymatic activity assays. Enzymatic activity of cytochrome *c* oxidase (a subunit of Complex IV of the mitochondrial electron transport system) was measured by a temperature controlled Ultrospec 2100 UV–visible spectrophotometer equipped with Biochrom Swift II software (Biopharmacia Biotech). Briefly, 0.02% lauryl maltoside was mixed with 10 µg purified mitochondria and incubated for 1 min before addition of 40 µM reduced cytochrome *c* and 50 mM KPi to the mixture. The resulting absorbance decrease of reduced cytochrome *c* at 550 nm was monitored for 2 min (Roy Chowdhury et al., 2012). Enzymatic activity of mitochondrial Complex I was measured according to the instruction manual of the kit (Cat #:K968-100, BioVision, California, USA). Data was collected at 5 min by reading the absorbance of the mixture (10 µg mitochondria, Complex I assay buffer, Decylubiquinone and Complex I dye) at 600 nm using a Ultrospec 2100 UV-visible spectrophotometer and the kinetic reduction of Complex I dye was calculated as Complex I activity.

Metabolomic analysis of nerve

The tibial nerve tissue from rats was utilized for biochemical analyses. The nerve (10-30 mg) was homogenized with 500µl ultrapure water (Milli-Q H2O, EMDMillipore, Billerica, USA) using a bead homogenizer (Omni Bead Ruptor 24, OMNI, USA). The same volume of methanol (500µl) was added to the homogenized tissue, and the mixture was vortexed, sonicated and centrifuged at 10500 g for 5 min. The supernatant was dried under a gentle flow of nitrogen, and

reconstituted in 100µl deionized water:methanol (1:1) containing 150 ng of each of the following internal standards: L-Tryptophan-d5, L-Valine-d8, L-Alanine-d4, L-Leucine-d10, Citric Acid-d4 and D-Fructose (all from Sigma, USA). Metabolomics analysis was performed on a 1290 Infinity Agilent high performance liquid chromatography (HPLC) system coupled to a 6538 UHD Accurate Quadrupole time-of-flight liquid chromatography/mass spectrometry (Q-TOF LC/MS) from Agilent Technologies (Santa Clara, CA, USA) equipped with a dual electrospray ionization source as described elsewhere (Hanson et al., 2018). A Zorbax SB-Aq 4.6×100mm, 1.8U, 600 bar column (Agilent Technologies) was used to separate metabolites while the column temperature was maintained at 55°C. In brief, a sample size of 2µl was injected into the Zorbax column by maintaining the HPLC flow rate at 0.6 ml/min. The mass detection was operated using dual electrospray with reference ions of m/z 121.050873 and 922.009798 for positive mode, and m/z 119.03632 and 980.016375 for negative mode. Targeted MS/MS mode was used to identify potential biomarkers using Agilent MassHunter Qualitative (MHQ, B.07) and Mass Profiler Professional (MPP, 12.6.1). The Molecular Feature Extraction (MFE) parameters were set to allow the extraction of detected features satisfying absolute abundances of more than 4000 counts. The data were normalized using a percentile shift algorithm set to 75 and were adjusted to the baseline values of the median of all samples.

Statistical analysis

Data were analyzed using two-tailed Student's t-tests or one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, as appropriate and indicated (GraphPad Prism 7, GraphPad Software). A P value < 0.05 was considered to be significant. The HeatMap was made using GraphPad (GraphPad Prism 7, GraphPad Software). The metabolomics data were analyzed using

One Way ANOVA ($P < 0.05$) followed by Benjamini-Hochberg multiple testing corrections (Mass Professional Profiler 12.6.1 and XLSTAT).

Results

IGF-1 enhances mitochondrial respiration and ATP production in cultured DRG neurons from control and diabetic rats

DRG neurons derived from control rats were cultured and treated with IGF-1 (10 nM) for 2-24 h. This concentration of IGF-1 does not cross-occupy the insulin receptor in neurons (Recio-Pinto and Ishii, 1988). Mitochondrial oxygen consumption rate (OCR) was enhanced at 24h but not at 2h or 6h (Figure 3.1A). Bioenergetic parameters of maximal respiration and spare respiratory capacity were significantly ($P<0.05$ and $P<0.01$, respectively) increased 24h after IGF-1 treatment (Figure 3.1B). Nevertheless, IGF-1 did not affect corrected oligomycin-insensitive mitochondrial respiration (proton leak) (Figure 3.11. Supplemental Figure). IGF-1 treatment of DRG neurons derived from age-matched diabetic rats showed up-regulation of mitochondrial maximal respiration at 2h and 24h treatment. Spare respiratory capacity was elevated at least 3-fold, although not reaching statistical significance, and respiratory control ratio was significantly increased at 2h and 24h of IGF-1 treatment (Figure 3.1C). To confirm that the mitochondrial OCR was reflected by similar alterations in cellular ATP production, DRG neurons from control rats were treated with various doses of IGF-1 for 24h and ATP production was measured in live cells. At doses of 1, 10 and 100nM, but not 0.1nM, IGF-1 augmented ATP production ($P<0.05$) (Figure 3.1D).

Genes linked to mitochondrial function were dysregulated in DRGs from control vs. diabetic rats and *in vitro* were up-regulated after IGF-1 treatment

The mRNA levels of a number of genes downstream of IGF-1 signaling that are linked to mitochondrial function, including Akt2, Akt3, AMPKα2, UQCRC2, ATP5a1, MFN1, RHOT1, IGF-1 and Kif5B, were down-regulated in DRG of diabetic rats when compared to control rats ($P<0.05$) (Figure 3.1F). Some mRNAs including those for Slc2a1 (Glut1), Ppargc1 β (PGC-1β) and DNAi1 were up-regulated in DRGs of diabetic rats (Figure 3.1F). IGF-1 (10nM) treatment of cultured DRGs from control rats for 6h up-regulated Akt1, Akt2, Akt3, AMPKα1, AMPKα2, IGF-1R, GSK3b, Beta-actin, RPS6Kb1 (P70S6K), CPT1a, Glut1, PFKp, P53, Ppargc1a (PGC-1α), PGC-1β, Srebp1c, MFN1, Nrf1 and VDAC1 mRNA levels when compared to untreated DRG neurons ($P<0.05$) (Figure 3.1E). DRGs derived from diabetic rats and treated *in vitro* with IGF-1 showed a significant ($P<0.05$) up-regulation in mRNA levels of Akt1, Akt2, Akt3, AMPKα1, AMPKα2, GSK3b, Beta-actin, RPS6Kb1 (P70S6K), Glut1, PFKp, P53, Ppargc1a (PGC-1α), PGC-1β, UQCRC2, MTCO1, ATP5a1, MFN1, Opa1, Drp1, Nrf1 and VDAC1 genes vs. untreated cultured DRG neurons (Figure 3.1G).

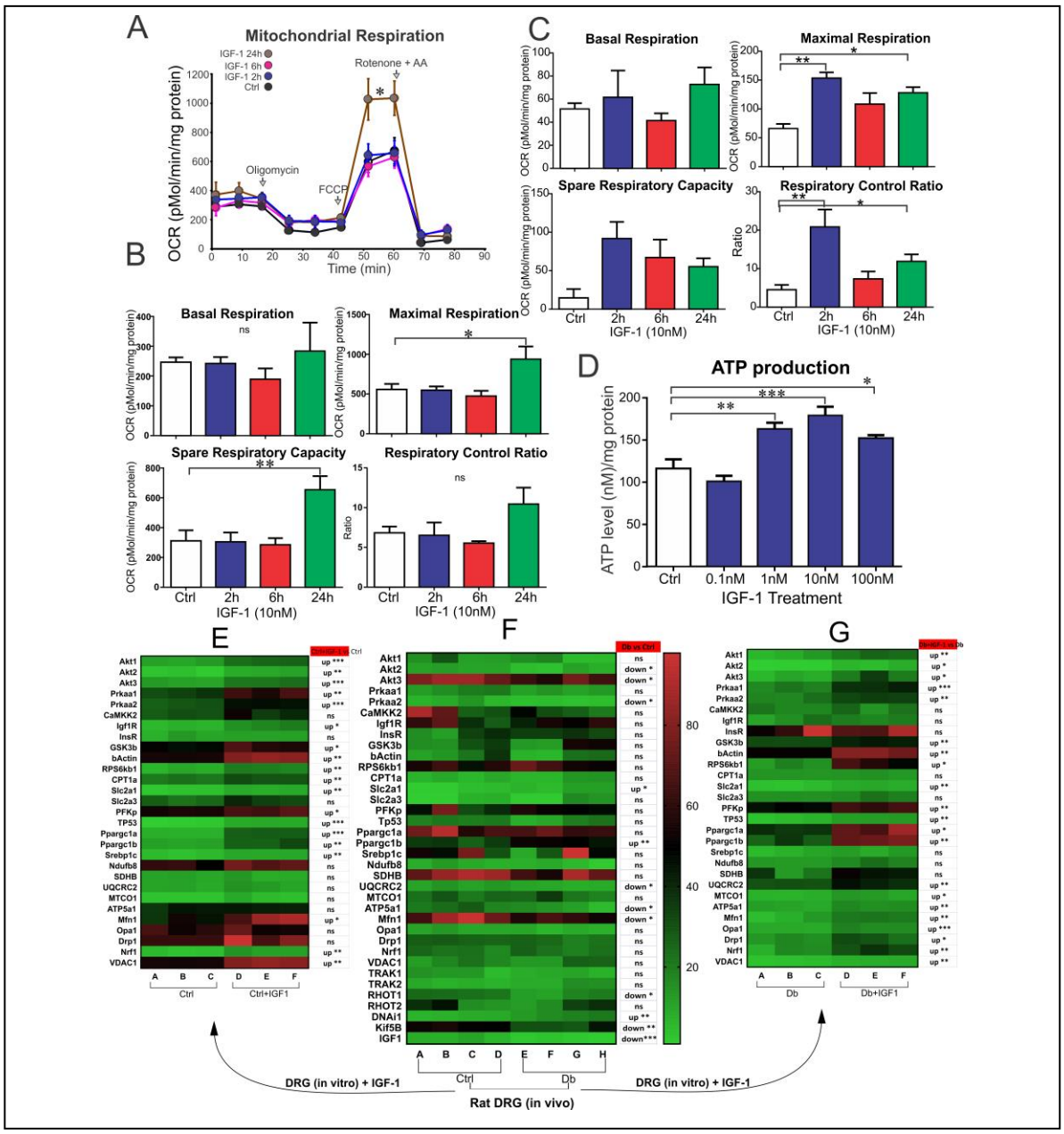

Figure 3.1. Mitochondrial function-related genes are dysregulated in DRG neurons from diabetic rats, and exogenous IGF-1 modulates them and upregulates mitochondrial respiration and ATP production in cultured adult sensory neurons. DRG neurons derived from (A, B, D) adult control or (C) diabetic rats were treated with/without IGF-1 for 2-24 h. In

(A, B, C) the culture plate was then inserted into the Seahorse XF24 Analyzer and oligomycin, FCCP and rotenone+AA (antimycin A) added sequentially. In (D) ATP concentration was calculated from proportional luminescent reads by GloMax®-Multi Detection System. Data were normalized to protein concentration units per well prior to statistical analysis. Real-time PCR array derived mRNA levels for (F) DRG tissues or (E and G) cultured DRG neurons treated with IGF-1 for 6h, from control (ctrl) and diabetic (Db) rats. All mRNA levels were calculated relative to *GAPDH* or *B2m* mRNA levels using delta dela Ct method. Data are mean ± SEM of N=3-5 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$; analyzed by unpaired Student's t-test or one-way ANOVA with Dunnett's *post-hoc* test.

IGF-1 augments AMPK, Akt, ACC phosphorylation and respiratory protein expression in cultured neurons from control rats

DRG neurons from age matched control rats treated with 10 nM IGF-1 exhibited elevated phosphorylation of Akt (at S473) within 15min (Figure 3.2A and B) and at 2h this was associated with enhanced phosphorylation of pP70S6K (a downstream substrate for P-Akt) (Figure 3.12D.Supplemental Figure). IGF-1 elevated phosphorylation of AMPK at T172 and also its phosphorylation target acetyl-Co-A carboxylase (P-ACC) at 2h and 6h (Figure 3.2A and B). These effects were dose dependent; with 10 nM IGF-1 giving the highest P-AMPK and P-ACC levels, whereas 100 nM induced the highest activation of Akt (Figure 3.12A and B. Supplemental Figure). Treatment for up to 24h with 10nM IGF-1 up-regulated the total protein levels for AMPK and Akt (Figure 3.12C. Supplemental Figure). The stimulatory effect of IGF-1 on total protein expression levels for AMPK and Akt at 6h and 24h was the reason we normalized all data to total protein levels. Mitochondrial OXPHOS proteins, components of the electron transport system (ETS), including Complex components IV-MTCO1 and V-ATP5a were significantly elevated after 6h to 24h of IGF-1 treatment (Figure 3.2C and D). IGF-1 also elevated these same Complex proteins and Complex II-SDHB in DRG neuron cultures derived from STZ-induced type 1 diabetic rats (Supplementary Figure 3.4A and B). Overall, the impact of IGF-1 on the level of expression of these respiratory chain proteins was greater in the diabetic cultures compared with control age-matched cultures.

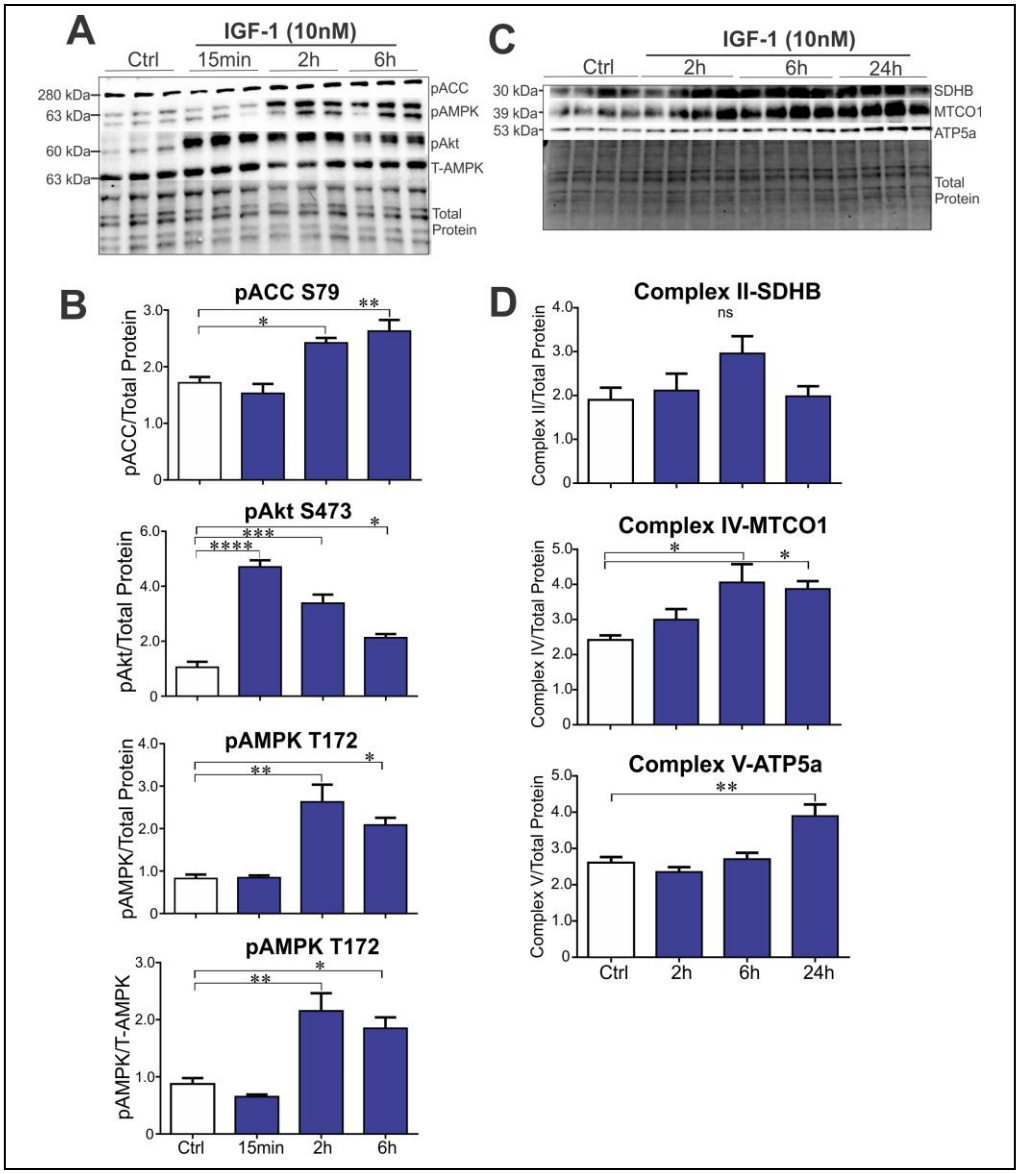

Figure 3.2. IGF-1 treatment increases Akt and AMPK phosphorylation, and the expression of electron transport chain proteins. DRG neurons derived from adult control rats were treated with/without 10 nM IGF-1 for (A, B) 15min-6h or (C, D) 2-24 h and lysates subjected to Western blotting. Specific proteins from each respiratory Complex were quantified and expressed relative to total protein. Complexes I-NDUFB8 and III-UQCRC2 subunit proteins were not detectable. Data are mean ± SEM of N=3-4 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

AMPK inhibitor, AMPKα1 knockdown or Akt inhibitor suppress the IGF-1 induced up-regulation of mitochondrial function in DRG neurons from control rats

The stimulatory effect of IGF-1 on AMPK activity and respiratory chain protein expression provided a good rationale to study putative effects on mitochondrial function. To identify the signaling pathway utilized by IGF-1 to modulate mitochondrial function DRG neurons from control rats were cultured and pretreated with 1 μM compound C (AMPK inhibitor), MK-2206 (Akt inhibitor) or STO-609 (CaMKKβ inhibitor) 2h prior to 10 nM IGF-1 treatment for 24h. Seahorse XF24 assay for oxygen consumption rate (OCR) revealed that IGF-1 up-regulated mitochondrial function with respect to augmented maximal respiration and spare respiratory capacity (Figure 3.3A and B). This effect was completely suppressed by the AMPK inhibitor and partially suppressed by the Akt inhibitor (Figure 3.3A and B). The CaMKKβ inhibitor STO-609 also prevented induction of mitochondrial function by IGF-1 but this effect did not reach statistical significance (Figure 3.3A and B). To specifically knockdown isoforms of AMPK, DRG neurons were also transfected with siRNAs specific to AMPK α1 or AMPK α2 (and in combination) for 24h prior to IGF-1 treatment and then for another 24h. AMPK knockdown efficiencies were 75-80% for the specific isoform mRNAs and 73% for the total AMPK protein when both siRNAs were combined (Figure 3.14 A-C. Supplemental Figure). 2D gel electrophoresis revealed that the isoforms of AMPKα1 (mw 63.97 kDa) and AMPK α2 (mw 62.26 kDa) could be visualized (Figure 3.14D. Supplemental Figure). Supplementary Figure 5D and E shows that siRNA knockdown for each isoform was relatively specific. SiRNA to AMPKα1 caused a 77% knockdown and only reduced AMPKα2 by 16% (Figure 3.14F. Supplemental Figure). For siRNA to AMPKα2, the values were 61% for its own isoform and 16% for the AMPKα1. SDS-PAGE revealed effective knockdown of phosphorylated AMPK and

its target P-ACC with the combined siRNAs in the presence/absence of IGF-1 for 24h (Figure 3.15A. Supplemental Figure). Under the same experimental conditions siRNA to AMPK α1 blocked phosphorylated AMPK. However, its target P-ACC was not significantly affected (Figure 3.15B. Supplemental Figure). With the same experimental paradigm OCR measurements showed suppression of IGF-1-driven up-regulation of maximal respiration and spare respiratory capacity in neurons treated with AMPK α1 siRNA (Figure 3.4A and B) and AMPK α1α2 siRNAs combined (data not shown), but not with siRNA to AMPK α2 (Figure 3.16A-D. Supplemental Figure).

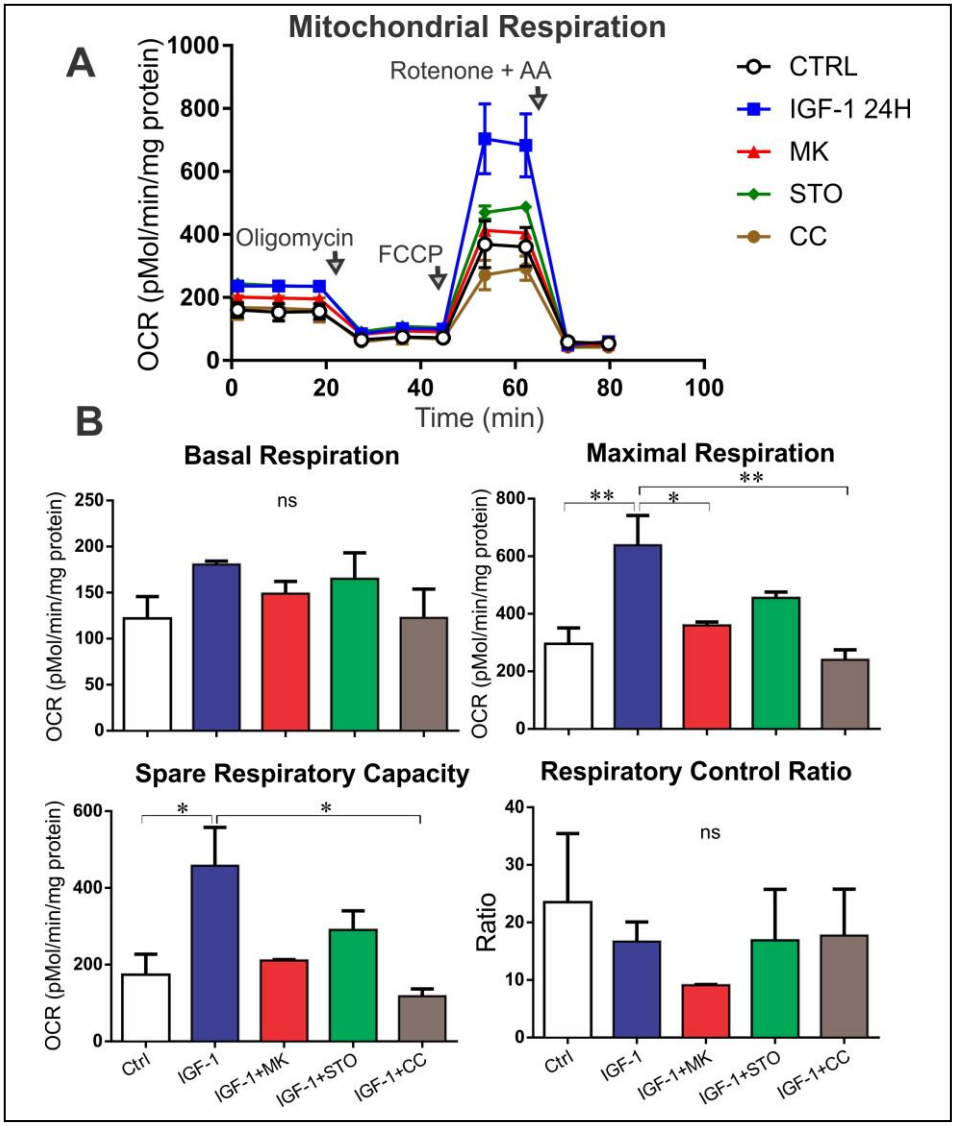

Figure 3.3. Compound C (AMPK inhibitor) suppresses the IGF-1 upregulation of mitochondrial respiration. (A, B) DRG neurons derived from control rats were cultured, pretreated with inhibitors 2h prior to IGF-1 treatment and maintained for 24 h. Mitochondrial respiration was measured using Seahorse XF24 Analyzer. CC (compound C, 1µM): AMPK inhibitor, MK (MK-2206, 1µM): Akt inhibitor and STO (STO-609, 1µM): CaMKKβ inhibitor. Data were normalized to protein concentration units per well prior to statistical analysis. Data are mean ± SEM of N=3-5 replicates; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

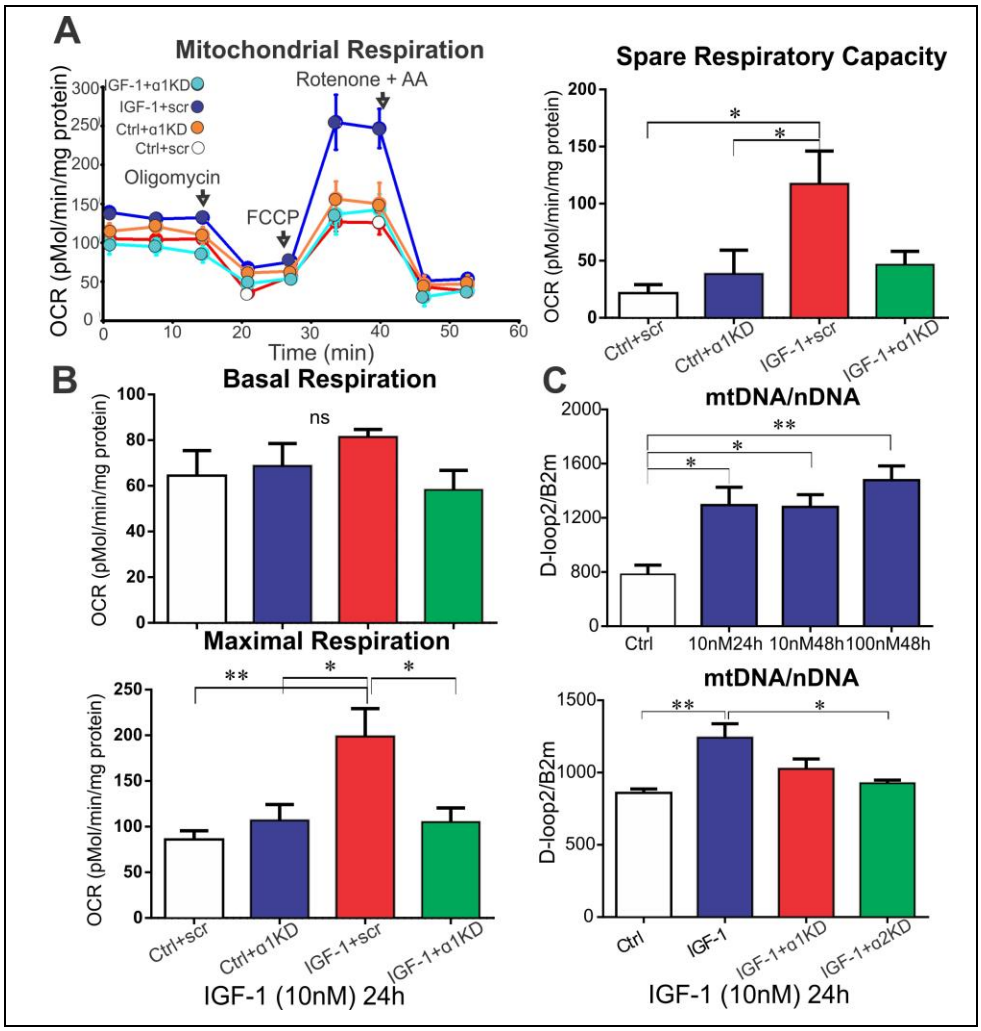

Figure 3.4. IGF-1 acts through AMPKα1 and α2 to augment mitochondrial function and mtDNA copy number. (A, B) DRG neurons derived from adult control rats were transfected with AMPKα1- or α2-specific siRNAs, cultured for 24 h and treated with/without 10 nM IGF-1 for another 24 h. Mitochondrial respiration was measured using Seahorse XF24 Analyzer. Data were normalized to protein concentration units per well prior to statistical analysis. (C) DRG neurons from control rats were cultured and treated with different IGF-1 doses (10nM and 100nM) for 24-48 or transfected with AMPKα1- or α2-specific siRNAs, cultured for 24 h and treated with/without 10 nM IGF-1 for another 24 h. mtDNA/nDNA ratio was analyzed using

Real-Time PCR and calculated using the ΔΔCt method. Data are mean ± SEM of N=3-5 replicates; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Tukey's or Dunnett's *post-hoc* test.

AMPK knockdown and Akt inhibitor impede IGF-1 enhancement of neurite outgrowth and mtDNA copy number in DRG cultures from control rats

To investigate the inhibitory effect of AMPK on mitochondrial DNA copy number and neurite outgrowth, DRG cultures derived from control rats were transfected with siRNA to AMPKα1 or α2 and treated with IGF-1. IGF-1 (10 nM or 100 nM) significantly ($P<0.05$) increased mtDNA copy number after 24h and 48h (Figure 3.4C). This effect of IGF-1 on mtDNA was significantly suppressed following treatment with siRNA to AMPKα2 (Figure 3.4C). Knockdown of either AMPKα1 or AMPKα2 isoforms also prevented the IGF-1 enhancement of neurite outgrowth (Figure 3.5A). In a complementary experiment, neurite outgrowth was enhanced by IGF-1 treatment ($P<0.01$) and pretreatment with Akt inhibitor or AMPK inhibitor Compound C suppressed this neuritogenic effect of IGF-1 (Figure 3.5B).

Topical IGF-1 to the eye of diabetic mice reversed the loss of corneal nerve profiles

To address whether the relatively rapid effects of IGF-1 on regulation of mitochondrial respiration and neurite outgrowth from adult sensory neurons *in vitro* predict neurotrophic and neuroprotective properties *in vivo,* we delivered IGF-1 topically to the eye of STZ-induced diabetic mice daily for 4 weeks, following 8 weeks of untreated diabetes. Nerve density of the sub-basal nerve plexus was measured iteratively by corneal confocal microscopy across the study period. Nerve density did not change over the 12-week study period in control mice (Figure 3.5C-D). Eight weeks of diabetes induced a mild reduction in nerve density that progressed in the subsequent 4 weeks. Initiation of topical IGF-1 treatment prevented this progression such that by the end of the study the vehicle-treated diabetic group had values that were significantly ($P<0.05$) lower than the IGF-1 treated group.

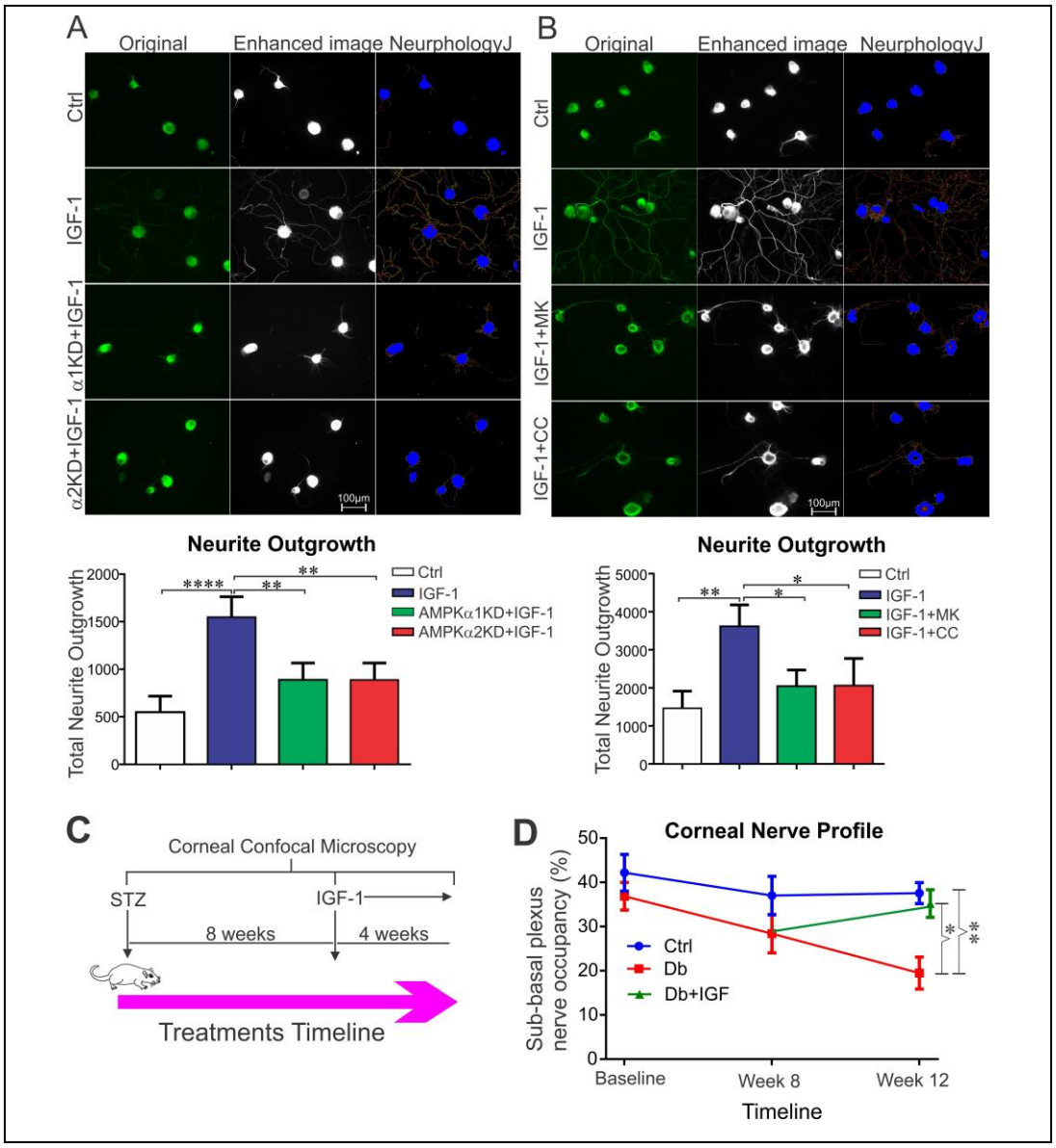

Figure 3.5. AMPK and Akt inhibition suppresses the axonal regeneration triggered by IGF-I in cultured DRGs from rats and topical IGF-1 prevents progressive loss of corneal nerves in diabetic mice. DRG neurons derived from adult control rats were (A) transfected with AMPK α1- or α2-specific siRNAs, cultured for 24 h or (B) pretreated with inhibitors for 2 h and treated with/without 10 nM IGF-1 for another 24 h. β-tubulin III immunostaining was used to stain sensory neurons and NeurphologyJ was used for automated neurite tracing. CC (compound

C, 1µM): AMPK inhibitor and MK (MK-2206, 1µM): Akt inhibitor. Total neurite outgrowth data is presented relative to neuron number. Data are mean ± SEM of N=4 replicates; *= $p<0.05$ or **= $p<0.01$ or ****= $p<0.0001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test. (C) Reveals the time line for the topical delivery of IGF-1 to the eye of STZ-induced diabetic mice. (D) Line chart showing nerve density (as % occupancy) in the sub-basal nerve plexus of control (blue line), diabetic (STZ: red line) and IGF-1 treated diabetic (STZ+IGF-1; green line) mice at 0, 8 and 12 weeks of diabetes. IGF-1 treatment was initiated at 8 weeks. Data are group mean ± SEM of N=9-10/group. At week 12 *=$p<0.05$ for Db vs Db+IGF-1 group and **p=<0.01 for Db vs age matched control group by two-way ANOVA with Tukey's *post-hoc* test.

IGF-1 therapy reverses thermal hypoalgesia and corrects impaired activities of mitochondrial Complexes I and IV in DRG of diabetic rats

Age-matched control and STZ-induced diabetic rats were maintained for 3 months, and then a cohort of diabetic rats received injections of human recombinant IGF-1 (20 μg/rat per day s.c.) for the final 11 weeks (Figure 3.6A). Body weight, blood glucose and glycated hemoglobin values were not any different between untreated and IGF-1-treated diabetic rats (Table 3.1.Supplemental Table). Thermal testing on the hind paw was performed at three time points and revealed development of hypoalgesia in diabetic rats and significant improvement in the IGF-1-treated diabetic rats at the end of study (Figure 3.6A). Analysis of IENF density and SNP levels in the hind paw revealed no significant loss of fibers at the end of this study and no effect of IGF-1 treatment on IENF whereas a significant rise in SNP profiles was observed compared to control rats (Figure 3.6B). Mitochondria isolated from DRGs of diabetic rats exhibited depressed activity levels of mitochondrial Complexes I and IV compared with age matched control and these activities were significantly elevated by IGF-1 therapy (Figure 3.6C).

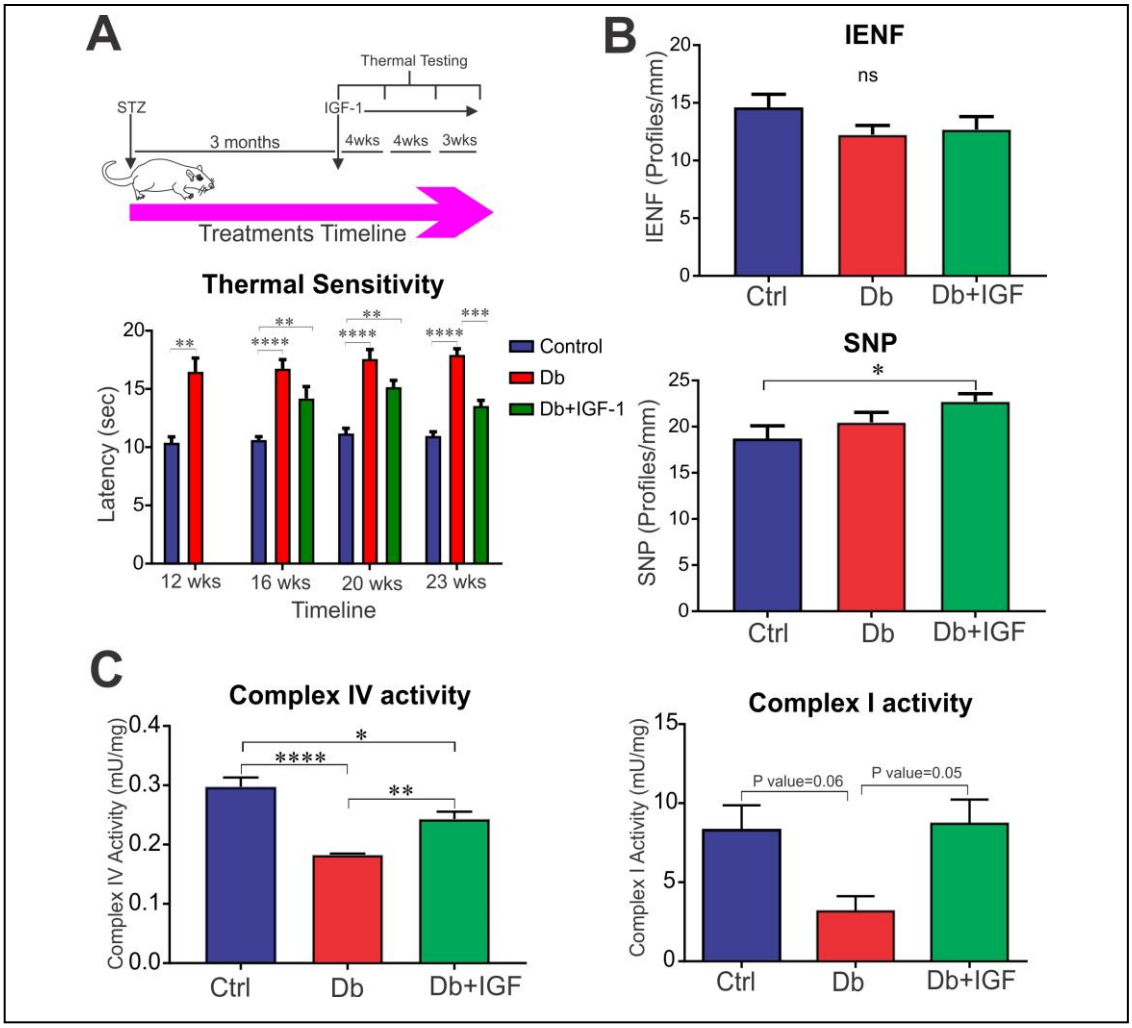

Figure 3.6. IGF-1 therapy restored the activity of Complexes I and IV, and prevented thermal hypoalgesia in diabetic rats. Control (ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were tested for their thermal response latency according to the timeline in (A). (B) IENF and SNP were analyzed in hind paw footskin in the three groups of animals. (C) DRG tissues from control (Ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were isolated and subjected to Complex I and Complex IV enzymatic activity assays. Data are in mU/mg tissue. In (A, B and C) data are mean ± SEM of N=5-10; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$ or ****= $p<0.0001$; analyzed by one-way ANOVA or two-way ANOVA with Tukey's *post-hoc* test.

IGF-1 therapy increases the expression of PGC-1α and respiratory chain proteins and up-regulates AMPK, Akt and P70S6K phosphorylation in DRG of diabetic rats

Due to low tissue availability, each animal group was divided into different sub-groups to perform Western Blotting, enzymatic assay and mRNA measurements on DRGs. Levels of expression of mitochondrial Complex IV-MTCO1 and Complex V-ATP5a, and PGC-1α proteins were suppressed in DRGs derived from diabetic rats by approximately 40% and these deficits were prevented by IGF-1 therapy (Figure 3.7A). IGF-I therapy also significantly increased the phosphorylation of critical effector proteins upstream of mitochondria including AMPK and P70S6K compared to both control and untreated diabetic rats, while a trend of elevated Akt activation was also observed (P=0.08 vs. control: Figure 3.7B). It should be noted, that only a partial depression of P-AMPK and P-P70S6K levels were observed in DRGs of the diabetic group versus age-matched control in this study. Consistent with Western blotting data, mRNA levels of AMPKα2 isoform, Complex III-UQCRC2 and Nrf-1 were restored in diabetic rat DRGs after IGF-I therapy (Figure 3.17. Supplemental Figure). AMPKα1, Akt2 and Akt3 mRNAs were not significantly different between groups (Figure 3.17. Supplemental Figure).

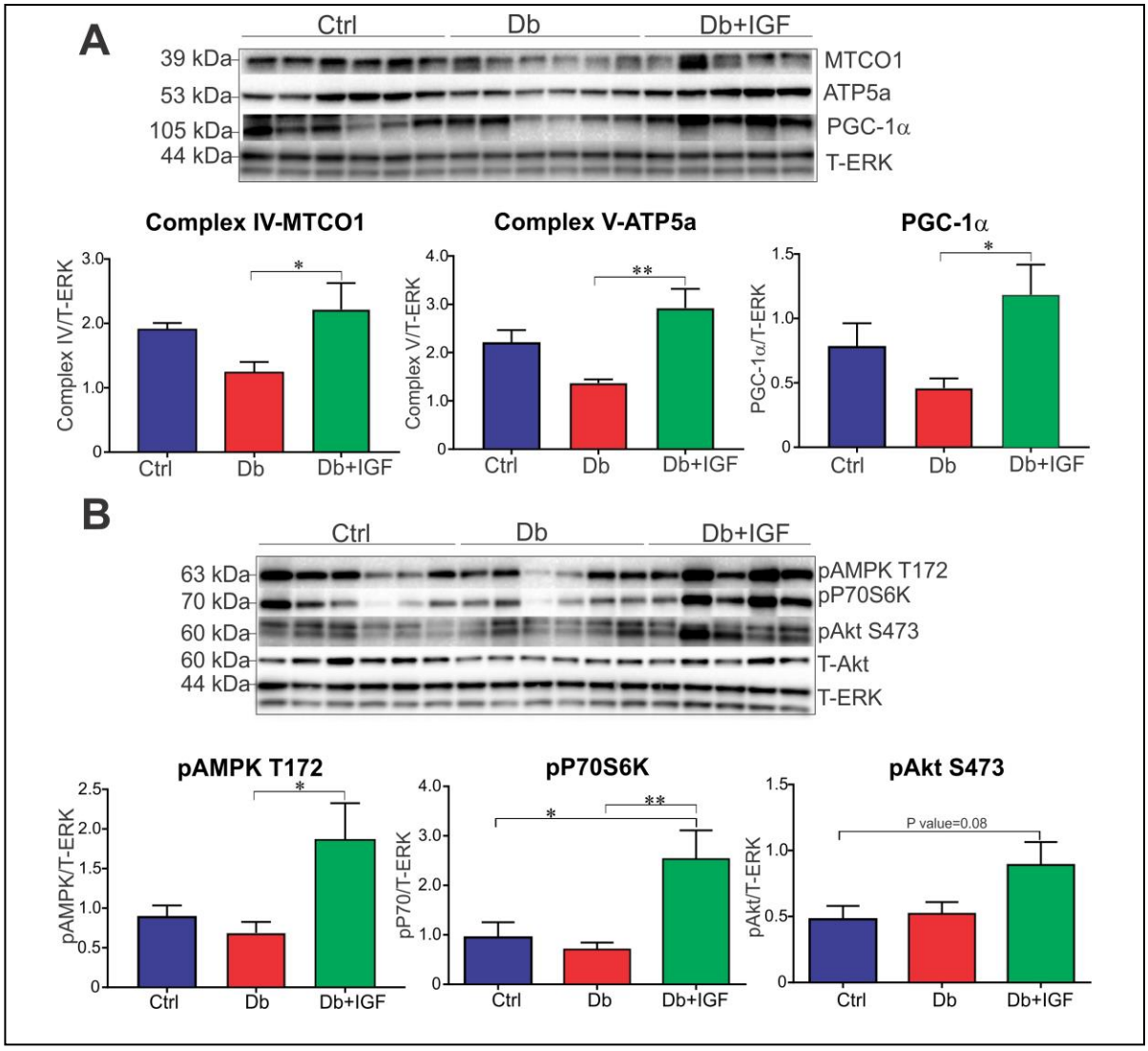

Figure 3.7. IGF-1 therapy restored the expression of PGC-1α and ETS proteins and up-regulated AMPK, P70S6K and Akt phosphorylation in diabetic rats. DRG tissues from control (Ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were isolated and subjected to (A, B) Western blotting. Western blot band intensity of target proteins was normalized to total ERK bands of the same blot. Data are mean ± SEM of N=5-7 animals; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

IGF-1 therapy reinstates components of the Krebs cycle, amino acid metabolism, ketosis and oxidant status, but does not correct aberrant glucose metabolism in tibial nerve of diabetic rats

A targeted metabolomics approach was used to monitor 35 selected metabolites (Table 3.2.Supplemental Table) related to energy generation by mitochondrial function, carbohydrate metabolism and protein metabolism, as recently reported (Freeman et al., 2016). A total of 13 metabolites were significantly different and exhibited a similar trend between the 3 groups (Figure 3.8A). The metabolomics analysis of tibial nerve tissues showed that Krebs cycle intermediates including fumaric acid, succinic acid, malic acid and citric acid, and free amino acids or fragmented peptides such as leucine, aspartic acid and Ala-Cys-Asp, were significantly ($P<0.05$) up-regulated in nerves of diabetic rats and were returned to the normal range with IGF-1 therapy (Figure 3.8A). IGF-1 injections did not correct the levels of sorbitol, glucose and myoinositol 4-phosphate, which all represent aberrant glucose metabolism (Figure 3.8A-B), indicating that IGF-1 did not alter glucose uptake or metabolism within the local environment of the nerve. However, diabetes-induced elevation of oxidized glutathione and ketone bodies including β-hydroxy butyric acid in nerve was reduced by IGF-1 treatment of diabetic animals (Figure 3.8A). Other metabolites such as isocitrate (TCA metabolite), homoserine (amino acid precursor) and acryloylglycine (fatty acid metabolism) did not differ or show any consistent trends between groups (Table 3.2.Supplemental Table). The Krebs cycle metabolites, free amino acids, ketone bodies and oxidized glutathione levels showed similar trends, as illustrated for citric and aspartic acid (Figure 3.8C-D).

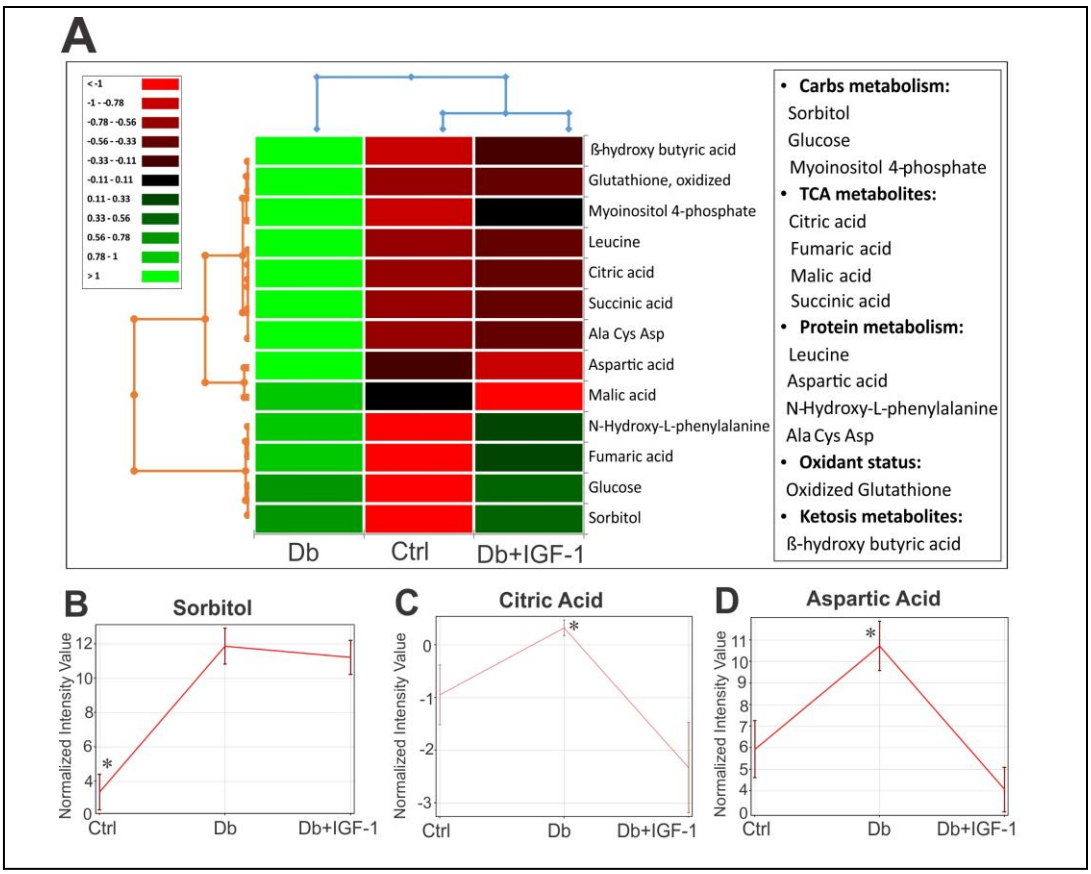

Figure 3.8. IGF-1 therapy reinstates the Krebs cycle, amino acid metabolism, ketosis and oxidant status in tibial nerve of diabetic rats. Tibial nerve tissues from control (Ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were dissected and weighed. Total metabolites were extracted using methanol-water mixture, dried and subjected to metabolomic analysis using HPLC system coupled to time-of-flight liquid chromatography/mass spectrometry. Normalized intensity value of each compound was estimated by using internal standards (L-Tryptophan-d5, L-Valine-d8, L-Alanine-d4, L-Leucine-d10, Citric Acid-d4 and D-Fructose). The final value for each metabolite was normalized to its corresponding tibial nerve weight and illustrated in (A) HeatMap. The trends for (B) sorbitol, (C) citric acid and (D) aspartic acid are shown as examples. Data are mean ± SEM of N=9-12 animals; *= $p<0.05$; analyzed by one-way ANOVA, followed by Benjamini-Hochberg multiple testing corrections.

Discussion

We demonstrate for the first time that IGF-1 activates and up-regulates AMPK to augment mitochondrial function, ATP production, mtDNA copy number and expression of ETS proteins in cultured rat DRG neurons (Figure 3.9). IGF-1 utilizes this AMPK pathway and, possibly in parallel, the Akt pathway to drive axonal outgrowth. Further, we show that IGF-1 therapy prevented diabetes-induced progressive loss of sensory nerves in the cornea and ameliorated paw thermal hypoalgesia. Correction of these clinically relevant endpoints was associated with suppression of build-up of TCA intermediates in nerve and optimization of mitochondrial phenotype in the DRG of type 1 diabetic rats. The stimulatory effect of IGF-1 upon mitochondrial oxygen consumption rate can be explained by signaling via the AMPK α1 isoform. The elevation in mtDNA copy number revealed the involvement of AMPK α2 isoform downstream from IGF-1 signaling.

For more than two decades, a variety of studies have demonstrated that IGF-1 promotes neurite outgrowth and protects from multiple functional and structural indices of diabetic neuropathy in animal models (Fernyhough et al., 1993, Zackenfels et al., 1995, Zhuang et al., 1997). The emergence of corneal confocal microscopy as a technique to visualize human sensory nerves *in situ* has provided a non-invasive and sensitive biomarker of DSPN that offers the opportunity to track progression of neuropathy and efficacy of therapeutic interventions (Tavakoli et al., 2013). Diabetic rodents also develop loss of corneal nerves (Chen et al., 2013, Davidson et al., 2012) and topical delivery of potential therapies to the eye provides a useful translational model that bridges *in vitro* and traditional *in vivo* therapeutic studies. Our demonstration that topical delivery of IGF-1 prevented progressive corneal nerve loss in diabetic mice provides *in vivo* validation of our *in vitro* model using adult sensory neuron cultures and

adds a novel index of the therapeutic potential of IGF-1 against a structural manifestation of DSPN using an assay that reflects the human condition.

The mechanisms through which IGF-1 operates to implement nerve growth and repair are not completely understood. It has been proposed that optimal mitochondrial function is a key factor for axonal outgrowth and repair (Chowdhury et al., 2013, Cashman and Hoke, 2015). For instance, modulating mitochondrial dynamics by overexpression of MFN-2 or inhibition of DRP-1 regulated the direction and rate of neurite outgrowth in cultured retinal ganglion cells from rat embryos (Steketee et al., 2012). Mitochondrial biogenesis triggered by the AMPK-PGC-1α-NRF1 pathway and energy supplementation was required for axonal outgrowth in rat cortical neurons (Vaarmann et al., 2016). Resveratrol, an activator of AMPK, drives axonal outgrowth and was protective against diabetic neuropathy in STZ-induced diabetic rats (Dasgupta and Milbrandt, 2007, Roy Chowdhury et al., 2012). Growth factors IL-1β, IL-17A, ciliary neurotrophic factor and insulin enhanced mitochondrial function and promoted neurite outgrowth, which correlated with protection from development of DSPN in animal models of type 1 diabetes (Habash et al., 2015, Saleh et al., 2013a, Saleh et al., 2013b, Aghanoori et al., 2017). Studies with modulators of heat shock protein function revealed that improved mitochondrial function was associated with prevention and reversal of diabetic neuropathy in type 1 and type 2 models of diabetes (Ma et al., 2014, Urban et al., 2010). In the present work in cultured DRG neurons, we demonstrate the novel finding that a physiologically relevant concentration of IGF-1, which does not stimulate the insulin receptor (Recio-Pinto and Ishii, 1988), up-regulated mitochondrial respiration together with a dose-dependent stimulation of ATP production.

Recent studies also report IGF-1-mediated optimization of mitochondrial phenotype in other cell types and diseases. IGF-1 therapy corrected mitochondrial ATPase activity and inhibited caspase 3 and 9 activation in liver cells from aging rats (Puche et al., 2008). Glutamine knock-in in striatal cells from the mutant Huntingtin (mHtt) mouse displayed a reduction in ROS production and caspase 3 activity, and an elevation in Tfam and mitochondrial-encoded cytochrome c oxidase II proteins when treated with IGF-1 (Ribeiro et al., 2014). In cancer cell lines, exogenous IGF-1 sustains cell viability by stimulating mitochondrial biogenesis and BNIP3-induced mitophagy (Lyons et al., 2017). In the current study in cultured DRG neurons derived from control or diabetic rats, IGF-1 exerted a novel up-regulatory effect on mitochondrial respiration, with a significant stimulatory effect on spare, or reserve, respiratory capacity. This effect occurred over 24h in control cultures and, in diabetic cultures, was triggered within 2h. This may suggest that IGF-1 enhances the electrochemical gradient, as observed previously with insulin in DRG cultures (Huang et al., 2003), and aerobic glucose metabolism to elevate mitochondrial oxygen consumption rate (Poburko et al., 2011, Cardoso et al., 2010, Okorodudu et al., 1995). In contrast to mitochondrial basal respiration, spare respiratory capacity is normally utilized when cells have excessive ATP demands or are stressed, as under chronic hyperglycemia. It is most likely that defects in expression or activity of respiratory chain complexes (or supercomplex formation) in diabetic conditions, essentially nutrient stress, drives suppression of spare respiratory capacity thus inducing an energy deficit (Sansbury et al., 2011, Rossignol et al., 2003, Fernyhough, 2015). IGF-1 may raise spare respiratory capacity through high ATP demand due to accelerated neurite outgrowth (thus optimizing the electrochemical gradient), and/or by normalizing aerobic glucose metabolism and/or via AMPK-dependent modulation of gene expression of ETS components.

Comparative mRNA quantitation of selective genes revealed that IGF-1, AMPK and Akt isoforms, ATP5a1, MFN-1 and RHOT-1 were suppressed in DRG in diabetic rats. IGF-1 treatment of cultured DRGs from both control and diabetic rats improved the expression of these dysregulated genes with a higher impact in DRG neurons derived from diabetic rats. The most prominent finding of our gene expression analysis was that IGF-1 up-regulated AMPK (α1 and α2) and Akt (Akt1, Akt2 and Akt3) functional isoforms in cultured DRGs derived from diabetic rats. The transcriptional upregulation of these genes by IGF-1 in 6h could be the main driving factor for enhanced mitochondrial respiration after 24h IGF-1 treatment in cultured DRGs from both control and diabetic rats. Nevertheless, this does not seem to be the case for enhanced mitochondrial respiration after 2h IGF-1 treatment which is observed in diabetic cultures implying the involvement of post translational modifications of metabolism-regulating proteins including AMPK and Akt.

AMPK and Akt pathways are modulated by IGF-1 in other cell types. In vascular smooth muscle cells, IGF-1 activates Akt to suppress AMPK activity by stimulating AMPK S485 phosphorylation, thus instigating AMPK T172 dephosphorylation (Ning et al., 2011). There are two common upstream kinases, LKB-1 and CaMKKβ, known to phosphorylate AMPK on its activation site, T172, and activate this enzyme (Hawley et al., 2005, Shaw et al., 2004). Our preliminary data (not shown) reveal that LKB-1 was not activated by IGF-1, and CaMKKβ inhibition by STO-609 did not significantly affect AMPK phosphorylation/activity (data not shown) or mitochondrial function in the presence of IGF-1 (Fig. 4B). In addition, we have preliminary data showing that IGF-1 did not induce a rise in intracellular Ca^{2+} in cultured DRG neurons (where a rise in Ca^{2+} could be surmised to trigger activation of CaMKKβ). AMPK is also reported to be phosphorylated on T172 and activated in an ATM-dependent manner by IGF-

1 in HeLa, PANC-1 and TIG103 cell lines (Suzuki et al., 2004a) and so future studies will focus on this pathway.

Among different pharmacological inhibitors used to block IGF-1 downstream effectors, the AMPK inhibitor, Compound C, completely abolished the IGF-1-mediated up-regulation of mitochondrial maximal respiration and spare respiratory capacity. Neurite outgrowth was also blocked (Fig. 6). SiRNA-based inhibition of the AMPK α1 isoform, but not AMPK α2 isoform, exhibited a similar suppression of the IGF-1 enhancement of mitochondrial respiration and neurite outgrowth. The IGF-1 stimulation of mitochondrial DNA copy number was blocked by either AMPK α2 or AMPK α1 knockdown, which implicates both isoforms in mtDNA replication. A variety of functions have been reported for these two AMPK isoforms in other tissues. In striatal cells in mice with Huntington's disease (HD), accumulated Huntingtin resulted in AMPK α1 over-activation and nuclear translocation causing cell death through down-regulation of Bcl-2 (Ju et al., 2011). AMPK α1 promotes contractile function of cardiomyocytes by phosphorylating troponin I (Chen et al., 2014), and myogenesis through myogenin gene expression (Fu et al., 2013). AMPK α2 knock-out mice show deficient Complex I activity and lower cardiolipin content correlating with decreased cardiolipin synthetic enzymes in cardiomyocytes (Athea et al., 2007). In endothelial cells, AMPK α2 activates anti-inflammatory pathways by phosphorylating PARP-1 and induction of Bcl-6 (Gongol et al., 2013).

Acute (30 min) intrathecal application of IGF-1 activated Akt in DRG and this effect was diminished in *ob/ob* type 2 diabetic mice (Grote et al., 2013a). In our *in vitro* studies the Akt inhibitor, MK-2206, suppressed the IGF-1 dependent augmentation of mitochondrial function and neurite outgrowth (Fig. 4B and Fig. 6). Previous work in smooth muscle cells and cancer cell lines revealed an inhibitory effect of Akt on AMPK via phosphorylation on S485/S487 and

subsequent suppression of phosphorylation on T172 (Ning et al., 2011, Hawley et al., 2014). Moreover, we also found that inhibiting Akt results in higher AMPK T172 phosphorylation in cultured DRGs (data not shown). However, we have observed that the Akt inhibitor suppressed the IGF-1 up-regulation of mtDNA copy number (data not shown). In the present study, IGF-1 enhanced phosphorylation of P70S6K (Supplementary Fig. 3D) and Akt directly elevates P70S6K activity and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) function to augment ETS gene expression (Goo et al., 2012). Thus, these studies utilizing pharmacological blockade of Akt reveal (i) in DRG neurons treated with IGF-1 Akt is acting upstream from AMPK to suppress AMPK activity, and (ii) upon IGF-1 treatment, Akt is mediating parallel or alternative pathways from AMPK that regulate mitochondrial function and neurite outgrowth.

Previous studies reveal a role of impaired IGF-1 signaling in the etiology of diabetic neuropathy, or as a protective factor (Ishii, 1995, Simon et al., 2015, Rauskolb et al., 2017). Mice with overexpression of neuron-specific IGF binding protein 5 (IGFBP5: an inhibitory IGF binding protein) or with depleted IGF1R expression displayed a progressive neurodegeneration similar to that seen in diabetic neuropathy. These deficits included motor axonopathy and sensory disorders such as thermal hypoalgesia and epidermal nerve fiber loss (Simon et al., 2015). Osmotic minipump implants releasing 4.2-4.8 µg/day IGF-1 or IGF-2 for two weeks prevented hyperalgesia and promoted nerve regeneration in STZ-diabetic rats that had a sciatic nerve crush injury (Zhuang et al., 1996, Ishii and Lupien, 1995). In a similar study, STZ-induced diabetic rats exhibited loss of IENF after three months of diabetes, which was prevented by one-month intrathecal delivery of IGF-1 (Toth et al., 2006). Five micromolar (5 µM) intrathecal

infusion of IGF-1 for one month also improved sensory and motor nerve conduction velocities in diabetic rats (Brussee et al., 2004).

We previously reported a significant loss of nerves of paw skin and concurrent hypoalgesia in 5 month diabetic rats when compared to controls, and that insulin implants for the last four months prevented IENF loss and thermal hypoalgesia without correcting hyperglycemia (Aghanoori et al., 2017). In the present study, maintaining diabetic rats for 5 months did not cause significant nerve fiber loss in the foot skin compared to age-matched control rats, despite concurrent thermal hypoalgesia. There is some precedence for this disassociation as thermal hypoalgesia precedes detectable IENF loss in STZ-diabetic mice (Beiswenger et al., 2008) while acute depletion of IENF in humans using topical capsaicin does not produce loss of heat discrimination until over 50% of IENF are ablated (Malmberg et al., 2004). The amelioration of thermal hypoalgesia by IGF-1 in diabetic rats could be due to enhanced sensitivity of afferents via such known properties as stimulation of T-type channels (Zhang et al., 2014) or sensitization and translocation of the vanilloid receptor (Van Buren et al., 2005). Alternately, as IGF-1 enhances substance P and CGRP expression in DRG cultures (Liu et al., 2010b), it may protect against the diabetes-induced depletion of sensory neuron neuropeptide expression (Diemel et al., 1994) and stimulus-evoked spinal release (Calcutt et al., 2000) that is associated with early stages of thermal hypoalgesia (Beiswenger et al., 2008).

Nutrient flux analysis in *db/db* type 2 diabetic mice at 24 weeks of age showed an increase in flux of TCA intermediates in kidney but a decrease in sciatic nerve (Sas et al., 2016). Metabolomic analysis of tibial nerves in the current study exhibited an increase in TCA intermediates in diabetic rats. Thus, if reduced flux of metabolites is occurring (Sas et al., 2016), this build-up of TCA intermediates suggests that a deficiency in ETS electron flow and/or

enzyme activities is causing impaired utilization of the excessive available energy sources provided by the Krebs cycle under diabetic conditions. The diabetes-induced suppression of spare respiratory capacity combined with reduced ETS protein expression and activity of Complexes I and IV support the last statement. Despite no effect on up-regulated intracellular sorbitol and glucose in nerve in diabetes, IGF-1 therapy reinstated ketosis, lowered TCA intermediate build-up, and normalized oxidant status. Moreover, increased free amino acids or fragmented peptides in tibial nerves of diabetic rats demonstrate disturbed protein synthesis and metabolism, which were restored by IGF-1 therapy, correlating with higher phosphorylation of P70S6K in DRGs.

Given the established role of IGF-1 in neurite outgrowth, differentiation and development, a plethora of nervous system-related diseases including Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, Fragile X syndrome and Rett syndrome have become candidates for IGF-1 therapy (Costales and Kolevzon, 2016). A double-blind clinical trial of IGF-1 therapy in ALS patients receiving IGF-1 (0.5-3 μg/kg every two weeks for 40 weeks) resulted in beneficial effects with no adverse events (Nagano et al., 2005). A phase 1 clinical trial of IGF-1 therapy (40-120 μg/kg twice daily for 4 weeks) in 12 girls with Rett syndrome improved measures of anxiety, mood and apnea with no serious adverse effects (Khwaja et al., 2014). IGF-1 therapy remains a potential therapeutic option in diabetic neuropathy but has been hindered by other potential systemic actions of the peptide. Our studies reveal a novel aspect of IGF-1 signaling to augment the AMPK pathway to drive nerve repair. Specific targeting of the IGF-1 signaling axis in neurons could offer an alternative or complementary approach to treating neurological disease.

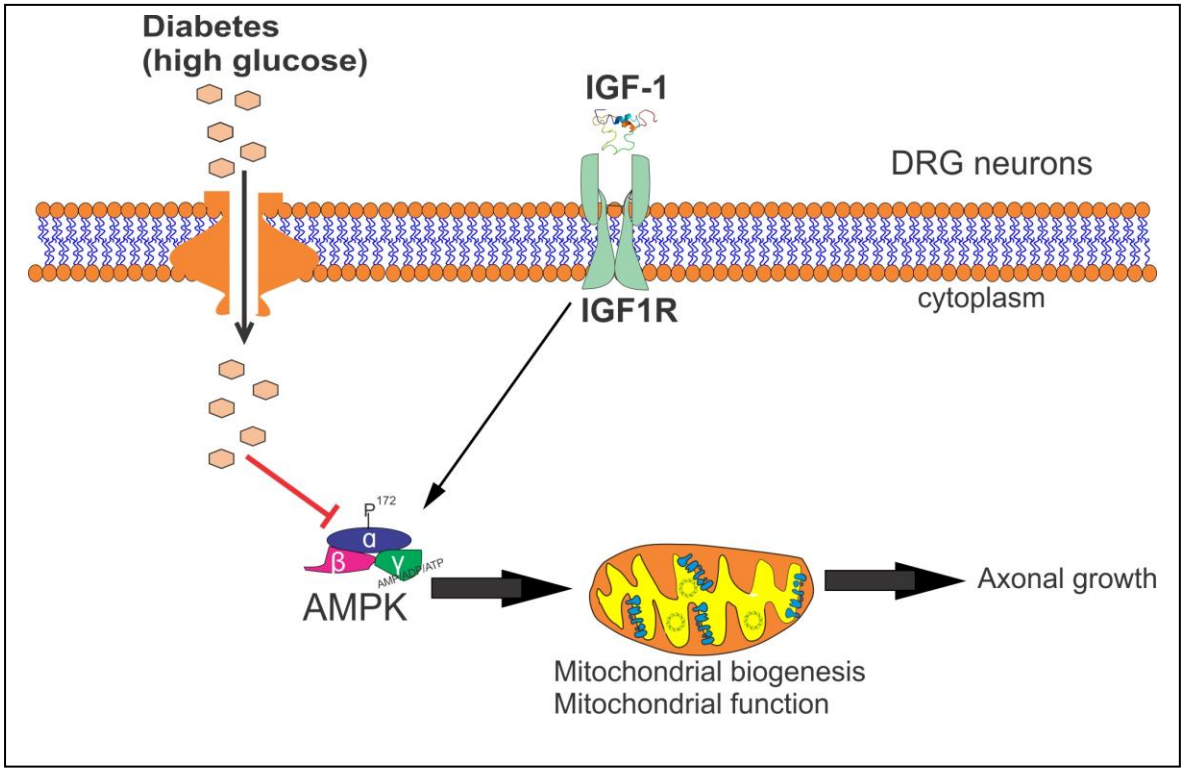

Figure 3.9. IGF-1 circumvents diabetes-induced suppression of AMPK to drive mitochondrial function and nerve repair. AMPK activity is downregulated under hyperglycemic condition. IGF-1 overcomes this suppression leading to increased mitochondrial function and improved nerve structure and function in diabetes. This figure is original and was prepared by myself.

Acknowledgments

We would like to thank Dr. Mohammad Golam Sabbir, St Boniface Research Centre, for his contribution to 2D gel set up and Alex Marquez and Katie Frizzi for excellent technical support. This work was supported by grant # MOP-130282 from the Canadian Institutes of Health Research to P.F. and grants NS081082 and 3DP3DK108245/T729934 from the National Institutes of Health to N.A.C. We are grateful to St Boniface Hospital Research for support.

Author contributions

M.R.A. designed and performed the in vitro studies and analyzed all tissues from the in vivo study. He aided in the metabolomic analysis and thermal sensitivity testing, performed data generation and analysis and contributed to writing of the manuscript. D.S. directed maintenance of diabetic rats, performed thermal sensitivity testing, supervised IGF-I therapy and aided in data analysis and statistics. A.A. performed in vitro studies identifying IGF-1 as a regulator of AMPK. M.A. and S.S. performed metabolomic analysis and bioinformatics. C.A.J. and F.D. maintained and treated diabetic mice and performed imaging studies on the cornea. A.N. performed terminal analysis of corneal confocal measurements. X.Z. performed IENF analysis. N.A.C. supervised skin histopathology and corneal confocal microscopy studies and aided in writing of manuscript. P.F. supervised all in vitro and in vivo work and wrote the manuscript.

Supplementary materials:

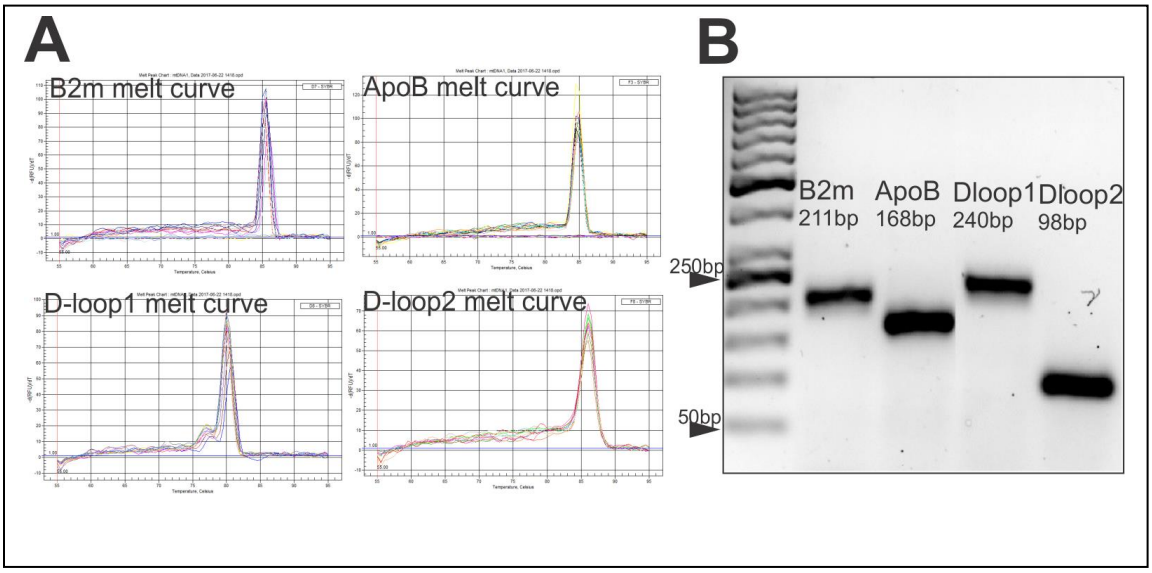

Figure 3.10.Supplemental Figure. PCR products of our mtDNA/nDNA assay are validated using melt curve and agarose gel analyses. DRG neurons derived from adult control rats were treated with IGF-1 for 24-48 h and underwent Real-Time PCR to measure mtDNA copy number relative to nuclear DNA. (A) Melt curve and (B) agarose gel of the PCR products shown here indicate the validity of the target sequences (two mtDNA and two nDNA sequences) and the method. Data are mean ± SEM of N=3-4 replicates.

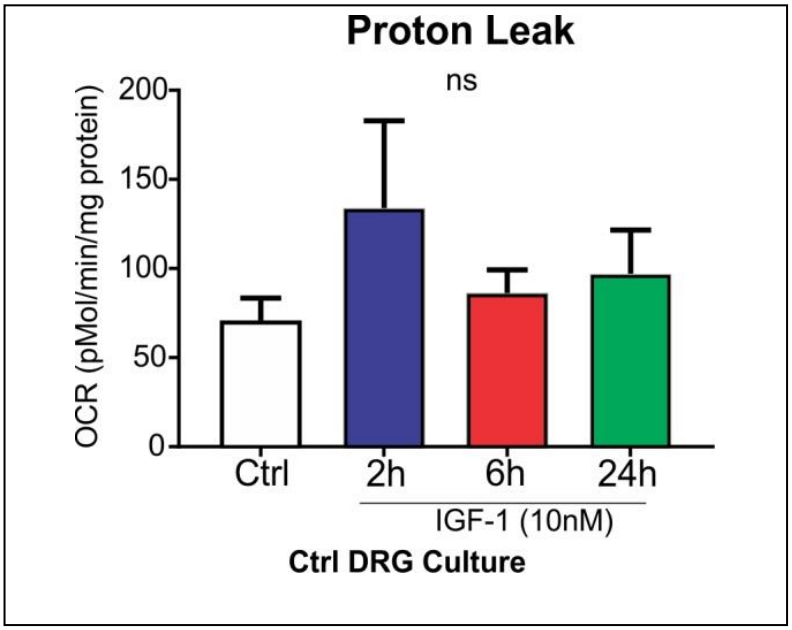

Figure 3.11.Supplemental Figure. IGF-1 does not change proton leak in DRGs from control rats. DRG neurons derived from adult control rats were treated with/without IGF-1 for 2-24 h. OCR was measured using Seahorse XF24 Analyzer. Proton leak was calculated by subtracting non-mitochondrial respiration from oligomycin-insensitive mitochondrial respiration. Data are mean ± SEM of N=4-5 replicates; analyzed by one-way ANOVA with Dunnett's *post-hoc* test.

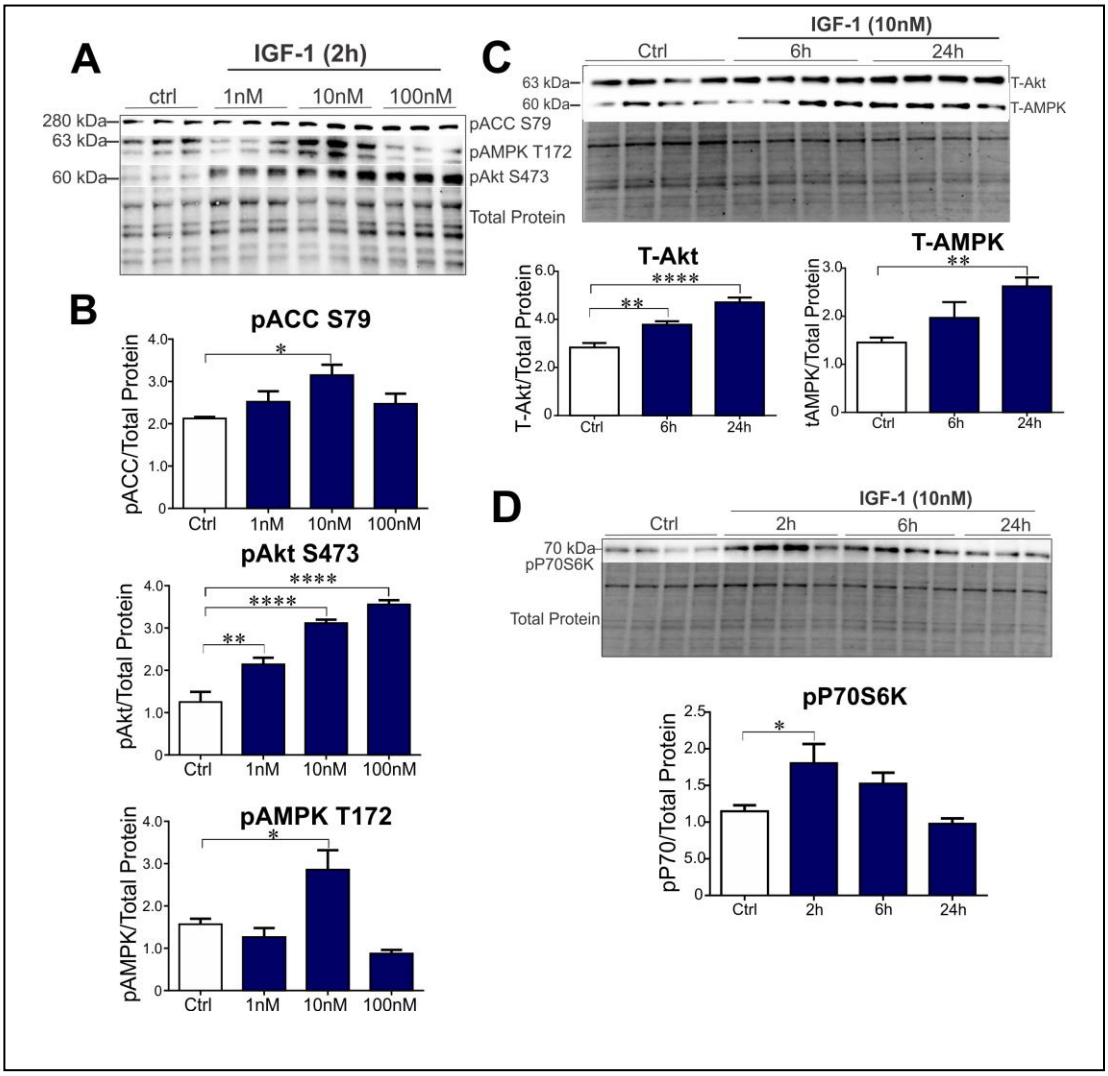

Figure 3.12.Supplemental Figure. IGF-1 doses increase Akt and AMPK activity, and total AMPK and Akt protein expression in long time treatment. DRG neurons derived from adult control rats were treated with/without (A, B) different IGF-1 doses for 2 h or (C, D) 10 nM IGF-1 for 2-24 h and lysates subjected to Western blotting. Specific bands from each protein was quantified and expressed relative to total protein of the same blot. Data are mean ± SEM of N=3-4 replicates; *= $p<0.05$ or **= $p<0.01$ or ****= $p<0.0001$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

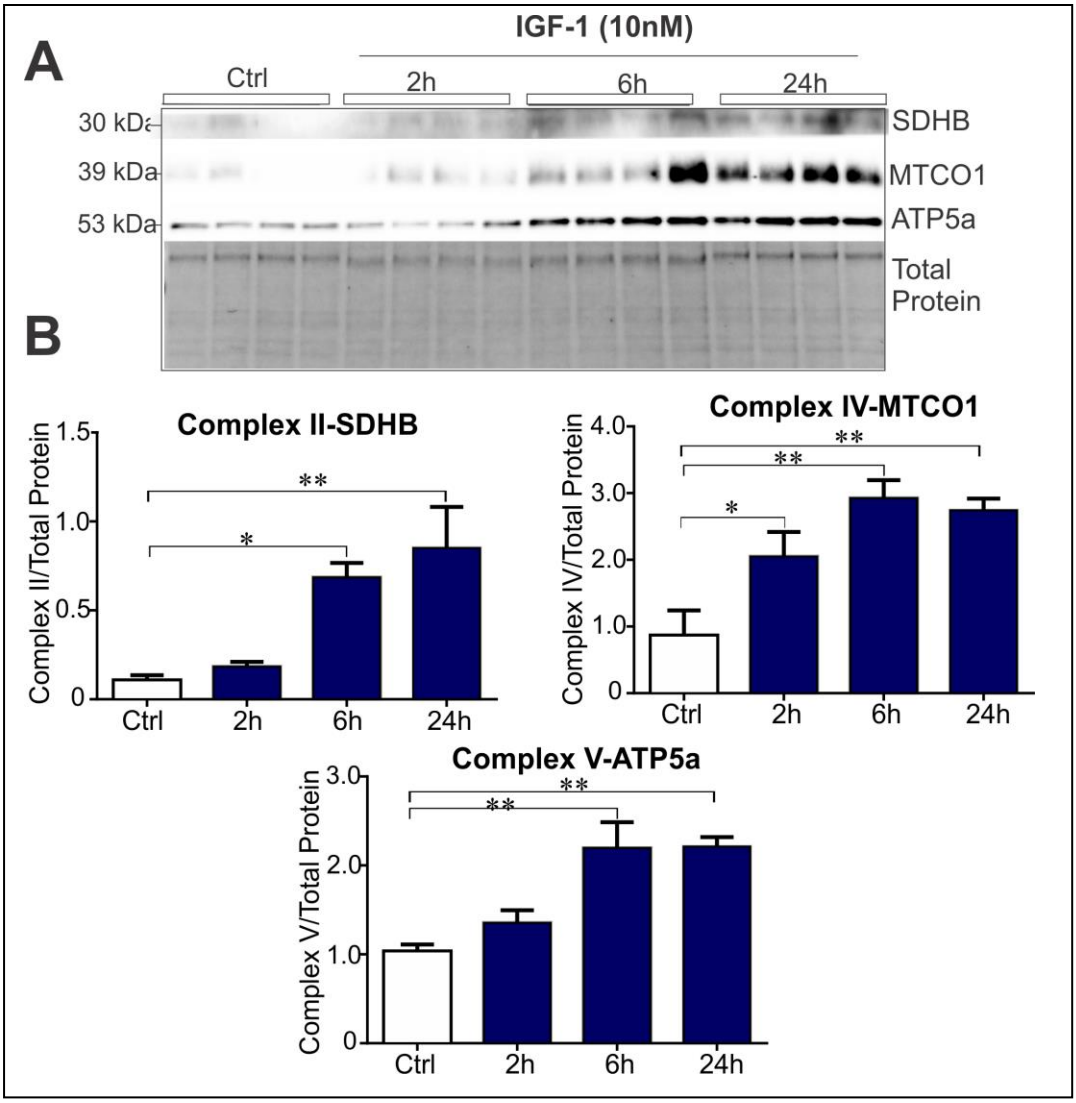

Figure 3.13.Supplemental Figure. IGF-1 treatment upregulates the expression of electron transport chain proteins in cultured DRGs from diabetic rats. (A, B) DRG neurons derived from adult diabetic rats treated with/without 10 nM IGF-1 for 24 h and lysates subjected to Western blotting. Specific proteins from each respiratory Complex were quantified and expressed relative to total protein. Complexes I-NDUFB8 and III-UQCRC2 subunit proteins were not detectable. All data are mean ± SEM of N=3-4 replicates; *= $p<0.05$ or **= $p<0.01$ vs ctrl by one-way ANOVA with Dunnett's *post-hoc* test.

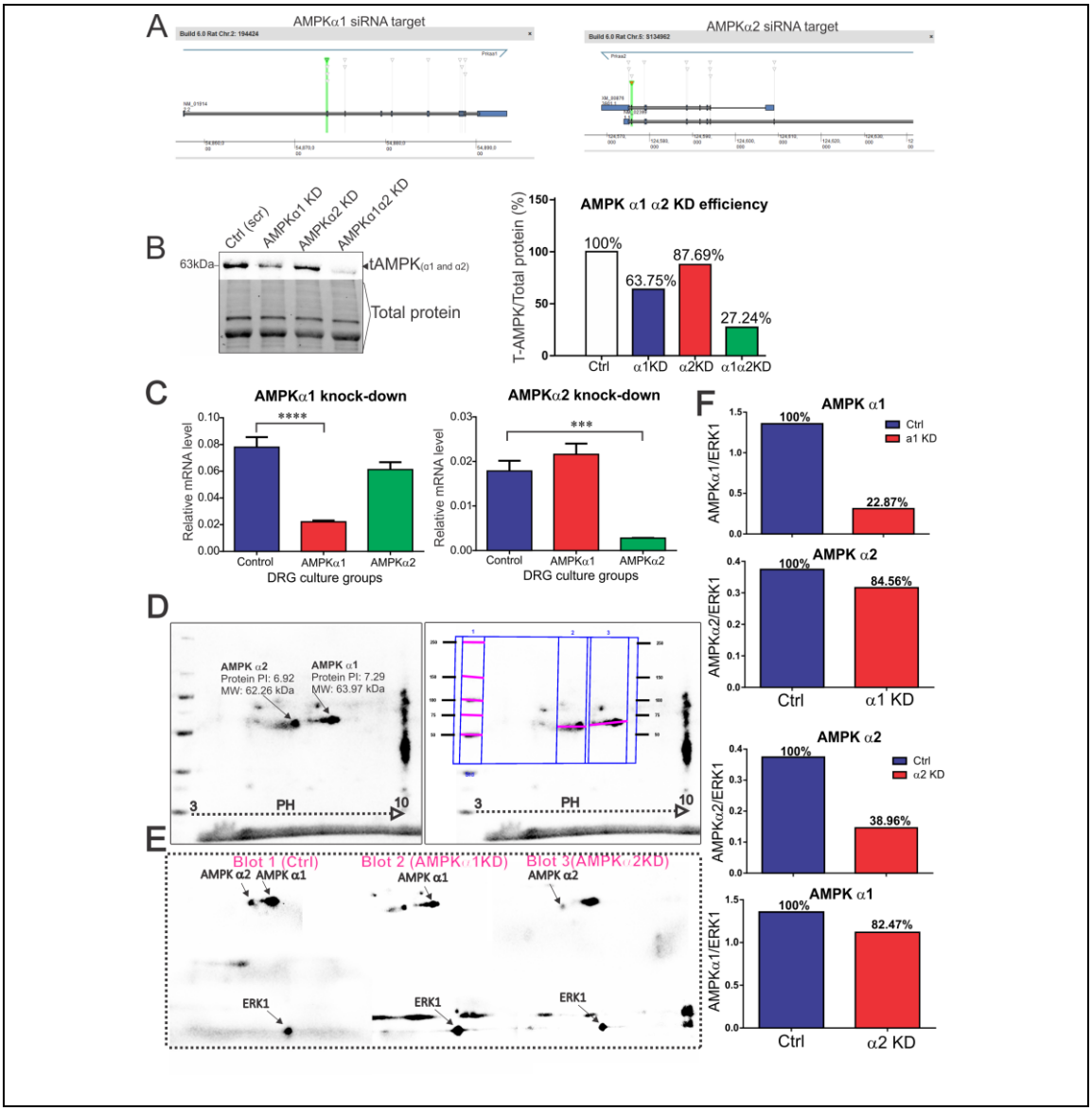

Figure 3.14.Supplemental Figure. Supplemental Figure 5: AMPK isoform knockdown efficiency and specificity in DRG neurons. DRG neurons derived from adult control rat transfected with (A) siRNAs specific to AMPK specific isoforms (α1 and α2) were cultured for 36-48 h and subjected to (B) Western blotting, (C) Real-Time PCR and (D, E, F) 2D gel followed by blotting. In (A), the target sequence map (AMPKα1 and α2 mRNAs) for each siRNA is shown. The image is adopted from Thermo Scientific (Thermo Scientific, Pittsburgh,

PA, USA) website. In (B), Western blotting for total AMPK was used to calculate the knock-down efficiency of AMPK isoforms alone and together. In (C), knock-down efficiency for each AMPK isoforms at mRNA levels was analyzed using Real-Time PCR and normalized to GAPDH mRNA level. In (D), theoretical molecular size and isoelectric point for AMPK isoforms was calculated using ExPASy software (ExPASy Bioinformatics Resource Portal) and validated using ImageLab software after 2D-gel-based isoform separation. In (E), all AMPK blots were imaged with the same amount of antibodies and exposure time, and normalized to corresponding ERK1 blots. In (F), normalized AMPK blot intensities were plotted. In (C) ***= $p<0.001$ or ****= $p<0.0001$, analyzed by one-way ANOVA with Dunnett's *post-hoc* test.

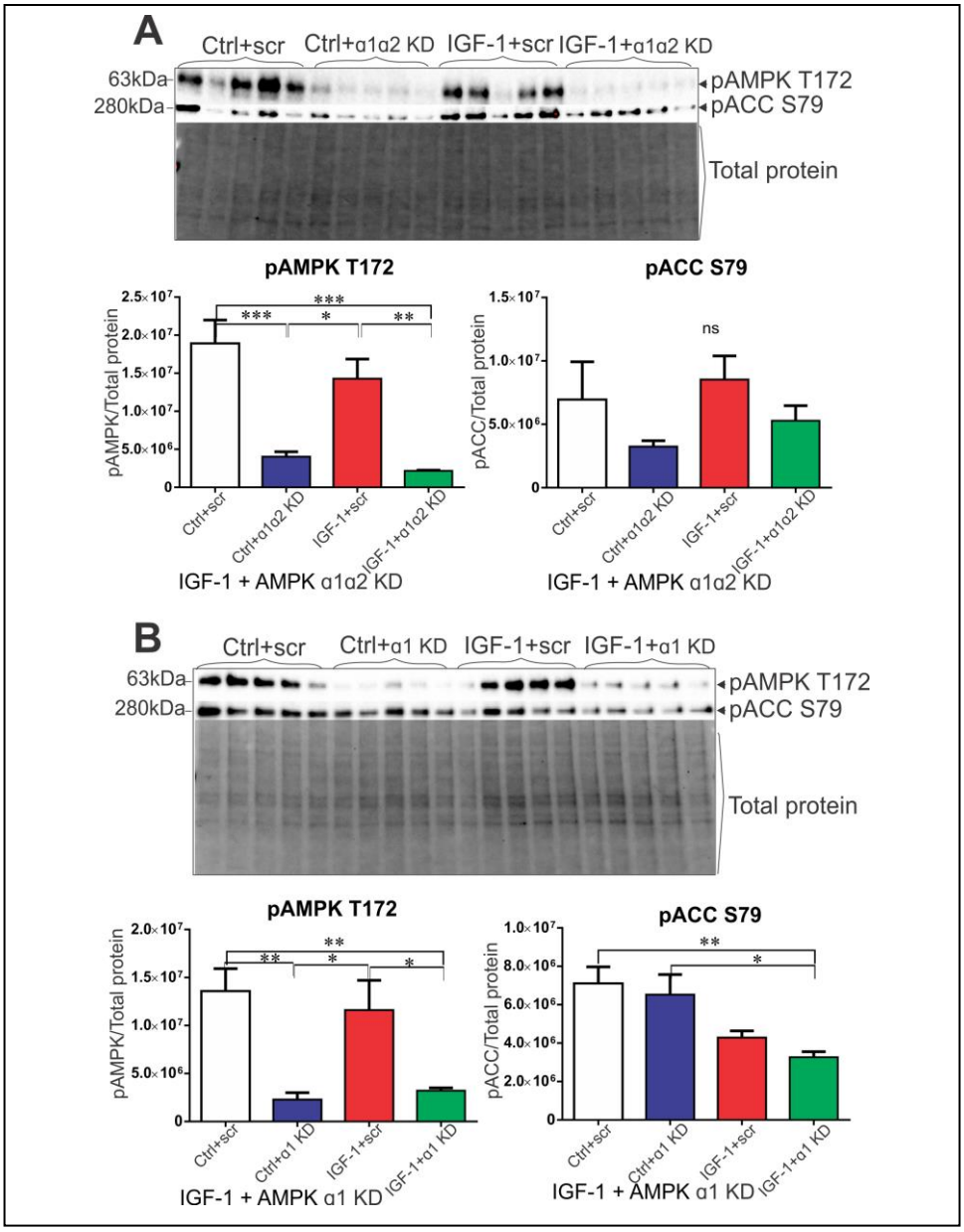

Figure 3.15.Supplemental Figure. DRG neurons used for Seahorse assay presented in Figure 5 were harvested and underwent Western blotting. (A) AMPKα1 and α2 siRNAs co-transfection or (B) AMPKα1 siRNA transfection was performed prior to seeding and IGF-1 treatment. Band intensity of proteins were normalized to total protein of the same blot. Data are mean ± SEM of N=5 replicates; *= $p<0.05$ or **= $p<0.01$ or ***= $p<0.001$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

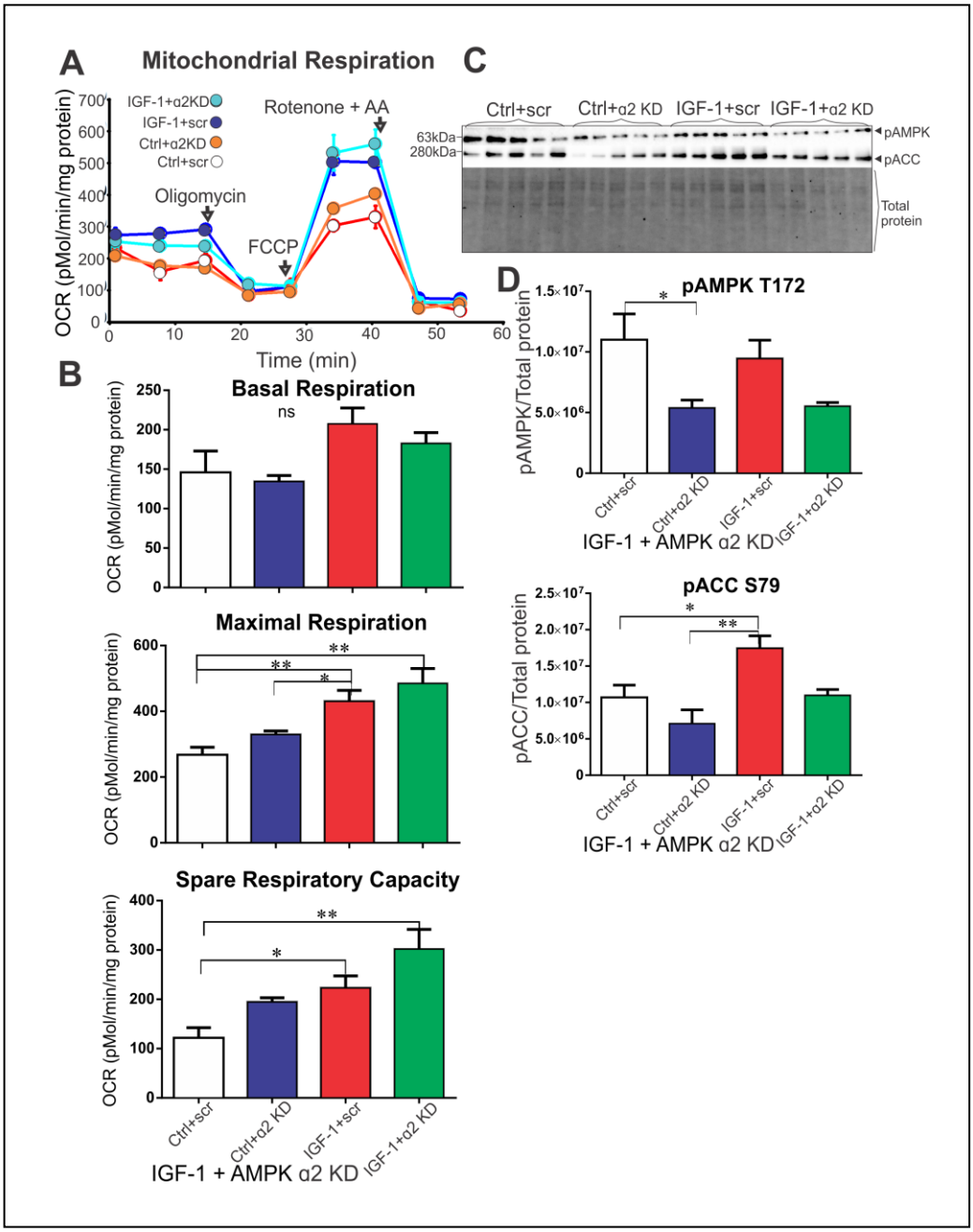

Figure 3.16.Supplemental Figure. AMPKα2 knockdown does not block IGF-1 up-regulation of mitochondrial respiration. DRG neurons derived from adult control rats were transfected with AMPKα2-specific siRNA, cultured for 24 h and treated with/without 10 nM IGF-I for another 24 h. (A, B) Mitochondrial respiration was measured using Seahorse XF24

Analyzer. Data were normalized to protein concentration units per well prior to statistical analysis. (C, D) DRG neurons were harvested and subjected to Western blotting. Data are mean ± SEM of N=4-5 replicates; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Tukey's.

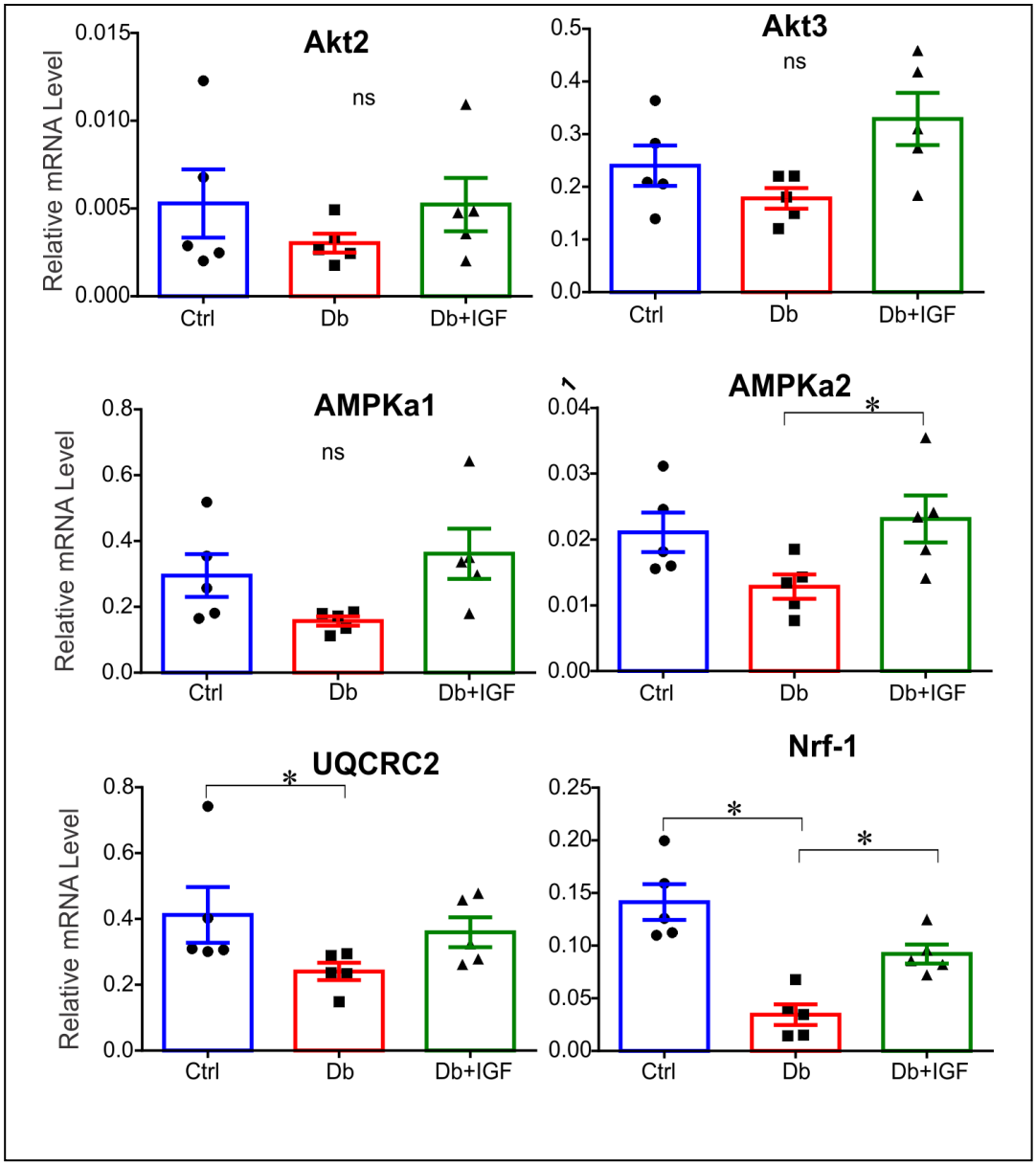

Figure 3.17.Supplemental Figure. IGF-1 therapy restores the mRNA level of AMPK alpha2, Nrf-1 and Complex III-UQCRC2 genes in diabetic rats. DRG tissues from control (Ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were isolated and subjected to Real-Time PCR. All mRNA levels were calculated relative to *GAPDH* mRNA levels using delta dela Ct method. Data are mean ± SEM of N=4-6 (duplicate test tubes in Real-Time PCR); *= $p<0.05$; analyzed by paired or unpaired one-way ANOVA with Tukey's or Dunn's *post-hoc* test.

Table 3.1.Supplemental Table: IGF-1-treated diabetic rats showed no change in their blood glucose and HbA1c compared to untreated diabetic rats. Animals were maintained for five and a half months. Blood glucose, HbA1C and body weight were recorded at tissue collection. Data are mean ± SEM of N=10-13; * = $p<0.001$ vs. other groups by one-way ANOVA with Tukey's *post-hoc* test.

Group	**N**	**Body weight (g)**	**Blood glucose (mmol/L)**	**HbA1c (%)**
Control	10	797.17±16.44 *	8.83±0.27 *	4.88±0.11 *
Diabetic	13	479.71±18.65	36.55±1.09	11.97±0.43
IGF-1-treated diabetic	12	451.18±15.39	35.87±1.90	11.39±0.37

Table 3.2.Supplemental Table. Targeted analysis of 35 metabolites linked to mitochondrial function, carbohydrates and protein metabolisms in tibial nerve of animals. Animals were maintained for 24 weeks. Tibial nerve tissues from control (Ctrl), diabetic (Db) and IGF-1-treated diabetic (Db+IGF-1) rats were dissected and subjected to targeted metabolomic analysis. Data are mean ± SEM of N=9-12 animals; $p< 0.05$; analyzed by one-way ANOVA followed by Benjamini Hochberg FDR and Tukey HSD tests (low case letters a, b and c indicates significant changes).

Notes: * indicates the detected sodium adduct [M+Na]+**,** £ indicates formate adduct [M+HCOO]-**,** ¥ indicates that all values are average Log 2 abundance for each individual metabolite.

No.	Metabolite	Formula	m/z	ESI Polarity	[Ctrl]	[Db]	[Db+IGF-1]	Corr. P value
	Carbohydrate metabolism							
1	Sorbitol	C6 H14 O6	183.0846	+	3.37[b]	11.88[a]	11.22[a]	0.028
2	Glucose	C6 H12 O6	203.0526	+	11.63[b]	15.65[a]	15.07[a]	0.029
3	Myoinositol 4-phosphate	C6 H13 O9 P	283.0188*	+	5.43[b]	10.00[a]	7.35[b]	0.028
4	D-Glycerate 2-phosphate	C3 H7 O7 P	184.9855	–	3.30	3.61	5.29	NS
5	α-D-Xylose 1-phosphate	C3 H7 O6 P	168.9908	–	17.75	18.07	15.28	NS
6	alpha-D-Glucose 1,6-biphosphate	C6 H14 O12 P2	338.9891	–	7.35	7.14	6.86	NS
7	D-Mannitol	C4 H10 O4	181.0720	–	13.37[b]	16.45[a]	16.00[a]	0.000001
8	D-Tagatose	C6 H12 O6	181.0709	+	11.63[b]	15.65[a]	15.07[a]	0.032
9	L-Fuculose	C4 H8 O3	163.0614	–	-0.59[b]	9.86[a]	6.54[a]	0.0001
10	Sorbose	C6 H12 O6	179.0561	–	15.37	15.63	13.16	NS
11	D-Hexose 6-phosphate	C6 H13 O9 P	259.0226	–	17.46[a]	18.40[a]	15.80[b]	0.032
	TCA metabolites							
12	Citric acid	C6 H8 O7	215.0160	+	6.88[b]	10.92[a]	7.37[b]	0.030
13	Succinic acid	C4 H6 O4	119.0354	+	1.32[b]	2.83[a]	1.56[b]	0.029
14	Fumaric acid	C4 H4 O4	134.0449	+	-0.36[b]	2.55[a]	1.62[b]	0.030
15	Malic acid	C4 H6 O5	135.0303	+	5.81[b]	12.09[a]	3.14[b]	0.032
16	Methylmalonic acid	C4 H6 O4	117.0192	–	2.73	5.92	4.41	NS
17	3-Dehydro-L-threonate	C3 H4 O3	133.0142[£]	–	8.47	7.84	9.97	NS
18	Isocitrate	C4 H4 O5	191.0195*	–	16.20	16.28	13.77	NS

	Protein metabolism							
19	Leucine	C6 H13 N O2	132.1016	+	6.51[b]	12.85[a]	7.28[b]	0.027
20	Aspartic acid	C4 H7 N O4	134.0450	+	5.94[b]	10.73[a]	4.07[b]	0.030
21	N-Hydroxy-L-phenylalanine	C9 H11 N O3	182.0812	+	10.35[b]	12.91[a]	12.27[a]	0.028
22	Ala Cys Asp	C10 H17 N3 O6 S	308.0909	+	7.19[b]	11.96[a]	7.58[b]	0.028
23	D-Aspartic acid	C4 H7 N O4	132.0302	–	12.27	13.26	12.16	NS
24	(R)-2-Amino-3-hydroxypropanoic acid	C3 H7 N O3	150.0419[£]	–	3.38	9.01	6.26	NS
25	DL-Homoserine	C4 H9 N O3	118.0509	–	8.92	3.10	6.07	NS
26	DL-β-Leucine	C6 H13 N O2	130.0870	–	1.21	0.05	0.29	NS
27	(3S)-3,6-Diaminohexanoate	C6 H14 N2 O2	147.1127	+	7.27	10.15	10.70	NS
28	N-Hydroxy-L-phenylalanine	C9 H11 N O3	180.0661	+	10.34[b]	12.91[a]	12.27[a]	0.032
	Oxidant status							
29	Glutathione, oxidized	C20 H32 N6 O12 S2	613.1591	+	5.49[b]	11.42[a]	6.81[b]	0.031
	Ketosis metabolites							
30	β-hydroxy butyric acid	C4 H8 O3	127.0378*	+	[¥]4.12[b]	7.78[a]	5.23[b]	0.027
31	Erythrono-1,4-lactone	C4 H6 O4	117.0192	+	1.32[b]	2.83[a]	1.55[b]	0.032
32	Dihydroxyacetone (glycerone)	C3 H6 O3	135.0300[£]	–	1.28	5.56	8.87	NS
33	4-(3-Pyridyl)-butanoic acid	C9 H11 N O2	164.0714	–	15.60	15.41	13.07	NS
	Fatty acid metabolism							
34	N-Acryloylglycine	C5 H7 N O3	128.0352	–	16.37[a]	16.66[a]	13.98[b]	0.032
35	14-Methylheptadecanoic acid	C18 H36 O2	285.2790	+	-0.36[a]	0.009[a]	1.34[b]	0.032

Transition Statement Two

Aims 1, 2 and 3 of the part 2 (chapter 3) of this thesis were to determine the effect of IGF-1 as a therapy on AMPK activity, mitochondrial function and nerve degeneration in sensory neurons in diabetes. The mechanism of IGF-1 regulation of mitochondrial function and nerve repair was also investigated. We demonstrated for the first time that IGF-1 activated and up-regulated AMPK to augment mitochondrial function, ATP production, mtDNA copy number and expression of ETS proteins in cultured rat DRG neurons. IGF-1 utilized this AMPK pathway and, possibly in parallel, the Akt pathway to drive axonal outgrowth. Further, we showed that IGF-1 therapy prevented diabetes-induced progressive loss of sensory nerves in the cornea and ameliorated paw thermal hypoalgesia. Correction of these clinically relevant endpoints was associated with suppression of build-up of TCA intermediates in nerve and optimization of mitochondrial phenotype in the DRG of type 1 diabetic rats. The stimulatory effect of IGF-1 on mitochondrial oxygen consumption rate can be explained by signaling via the AMPK α1 isoform. The elevation in mtDNA copy number revealed the involvement of AMPK α2 isoform downstream from IGF-1 signaling. However, endogenous IGF-1 in sensory neurons, its regulation and function were not addressed in chapter 3. Since the level of endogenous IGF-1 in the liver and DRGs is low in animal models of diabetic neuropathy, we investigated if endogenous IGF-1 contributes to the pathogenesis of diabetic neuropathy. In three aims of part 3 (chapter 4) of this thesis, we address how endogenous IGF-1 in DRG tissue is suppressed in diabetic condition and which cells in DRG tissue produce IGF-1 and what the function is.

Chapter 4: Sensory neuron-derived IGF-1 augments neurite outgrowth and this autocrine/paracrine pathway is suppressed in diabetes

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Abstract

The level of insulin-like growth factor 1 (IGF-1) in serum of diabetic patients and animal models with type 1 and 2 diabetes declines significantly as the disease progresses. Recently, IGF-1 has been used for treatment of neurodegenerative disorders including Alzheimer's disease and amyotrophic lateral sclerosis. We hypothesized that impaired autocrine/paracrine IGF-1 in dorsal root ganglia (DRG) was a contributing factor to progressive neurodegeneration and impaired nerve regeneration in diabetic sensory neuropathy. DRG neuron cultures and tissues from age-matched control or streptozotocin (STZ)-induced type 1 diabetic rats were used for *in vitro* and *in vivo* studies. Despite no difference in IGF-1 receptor level, IGF-1 protein and mRNA levels in liver and DRG tissues were significantly ($P<0.05$) lower in type 1 diabetic rats vs age-matched control rats. DRG neurons derived from control rats secreted a higher amount of IGF-1 into the culture media compared to cultures from diabetic rats ($P<0.05$). IGF-1 mRNA was expressed in neurons of the DRG and brain rather than in glial cells or sciatic nerve tissue as determined by RNA-FISH and Northern blot analysis. The hyperglycemic state suppressed IGF-1 mRNA expression in DRG neurons after 2 days which was relieved by treatment with (10nM) IGF-1 or an aldose reductase inhibitor, Sorbinil (blocks polyol pathway activity under high [glucose]). Bioinformatic screening and chromatin immunoprecipitation assay revealed NFAT1 and CEBP-β functional binding sites on the IGF-1 gene promoter in rat DRG neurons. In growth factor-free media, either IGF-1 neutralizing antibody or two IGF-1-targeting encapsulated siRNAs (in cationic nanoparticles) downregulated IGF-1 receptor and Akt S473 phosphorylation, and lowered background neurite outgrowth in cultured DRG neurons. In conclusion, downregulation of endogenous IGF-1 in DRG neurons in diabetes may contribute to pathogenesis of progressive

distal dying-back neurodegeneration and up-regulation of neuronal IGF-1 at the mRNA level may be a promising target for therapy.

Key words: Endogenous IGF-1; dorsal root ganglia; axon regeneration; diabetic neuropathy; neurite outgrowth.

Introduction

Insulin-like growth factor-1 (IGF-1) functioning as a neurotrophic factor has been a therapeutic target in many disorders including Alzheimer's disease, Fragile X syndrome, Rett syndrome, amyotropic lateral sclerosis (ALS) and Parkinson's disease (Costales and Kolevzon, 2016). In a clinical trial, ALS patients who received IGF-1 twice weekly for 40 weeks exhibited improved motor nerve function and there were no adverse side-effects (Nagano et al., 2005). IGF-1 therapy in 12 female Rett syndrome patients improved mood stability, apnea and anxiety after 4 weeks of treatment in a phase 1 clinical trial (Khwaja et al., 2014).

Suppressed IGF-1 expression and subsequent depression of its signaling pathway have also been proposed to contribute to neurodegeneration in diabetes (Ishii, 1995, Rauskolb et al., 2017, Zochodne, 2016b) since the level of systemic or background IGF-1 is markedly diminished in humans and animal models of type 1 and type diabetes (Zhuang et al., 1997, Palta et al., 2014, Ishii et al., 1994, Ekstrom et al., 1989). Overexpression of IGF binding protein 5 (an intrinsic IGF-1 inhibitor) or depletion of IGF-1 receptor (IGF1R) in mice enhanced the development of neurodegeneration which resembled the nerve damage observed in diabetic sensorimotor polyneuropathy (DSPN) (Simon et al., 2015).

IGF-1 therapy has been a tool utilized to augment nerve regeneration and neuro-protection in various studies. For example, IGF-1 peptide improved nerve regeneration in normal and streptozotocin (STZ)-induced type 1 diabetic rats after sciatic nerve crush (Ishii and Lupien, 1995, Ekstrom et al., 1989, Sjoberg and Kanje, 1989). Nerve regeneration was accelerated in STZ-induced type 1 diabetic rats with sciatic nerve injury following IGF-1/IGF-2 therapy using mini-osmotic pump implants (Ishii and Lupien, 1995; Zhuang et al., 1996). Hyperalgesia was also reversed in these animals. Intrathecal delivery of IGF-1 reversed sensory and motor nerve

conduction velocities and prevented intra-epidermal nerve fiber (IENF) loss and sural nerve axonal degeneration in STZ-induced diabetic rats (Toth et al., 2006, Brussee et al., 2004). IGF-1 gene therapy using adenovirus-based delivery of IGF-1 improved indices of neuropathy including myelination, nerve conduction velocities and nerve regeneration in mice with diabetes (Homs et al., 2014, Chu et al., 2008).

In vitro, IGF-1 promoted neurite outgrowth of axotomized DRG neurons (Fernyhough et al., 1993, Recio-Pinto et al., 1986), motor (Caroni et al., 1994) and sympathetic (Zackenfels et al., 1995, Recio-Pinto et al., 1986) neurons. IGF-1 and IGF1R signaling are also implicated in survival, proliferation, migration and myelinating properties of Schwann cells (Syroid et al., 1999, Chattopadhyay and Shubayev, 2009, Russell et al., 2000, Cheng et al., 2000). Upon IGF-1 binding to IGF1R, the receptor was phosphorylated/activated and recruited insulin receptor substrate-1 (IRS-1) to further activate two survival/growth pathways, the Akt/phosphoinositide-3 kinase (PI-3K) and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathways (Le Roith and Zick, 2001, Cheng et al., 2010). In neurons, IGF1Rs are expressed by all cell types (Fernyhough et al., 1993, Huang et al., 2005, Singh et al., 2012, Grote et al., 2013b) and can activate the same survival messengers and signaling pathways (Grote et al., 2013b; Huang et al., 2005; Kim et al., 2011).

Despite a plethora of evidence of systemic IGF-1 effects on cell phenotypes such as neuronal and non-neuronal survival and growth, less is known about *IGF-1* gene regulation and its function, especially with regard to the role of endogenous IGF-1 in sensory neurons. The *IGF-1* gene consists of 6 exons and 5 introns in human (chromosome 12) and rodents which is differentially spliced to produce six different protein precursors from various transcript variants (Rotwein, 2017). IGF-1 produced in the liver makes up the major portion of IGF-1 in the circulation;

however, other peripheral tissues produce and secrete autocrine/paracrine IGF-1 (Laron, 2001). In the liver, growth hormone (GH) stimulates *IGF-1* gene expression via mobilizing STAT5b transcription factor which triggers its binding to enhancer elements on the *IGF-1* gene (Rosenfeld and Hwa, 2009). GH receptor or *IGF-1* gene mutations can cause growth failure such as Dwarfism, and overproduction of GH and IGF-1 cause Acromegaly syndrome (Giustina et al., 2014, Ren et al., 2016). Several other transcription factors including hepatocyte nuclear factors (HNF)-1, HNF-3, CCAAT/enhancer-binding protein (C/EBP) α, β and δ have binding sites on *IGF-1* gene and regulate its transcription (Nolten et al., 1994, Nolten et al., 1996, Nolten et al., 1995, Umayahara et al., 1999).

Endogenous IGF-1 promotes neuronal growth and differentiation in cultured brain stem cells from adult mice (Brooker et al., 2000). Explants from the lateral ventricle of bovine brain produce and release endogenous IGF-1 which promotes survival in proliferating neurons (Perez-Martin et al., 2003). IGF-1 release from Muller cells stimulated by trans-corneal electrical stimulation (TES) delays the neuronal degeneration in retina in adult Wistar rats (Morimoto et al., 2005).

We hypothesized that impaired autocrine/paracrine IGF-1 in dorsal root ganglia (DRG) was a contributing factor to progressive neurodegeneration and impaired nerve regeneration in diabetic sensory neuropathy. The mechanism of suppression of IGF-1 under hyperglycemic condition was also tested. Thus, in this study we addressed the regulation of endogenous IGF-1 gene in DRGs from control and STZ-diabetic rats under normal and hyperglycemic conditions and its effect on sensory neuron phenotypes.

Materials and methods

Animals

As mentioned in chapter 1, we used type 1 diabetic rats because of low cost of maintenance and ease of use while reflecting most of neuropathy indices seen in human. To be consistent throughout our study, we focused on one gender (male) in the present study. Male Sprague-Dawley rats (275–325 g) were used as a model of type 1 diabetes after delivery of a single intraperitoneal injection of 90 mg/kg STZ (Sigma, St Louis, MO, USA) and were compared with age-matched control rats. A subgroup of diabetic animals received 20µg hIGF-1 peptide (peritoneal injection) thrice weekly for 11 weeks after 3 months of diabetes. Fasting blood glucose concentration was monitored half-way through the injection period and at study end using an AlphaTRAK glucometer (Abbott Laboratories, Illinois, USA) to ensure that our treatment did not affect hyperglycemia. At the study end, blood glucose, glycated hemoglobin (HbA1c Multi-test system, HealthCheck Systems, Brooklyn, NY, USA) and body weight were recorded before tissue collection. Animal procedures were approved by the University of Manitoba Animal Care Committee and followed Canadian Council of Animal Care (CCAC) rules.

Adult DRG sensory neuron, Schwann cell and SH-SY5Y cell culture

DRGs were isolated from adult male Sprague-Dawley (300-350g) rats and dissociated using previously described methods (Calcutt et al., 2017). Neurons were cultured in no-glucose Hams F12 media supplemented with Bottenstein's N2 without insulin (0.1 mg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite 0.1 mg/ml BSA; all additives were from Sigma, St Louis, MO, USA; culture medium was from Caisson labs, USA). DRG neurons

from control rats were cultured in the presence of 5 mM D-glucose and DRG neurons derived from STZ-induced diabetic rats with 25 mM D-glucose. No neurotrophins or insulin were added to any DRG cultures. For Schwann cell culture, a previously described method was used (Syroid et al., 1999). Briefly, freshly dissected sciatic nerve from adult control rats were cut into 1-5mm pieces, incubated with collagenase and trypsin and seeded on a poly-D-ornithine-coated plate. Dulbecco's Modified Eagle's Medium (DMEM) medium (D6046, (Sigma, St Louis, MO, USA) containing 5% fetal bovine serum (FBS), 2μM forskolin (F3917, Sigma, St Louis, MO, USA) and 10nM IGF-1 (Schumacher et al., 1993) was used to culture Schwann cells. Every 2-3 days medium was changed up to one week until when Schwann cells reached more than 70% confluence. The human neuroblastoma SH-SY5Y cell line (ATCC CRL-2266, Virginia, USA; we thank Dr. Jun-Feng Wang, University of Manitoba for providing this cell line) was cultured in DMEM/F12 (1:1) media containing 10% FBS. The following pharmacological inhibitors were used in this study: Sorbinil, an inhibitor of aldose reductase (Sigma, St Louis, MO, USA), MK-2206, a highly selective pan-Akt inhibitor (Santa Cruz Biotechnology, Texas, USA), U0126, a selective non-competitive inhibitor of MAP kinase kinase (Abcam, Cambridge, MA, USA).

Nanoparticle-siRNA packaging for IGF-1 knockdown

Nanoparticle-siRNA packaging was used to knock down endogenous IGF-1 in DRG neurons. For this purpose, a mixture of siRNA-29, 5'-GCUGAAGCCUACAAAGUCAtt-3' (siRNA ID: s127929, Thermo Scientific, Pittsburgh, PA, USA) and siRNA-31, 5'-GAAGUACACUUGAAGAACAtt-3' (siRNA ID: s127931, Thermo Scientific, Pittsburgh, PA, USA) specific to rat IGF-1, or a scrambled siRNA (Cat #:4635, Thermo Scientific, Pittsburgh, PA, USA) as a negative control were used. SiRNA benchtop transfection kit (Precision NanoSystems, Vancouver, BC, Canada) was used to encapsulate siRNAs according to the

instructions. Briefly, 20 nmol of each siRNA was reconstituted in 1 ml buffer 1, was drawn into a 3 ml syringe and inserted into the left inlet of the NanoAssemblr microfluidic cartridge. The 1 ml syringe was filled with 375 µl siRNA nanoparticle mix solution and inserted into the right inlet of the cartridge. The NanoAssemblr instrument mixed both solutions into the microfluidic chip and the encapsulated siRNAs were collected, mixed with buffer 2 and centrifuged for purification. The concentrations of encapsulated siRNAs were measured using NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA, USA) before adding to the treatment groups in cultured DRG neurons.

Quantitative Western blotting

Rat DRG neurons were harvested from culture or isolated intact from adult rats and then homogenized in ice-cold RIPA buffer containing: 25mM Tris pH=8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100 and protease phosphatase inhibitors. Proteins (2-20 µg total protein/lane) were resolved and separated via 4-20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently transferred to a nitrocellulose membrane (Bio-Rad, CA, USA) using Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA) and immunoblotted with specific antibodies against pAkt S473 (1:1000, Santa Cruz Biotechnology, Texas, USA), total Akt (1:1000, Abcam, Cambridge, MA, USA), pP70S6K T389 (1:1000, Cell Signaling Technology, Massachussetts, USA), total OXPHOS (1:2000, MitoSciences, Abcam, Cambridge, MA, USA) and total ERK (1:1000, Santa Cruz Biotechnology, Texas, USA). Total protein bands were captured by chemiluminescent imaging of the blot after gel activation (TGX Stain-Free™ FastCast Acrylamide Solutions, Bio-Rad, CA, USA) in addition to the use of T-ERK levels for target protein normalization (to adjust for loading). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG (H+L) or goat

anti-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, PA, USA. The blots were incubated in ECL Advance (GE Healthcare) and imaged using a Bio-Rad ChemiDoc image analyzer (Bio-Rad, CA, USA).

ELISA assay

Homogenized tissues or collected media from DRG cultures were assayed using the Mouse/Rat IGF-1 Quantikine ELISA kit (R&D Systems, Minnesota, USA) according to the kit instructions.

Real-Time PCR

RNA was extracted from cultured neurons or previously frozen tissue samples using TRIzol® Reagent (Invitrogen, California, USA). Complementary DNA (cDNA) was synthesized from RNA samples by using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) was performed using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA) or Bright Green Master mix (Abmgood Co., Richmond, Canada) compatible with the iQ5 Cycler machine (Bio-Rad, CA, USA). The ΔΔCt method was used to quantify gene expression. The mRNA level of GAPDH, 18s rRNA and B2m were used for normalization.

Chromatin immunoprecipitation (ChIP) assay

DRG tissues from rats were dissected, weighed and underwent ChIP analysis assay using the ChromaFlash™ High-Sensitivity ChIP Kit (Catalog # P-2027, Epigentek, Farmingdale, NY, USA). Briefly, 100mg amounts of DRG tissues were cut into small pieces, cross-linked using 1% formaldehyde for 15 min, quenched by 1.25mM glycine, centrifuged, washed and homogenized in lysis buffer using a Dounce homogenizer. The chromatin pellet was resuspended in ChIP buffer and sonicated three times (15sec each) with 40sec intervals. Chromatin from this step was

added to pre-incubated antibodies (non-immune IgG or CEBP Beta antibody-ab32358 or NFAT1 antibody-ab2722 by Abcam, Cambridge, MA, USA) in each assay strip well and incubated on an orbital shaker for 2 hours. A portion of this chromatin (10% dilution) was also saved to use as input for Real-Time PCR experiments as control. Following stringent washes, DNA fragments were collected in RNase A-Proteinase K-containing DNA releasing buffer. Different regions of rat IGF-1 promoter using specific primers and DNA samples from each group were used for Real-Time PCR. Finally, fold enrichment was calculated using the formula $FE=2^{(IgG\ CT\ -\ sample\ CT)}$ to test if there were binding sites for NFAT1 or CEPB Beta on the IGF-1 promoter.

RNA FISH

We designed 28 oligonucleotide probes, 18-26 bp in length, spanning the whole rat IGF-1 mRNA. They were fluorescently labeled with Quasar 570 (Stellaris RNA FISH, Biosearch Technologies, Petaluma, CA, USA) so that we could image using a Carl Zeiss Axioscope-2 upright fluorescence microscope equipped with AxioVision3 software. To visualize IGF-1 mRNA inside the cultured DRGs, we used the protocol for adherent cells (Stellaris RNA FISH, Biosearch Technologies, Petaluma, CA, USA). Briefly, cells seeded on coverslips were fixed with 3.7% formaldehyde for 10 min, permeabilized with 70% ethanol for 1 hour at 4°C and washed. Then, coverslips were mounted onto a 100µl hybridization buffer (made of sodium citrate buffer, formamide, salmon sperm DNA and dextran sulfate in nuclease-free water) containing 125nM probe mixture in a humidified chamber for 16 hours at 37°C. Coverslips were washed in wash buffer A (made of sodium citrate buffer and formamide in nuclease-free water) for 30min at 37°C, stained with Hoechst and washed in buffer B (made of sodium citrate buffer, Tween-20 and formamide in nuclease-free water) for 5min prior to mounting on slides for imaging. To visualize IGF-1 mRNA in DRG and liver tissues, we snap froze the OCT-embedded

tissues on dry ice, sectioned at a thickness of 10µm using a cryostat and followed the protocol for frozen tissues (Stellaris RNA FISH, Biosearch Technologies, Petaluma, CA, USA). Finally, we captured 20 images per group with a magnification of 63X. As a negative control we used a culture/section group without any probe or treated with RNase A (50 µg/mL) for 30 min at 37 °C, prior to the hybridization step.

Northern blotting

RNA was extracted from DRG, sciatic nerve and brain tissues from rat using TRIzol® Reagent (Invitrogen, California, USA). A modified protocol from the Hackett lab (https://cbs.umn.edu/hackett-lab/protocols/northern-blotting) for Norther blotting and hybridization was used. In brief, twenty micrograms (20µg) of extracted RNAs were mixed in RNA sample buffer containing 62.5% formamide, 1.14M formaldehyde, 1.25X TAE buffer, 200 µg/ml Xylene Cyanol FF and 200 µg/ml bromophenol blue, heated at 65°C for 15min and kept on ice to prevent renaturation of RNA. Denaturing gels were made by adding 1.2g agarose into 72ml nuclease-free water, heated and mixed with 10ml 10X TAE buffer and 18ml 37% formaldehyde (12.3M). Samples were run on the gel at 5V/cm in 1X TAE buffer. Gel was washed in RNase-free water and in transfer buffer (20X sodium citrate buffer) for 20min. In a pool of 20X transfer buffer, RNAs were transferred from the gel to nitrocellulose overnight, baked in oven at 80°C for 2 hours and subjected to hybridization (IGF-1 fluorescent probe mix-see RNA FISH above) for 16 hours at 42°C. The nitrocellulose filter was washed in 0.2X sodium citrate buffer containing 0.1% SDS for multiple times before visualization using a Bio-Rad ChemiDoc image analyzer (Bio-Rad, CA, USA). Two micrograms (2µg) of total RNA was used and run on a 1.2% agarose gel to visualize 5S, 18S and 28S rRNA from each tissue sample for normalization of Real-Time PCR and Northern blotting results.

Immunocytochemistry

DRG neurons were cultured on glass coverslips and were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Neurons were incubated with 5% BSA in PBS for 1 h and with neuron-specific β-tubulin III antibody (1:1000; from Sigma, St Louis, MO, USA) or IGF-1Rβ antibody (Santa Cruz Biotechnology, Texas, USA) overnight. Cells were incubated with Cy3-conjugated secondary antibody (1:1000, Jackson ImmunoResearch Laboratories Inc., PA, USA) for 1 h at room temperature following three washes with PBS. Coverslips were mounted on slides using VECTASHIELD antifade mounting medium with DAPI (Vectorlabs, inc. CA, USA) and imaged using a Carl Zeiss Axioscope-2 upright fluorescence microscope equipped with AxioVision3 software. Alternatively, confocal microscope (Zeiss LSM 510) was used to image live cells transfected with tubulin-GFP plasmid in the Amaxa® Rat Neuron Nucleofector Kit (Lonza Inc., Basel, Switzerland) using Amaxa Nucleofector machine (Lonza Inc., Basel, Switzerland). To quantify neurite outgrowth, the fluorescent signal was collected as total pixel area for neurites and was measured by the high throughput NeurphologyJ plugin in ImageJ software after image enhancement. Total pixel area was normalized to number of cell bodies to calculate total neurite outgrowth per neuron.

Statistical analysis

Data were analyzed using two-tailed Student's t-tests or one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, as appropriate and indicated (GraphPad Prism 7, GraphPad Software). A P value < 0.05 was considered to be significant.

Results

All cell types in the DRG express IGF-1R subunits, and the receptor level is not different in DRG from control or type 1 diabetic rats

IGF-1 receptor subunits (α and β) in cultured DRG neurons, Schwann cells or human neuroblastoma SH-SY5Y cell line were analyzed using Western blotting. All these cells express IGF1R subunits enabling them to respond to IGF-1 peptide (Figure 4.1A). In DRG neuron culture, all cell types including neurons, satellite cells and Schwann cells were positive when stained for IGF-1Rβ (Figure 4.1B). The level of IGF-1Rβ was not different between DRG tissues isolated from control, hIGF-1-treated and untreated type 1 diabetic rats (Figure 4.1C-D). However, a significant increase ($P<0.05$) in the level of pre-pro-IGF-1 protein was observed in the DRG of hIGF-1-treated diabetic rats vs. other groups (Figure 4.1C-D).

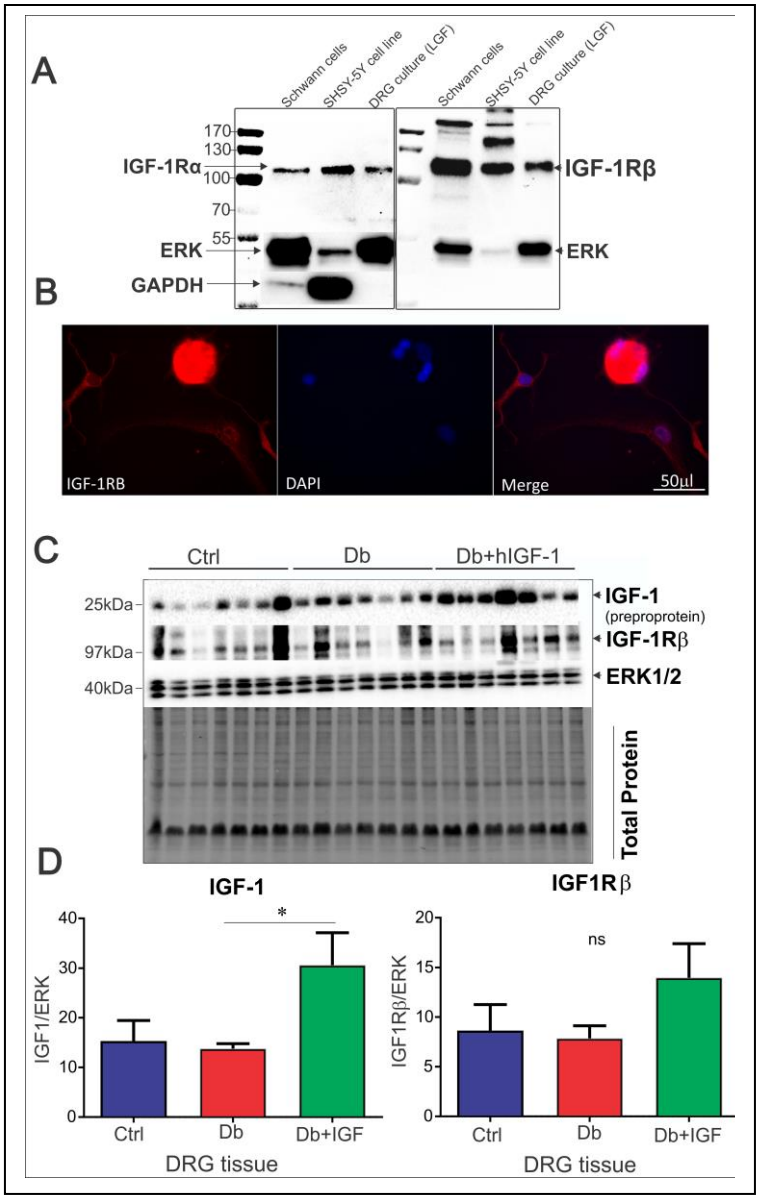

Figure 4.1. All cell types in the DRG express IGF1R subunits, and the receptor level was not different in DRG tissue from control and diabetic rats. (A) IGF-1 receptor subunits (α and β) in cultured DRG neurons, Schwann cells or SHSY-5Y cell lines were analyzed using Western blotting. In (B), DRG neurons from control (Ctrl) rats were cultured stained for IGF-1Rβ. In (C) and (D), DRG tissues from control (Ctrl), hIGF-1-treated (Db+hIGF-1) and untreated diabetic (Db) rats were homogenized and underwent Western blotting for IGF-1 and

IGF-1Rβ proteins. ERK band intensity was used for normalization. Data are mean ± SEM of N=7 replicates; *= $p<0.05$; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

The level of IGF-1 is reduced in the liver and DRG tissue from diabetic rats and is restored by treatment with exogenous hIGF-1

There was a suppression of the level of IGF-1 protein in the liver from hIGF-1-treated and untreated diabetic rats compared to control ($P<0.01$) (Figure 4.2A). Endogenous IGF-1 protein was restored in DRG from hIGF-1-treated diabetic rats (Figure 4.2B-C). Importantly, DRG expressed mRNA for IGF-1, and this endogenous expression was reduced in the diabetic state and significantly up-regulated by exogenous hIGF-1 treatment. We collected the conditioned media from DRG cultures derived from control or diabetic rats and measured endogenous IGF-1 production and secretion using an IGF-1 ELISA assay. There was a significant reduction ($P<0.05$) of IGF-1 protein secretion in DRG neuron cultures derived from diabetic rats when compared to control rats (Figure 4.2D). Of the note, the IGF-1 transcript variants 3 and 4 were expressed at higher levels compared with transcript variants 1 and 2 in rat DRG tissue (Figure 4.10A.Supplemental Figure).

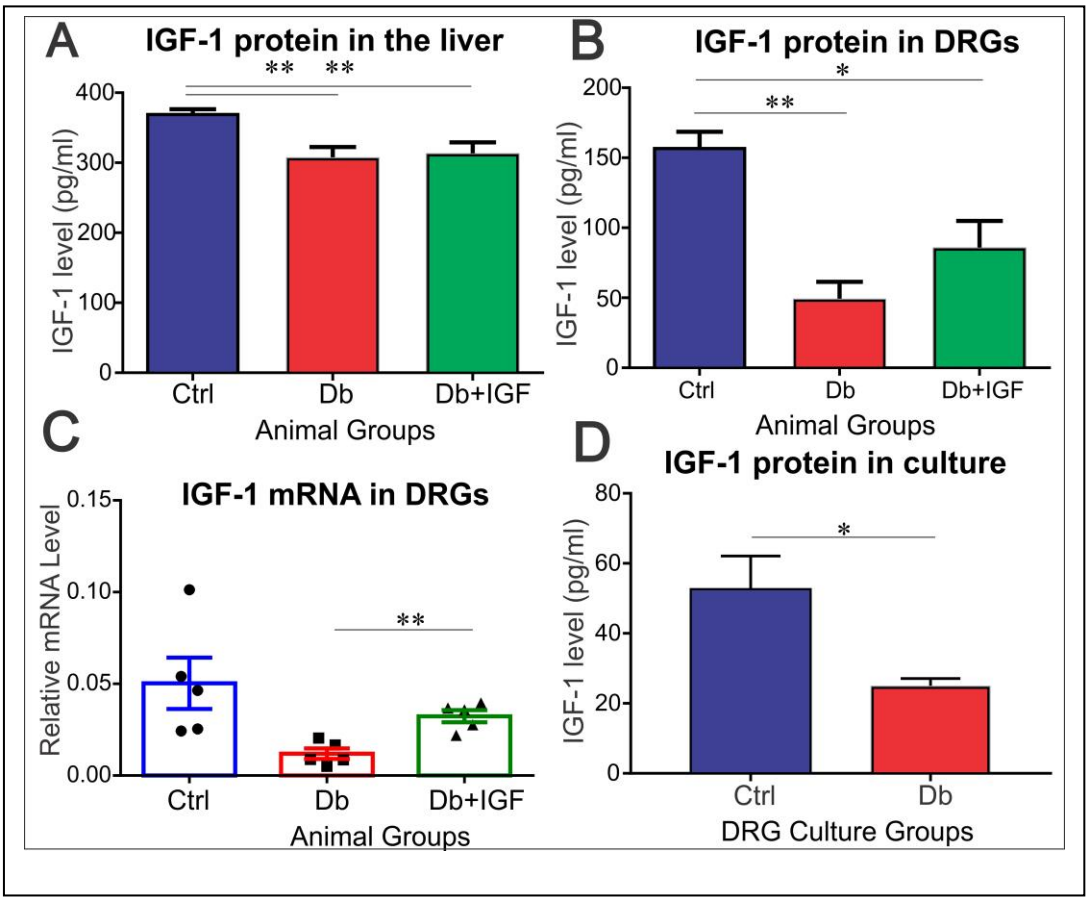

Figure 4.2. The level of endogenous IGF-1 was reduced in the liver and DRG tissue from diabetic rats and was restored by exogenous hIGF-1. In (A) and (B), liver and DRG tissues from control (Ctrl), hIGF-1-treated (Db+hIGF-1) and untreated diabetic (Db) rats were homogenized and underwent ELISA for IGF-1 detection. In (C), DRG tissues were used for IGF-1 mRNA measurement using Real-Time PCR. In (D), DRG neurons derived from Ctrl and Db rats were cultured and media was collected after 2 days to measure IGF-1 protein levels in the media using ELISA. Data are mean ± SEM of N=4-6 replicates; *= $p<0.05$ or **= $p<0.01$; analyzed by Student t-test or one-way ANOVA with Tukey's *post-hoc* test.

Endogenous IGF-1 mRNA is expressed at higher levels in DRG neurons compared with associated satellite cells within the ganglia

To investigate the expression of endogenous IGF-1 by DRG neurons, we conducted RNA-FISH assay to detect IGF-1 mRNA using specific fluorescent probes within DRG neurons and associated satellite cells. Endogenous IGF-1 mRNAs were detected in liver sections from control rats (Figure 4.3A). A punctate pattern of endogenous IGF-1 mRNA was also clear in DRG sections from control rats (Figure 4.3B). As a control, tissue sections and cells were exposed to RNase enzyme before hybridizing with IGF-1 probes. In cultured DRG neurons, endogenous IGF-1 mRNAs were expressed most highly in neurons, especially large diameter sensory neurons, compared with satellite cells and other non-neuronal cell types within the ganglia (Figure 4.4A-B). Sciatic nerve is comprised of mostly Schwann cells and connective tissue. Using quantitative Northern blotting we found that brain cortex tissue and DRG tissue expressed higher levels of IGF-1 mRNA compared to sciatic nerve (Figure 4.5A). The RNA integrity of brain cortex, DRG and sciatic nerve tissues were assessed and the 5S rRNA, 18S rRNA and 28S rRNA bands were used for normalization (Figure 4.5B). IGF-1 mRNA detected by Real-Time PCR, was significantly ($P<0.001$) higher in DRG tissue versus sciatic nerve (Figure 4.5C). The highest level of IGF-1 mRNA was detected in the brain cortex compared to DRG tissue and sciatic nerve (Figure 4.5D).

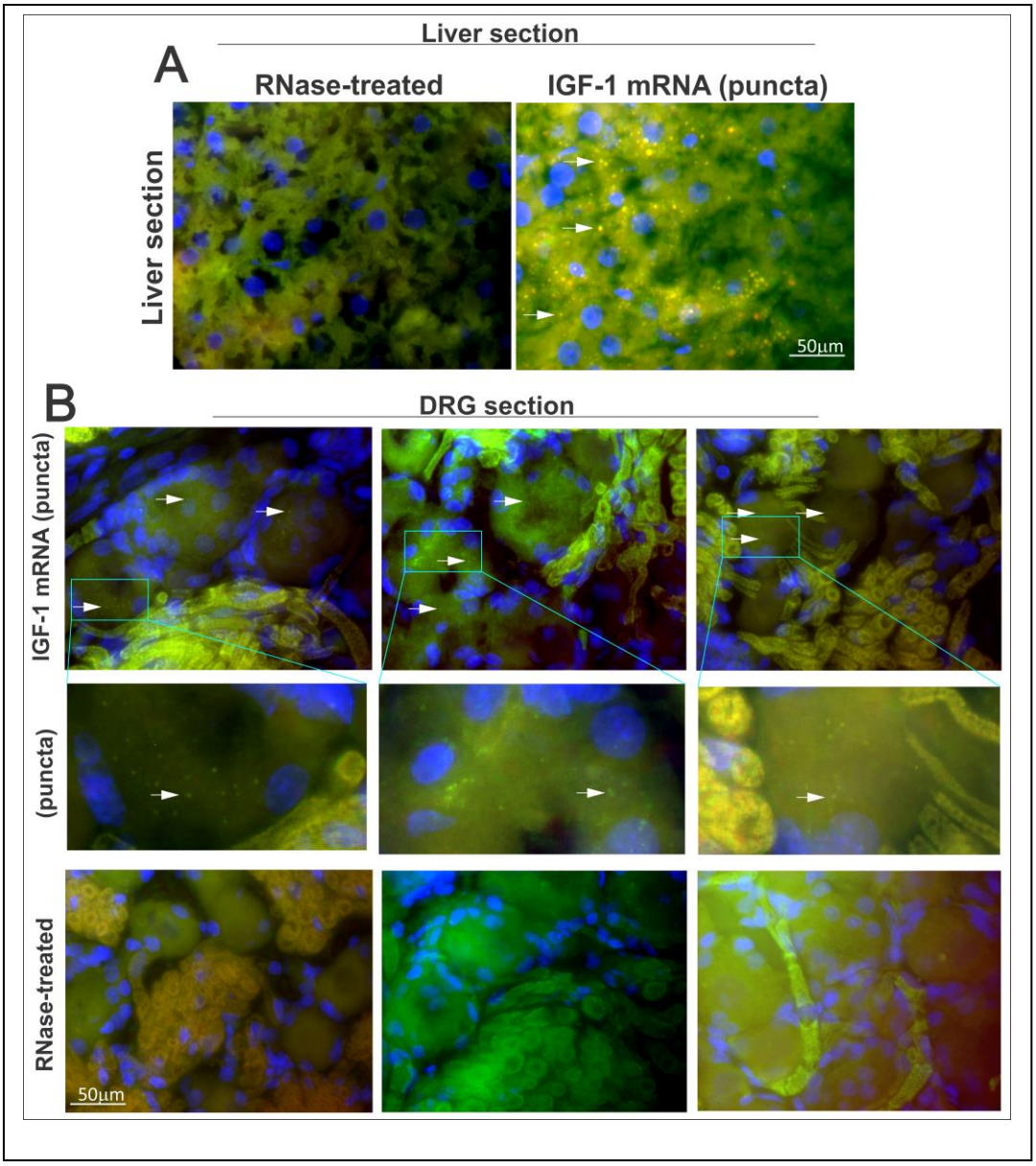

Figure 4.3. Localization of endogenous IGF-1 mRNA in the liver and DRG tissues from rats. (A) Liver or (B) DRG sections from control rats underwent RNA-FISH assay for IGF-1 mRNA detection and localization. A punctate pattern of IGF-1 mRNA was clear in these sections. As a control, tissue sections and cells were exposed to RNase enzyme before hybridizing with IGF-1 probes. Representative images from each group are shown. Data are mean ± SEM of N=10-15 (sections).

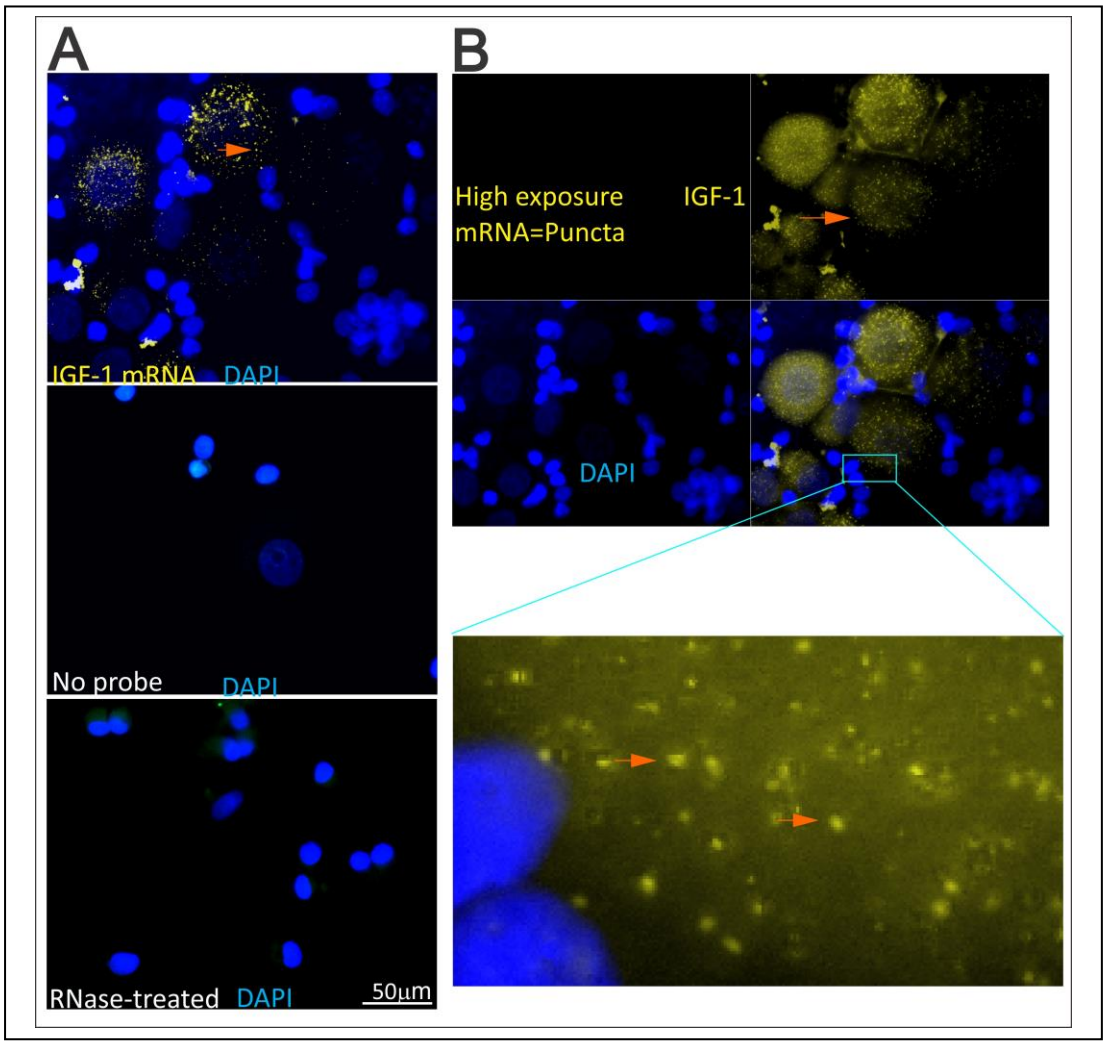

Figure 4.4. Endogenous IGF-1 mRNA expression was enriched in neurons compared to non-neuronal cells in DRG cultures from normal adult rats. DRG neurons from control rats were cultured and underwent RNA-FISH assay for IGF-1 mRNA detection and localization. (A) A punctate pattern of IGF-1 mRNA was clear in DRG neurons. As a control, cultured DRG neurons were exposed to RNase enzyme before hybridizing with IGF-1 probes. In (B), image was taken with higher exposure to visualize the cell dimensions. Each puncta representing one IGF-1 mRNA signal is shown with the orange arrow. Representative images from each group are shown. Data are mean ± SEM of N=10-15 (images).

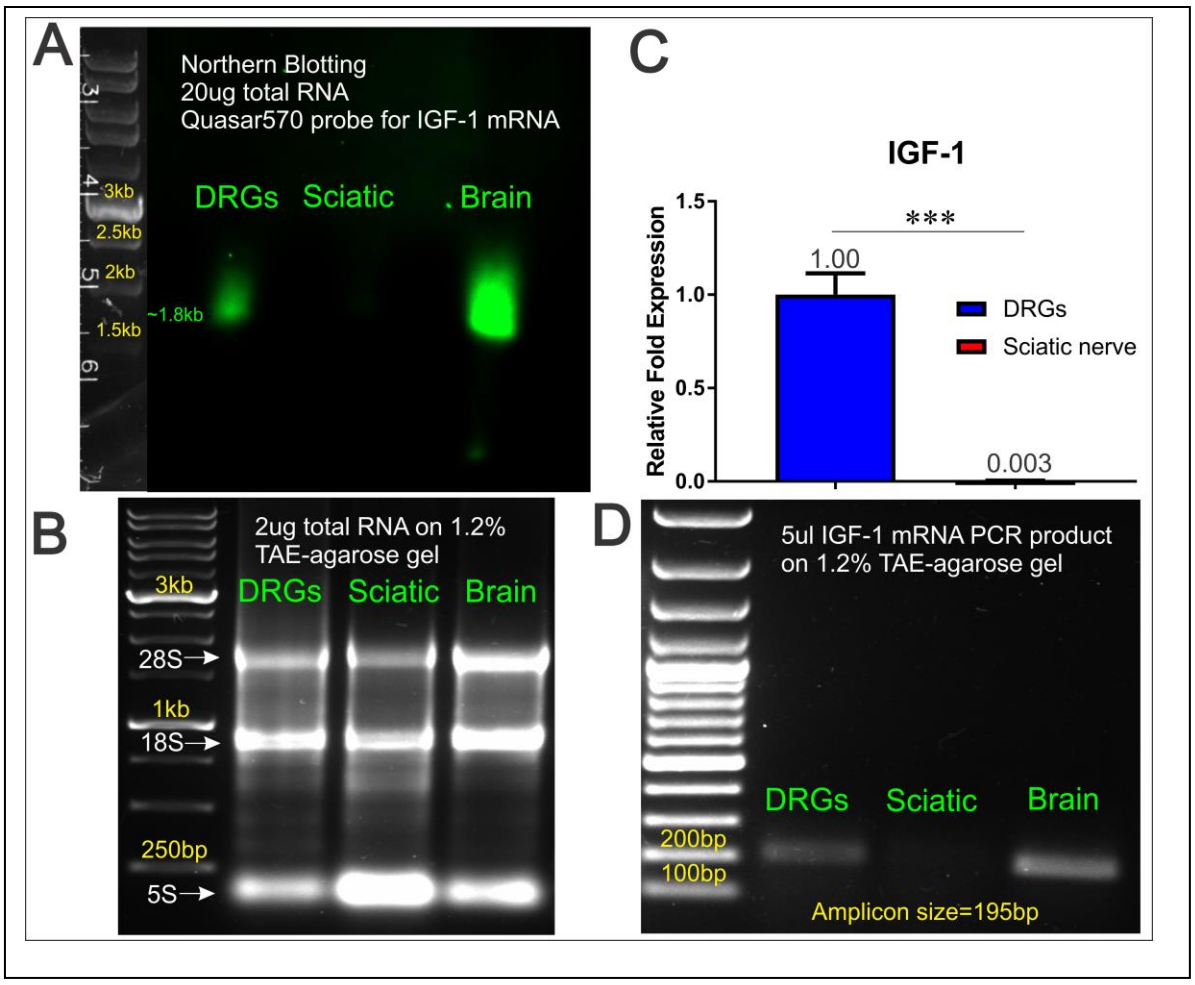

Figure 4.5. Endogenous IGF-1 mRNA was expressed at a higher level in brain cortex and DRG tissue compared with sciatic nerve from normal rats. DRG, sciatic nerve and brain cortex tissues were obtained from control rats and underwent (A) Northern blotting or (C) Real-Time PCR assay. In (B), 2µg RNA was run on 1.2% TAE-agarose gel for quality control in (A) and normalization purposes in (C). IGF-1 fluorescent probes were used for hybridization in (A). In (D), PCR products from (C) were run on 1.2% TAE-agarose gel to compare the band intensities. In (C), data are mean ± SEM of N=3-4 replicates; ***= $p<0.001$; analyzed by Student t-test.

Endogenous IGF-1 gene expression in DRG neuron culture is suppressed by hyperglycemia-dependent activation of the polyol pathway and restored by exogenous hIGF-1

To further investigate the mechanism through which IGF-1 gene expression is regulated in diabetic conditions, we measured IGF-1 mRNA in different cell culture groups. DRG neurons derived from diabetic rats were cultured in the following groups: a culture group with 25mM D-glucose (no insulin or hIGF-1), a culture group with 5mM D-glucose (no insulin or hIGF-1), a culture group with 25mM D-glucose treated with 10nM insulin for 24h, a culture group with 25mM D-glucose treated with 10nM hIGF-1 for 24h. Exogenous hIGF-1 (10nM) treatment but not insulin (10nM) increased ($P<0.0001$) the level of endogenous IGF-1 in cultured DRG neurons derived from diabetic rats and in the presence of 25mM D-glucose (Figure 4.6A). The 5mM D-glucose DRG neuron culture group (derived from a diabetic rat) exhibited significantly higher levels of IGF-1 mRNA compared with the 25mM D-glucose group revealing that the inhibitory effect of hyperglycemia on endogenous IGF-1 gene expression could be relieved by transition to normoglycemia (Figure 4.6A). A significant ($P<0.05$) suppression of endogenous IGF-1 gene expression was observed when cultured DRG neurons derived from control rats were exposed to 25mM or 50mM D-glucose for 2 days (Figure 4.6B). Exogenous hIGF-1 treatment (for 2 days) restored the level of endogenous IGF-1 mRNA in DRGs derived from control rats and exposed to 25mM D-glucose (Figure 4.6C). The was no restoration of the IGF-1 mRNA level with short term (6h) treatment with IGF-1 (Figure 4.10B and 4.10C.Supplemental Figure), and neither the Akt or ERK pathways were independently involved in the long term hIGF-1 upregulation of endogenous IGF-1 mRNA in cultured DRG neurons (Figure 4.10D.Supplemental Figure). Both concentrations (10 and 100μM) of sorbinil, an aldose reductase inhibitor (ARI),

prevented the suppression of IGF-1 transcript levels in cultured DRG neurons from control rats under hyperglycemic conditions suggesting polyol pathway activity was inducing an inhibitory effect on IGF-1 at the transcriptional or post-transcriptional level (Figure 4.6D).

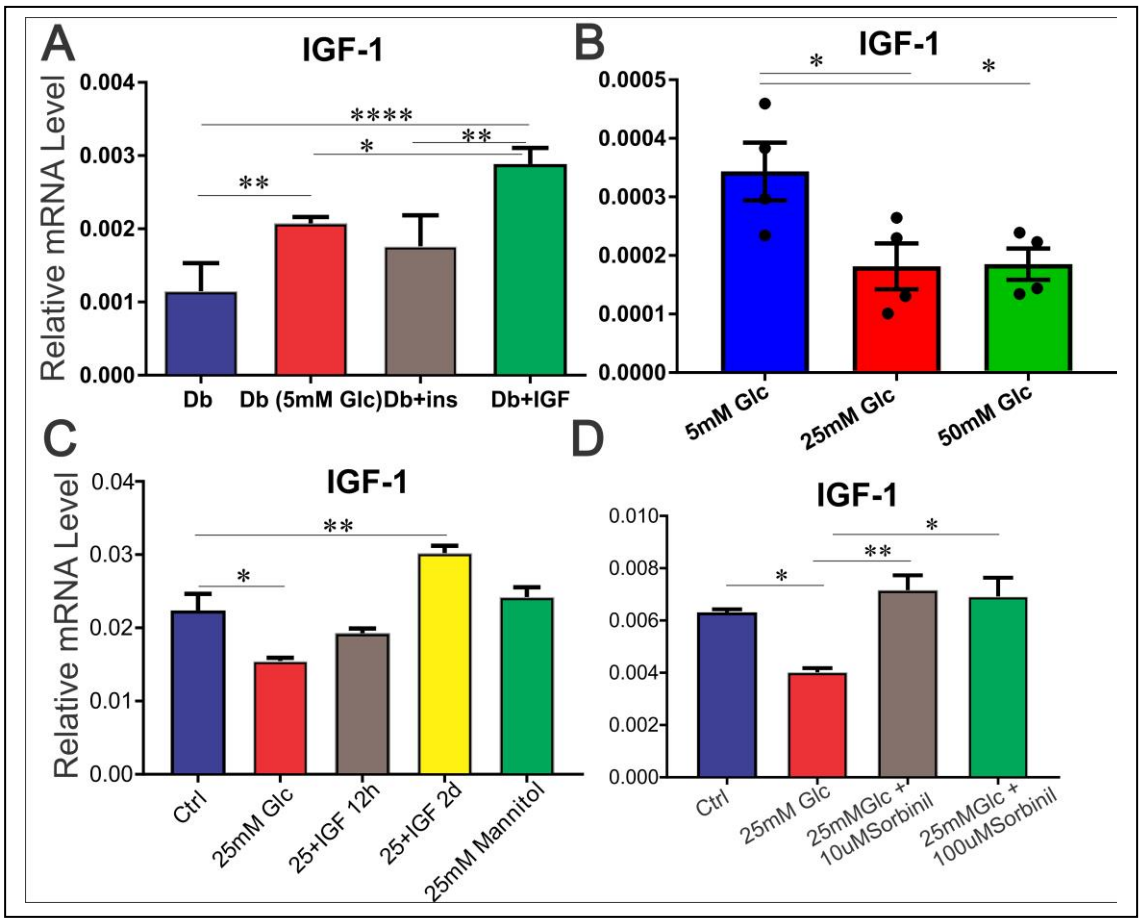

Figure 4.6. Endogenous IGF-1 gene expression in DRG cultures was suppressed by hyperglycemia-activated polyol pathway and restored by exogenous hIGF-1. DRG neurons from (A) diabetic or (B, C and D) control rats were cultured in the presence of 25mM glucose with/without insulin or hIGF-1 treatment or in the presence of 5mM glucose. RNA was extracted and utilized for Real-Time PCR assay. In (D), sorbinil (10 and 100µM), an aldose reductase inhibitor (ARI), was used. In (C and D), 25mM mannitol was used to control for osmotic pressure compared with 25mM D-glucose. Data are mean ± SEM of N=3-5; *=$p<0.05$ or **= $p<0.01$ or ****=$p<0.0001$; analyzed by one-way ANOVA with Dunnett's or Tukey's *post-hoc* test.

Binding of NFAT1 and CEBP-β transcription factors to the IGF-1 promoter is diminished in DRG tissue from diabetic rats

Bioinformatics screening of the IGF-1 promoter revealed several transcription factor binding sites from which the most abundant and comprehensive binding sites, specific to NFAT1 and CEBP-β transcription factors, were tested (Figure 4.11.Supplemental Figure). Of note, chromatin fragmentation was optimized before determination of the ChIP assay (Figure 4.7A). Five amplicons (five promoter regions) covering 1.2 kb upstream of rat IGF-1 gene were considered for Real-Time PCR (Figure 4.7B), and the specificity of the corresponding primers was validated on an agarose gel (Figure 4.7C). One out of five regions of the IGF-1 promoter (promoter region 5) was enriched for NFAT1 and CEBP-β binding in DRG tissues from control rats when compared to DRG tissue from diabetic rats ($P<0.05$) (Figure 4.7D). However, the level of both transcription factors remained unchanged in DRG tissue derived from diabetic rats compared with control rats (Figure 4.12A and 4.12B.Supplemental Figure).

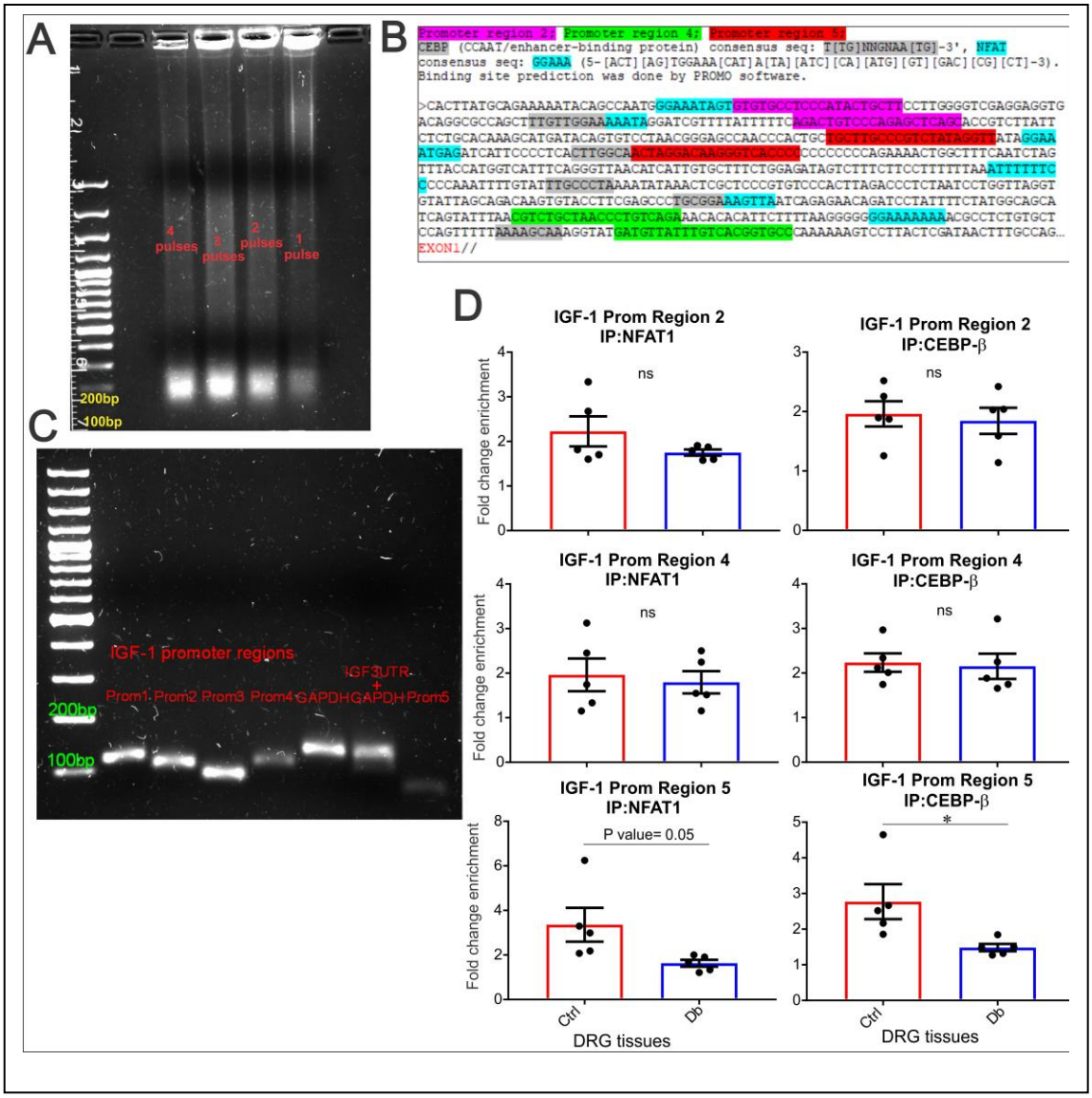

Figure 4.7. Binding of NFAT1 and CEBP-β transcription factors to the IGF-1 promoter was diminished in DRG tissues derived from diabetic rats. (A) Chromatin fragmentation optimization was performed prior to ChIP experiments. In (B), transcription factor (NFAT1 and CEBP-β) binding sites on promoter regions of IGF-1 gene are shown. Predicted binding sites for the two transcription factors on the IGF-1 gene promoter were determined in DRG tissues from control and diabetic rats, based on which five promoter regions were amplified. (C) The validity

of PCR products from five promoter regions were tested on an agarose gel. (D) DRG tissues derived from adult control and diabetic rats underwent ChIP assay using NFAT1 and CEBP-β antibodies for pull down followed by IGF-1 promoter region amplification using Real-Time PCR. Data are mean ± SEM of N=4 animals; *= $p<0.05$; analyzed by Student t-test.

The knock-down of expression or inhibition of endogenous IGF-1 signaling impeded background levels of neurite outgrowth in cultured DRG neurons

DRG neurons from age matched control rats treated with 1μM IGF-1 neutralizing antibody exhibited suppression of phosphorylated Akt (at S473) and IGF-1Rβ within 2 days of treatment (Figure 4.8A and 4.8B). This effect was associated with concomitant inhibition of neurite outgrowth in DRG neuron cultures from control rats (Figure 4.8C and 4.8D). To confirm the importance of endogenous IGF-1 secretion for neurite outgrowth, we used two specific IGF-1 siRNAs encapsulated in lipid nanoparticles (LNPs) for higher knock-down efficiency and lower toxicity (Figure 4.9A). These effects were dose dependent; with 180nM mix siRNAs giving the highest level of IGF-1 knock-down. The 180nM siRNA dose reduced the background neurite outgrowth in DRG neuron cultures derived from control rats ($P<0.05$) (Figure 4.9B and 4.9C). The suppressed neurite outgrowth was significantly elevated when treated with exogenous hIGF-1 ($P<0.05$). Similar enhancement of neurite outgrowth was observed when hIGF-1 was used as the only treatment (Figure 4.9B and 4.9C).

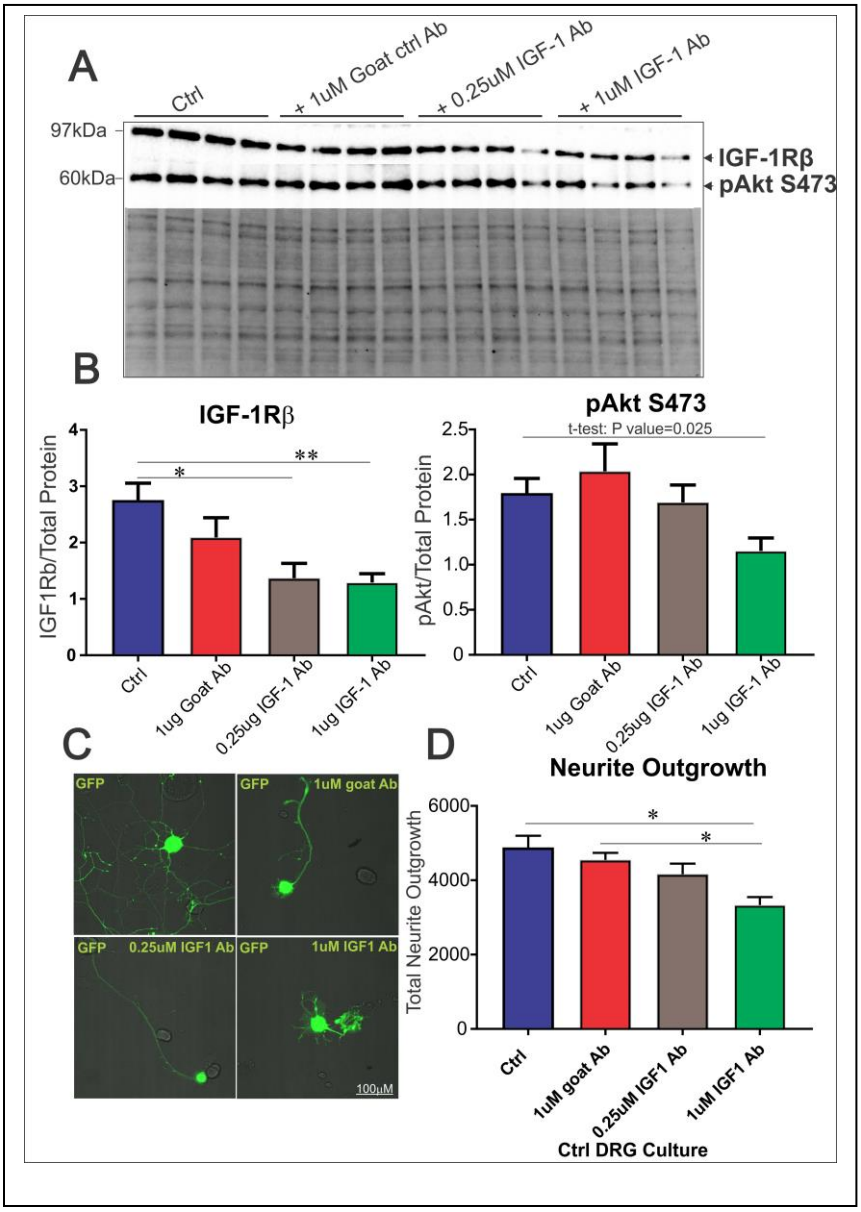

Figure 4.8. IGF-1 neutralizing antibody reduced IGF-1Rβ and Akt phosphorylation and was associated with diminished neurite outgrowth. DRG neurons from control rats were cultured, treated with different doses of IGF-1 neutralizing antibody and underwent (A and B) Western blotting for Akt phosphorylation and IGF-1Rβ or (C and D) neurite outgrowth measurement. In (A and B), total protein bands were used for normalization. In (C and D), normal goat IgG was used as a control antibody. Data are mean ± SEM of N=4; *= $p<0.05$ or **= $p<0.01$; analyzed by one-way ANOVA with Dunnett's *post-hoc* test.

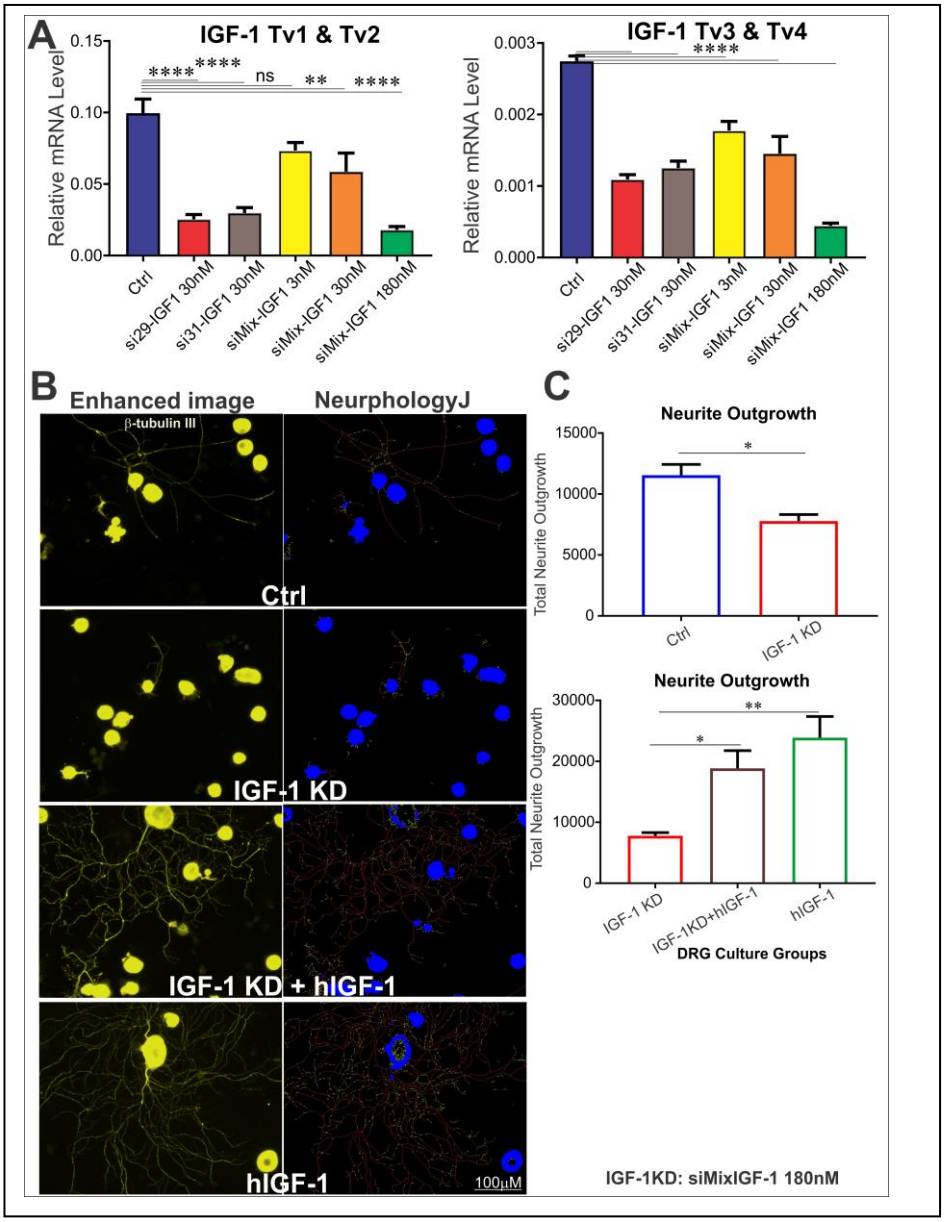

Figure 4.9. IGF-1 knock-down diminished neurite outgrowth in DRG neurons derived from control rats. DRG neurons from control rats were cultured, treated with different doses of two IGF-1 targeting siRNAs and underwent (A) Real-Time PCR assay for IGF-1 or (B and C) neurite outgrowth measurement. In (B), exogenous hIGF-1 was treated alone or along with IGF-1 knock-down as control groups. Data are mean ± SEM of N=4; *= $p<0.05$ or **= $p<0.01$ or ****= $p<0.0001$; analyzed by Student t-test or one-way ANOVA with Dunnett's *post-hoc* test.

Discussion

We show for the first time that DRG neurons synthesize and secrete their own IGF-1 that can act as an autocrine/paracrine neurotrophic factor to play a key role in neuronal maintenance and axonal outgrowth. This endogenous production of IGF-1, detected at both the mRNA and protein levels in DRG tissue and culture, is suppressed under hyperglycemic conditions via effectors mediated via the polyol pathway. We further demonstrate that exogenous hIGF-1 exerted a positive feedback on endogenous IGF-1 transcript levels under hyperglycemic conditions and a negative feedback under normoglycemic conditions in cultured DRG neurons. The endogenous IGF-1 was expressed at a higher level in DRG tissue compared with sciatic nerve, and more specifically higher mRNA expression was detected in neurons compared with non-neuronal cells of the DRG. We also found more than a 2-fold enrichment of the transcription factors NFAT1 and CEBP-β binding to the IGF-1 promoter in DRG tissue from control rats but not from diabetic rats. In addition, we provide evidence that IGF-1 ablation in DRG neuron cultures attenuated the background neurite outgrowth.

IGF-1 acts as a hormone in cooperation with growth hormone (GH) for growth and development, and is primarily produced by the liver and declines as we age (Sattler, 2013). In the adult human, IGF-1 is produced by almost all tissues including liver, fat, endometrium, placenta, prostate, heart, appendix and brain (Fagerberg et al., 2014). In adult rats and mice, IGF-1 is mainly produced by the liver and minimal amounts by other tissues (Yu et al., 2014, Yue et al., 2014). To fulfill its function, IGF-1 has to bind its receptor (IGF-1R) and trigger two survival pathways, Akt and ERK pathways. IGF-binding proteins (IGFBPs) in the circulation and insulin receptors with low affinity to IGF-1 may bind to IGF-1 and counteract its binding to IGF1R and thus alter function (Holly and Perks, 2012, Novosyadlyy and Leroith, 2012). Interestingly, the

IGF-1R is expressed in all tissues including brain, kidney, heart and muscle but with lowest expression in the liver tissue in man and rodents (Fagerberg et al., 2014, Yu et al., 2014, Yue et al., 2014). We and others have shown that both IGF-1 and IGF1R are expressed in DRG tissues and cultured DRG neurons (Kamiya et al., 2005, Lin et al., 2016, Miura et al., 2011, Tang et al., 2019).

Dysregulation of IGF-1 or IGF1R gene expression is associated with a plethora of disorders. Overexpression of IGF-1 leads to Acromegaly syndrome and its downregulation is associated with abnormal growth and development (Giustina et al., 2014, Ren et al., 2016). Higher levels of IGF-1 and its receptor are correlated with multiple types of cancer, and a range of cancer therapies have been successful in terms of antitumor effects by targeting IGF1R and inhibition of its tyrosine kinase activity (Li et al., 2009). On the other hand, lower levels of IGF-1 or impaired IGF-1 signaling have been proposed to contribute to development of cognitive impairment (Doi et al., 2015, Wennberg et al., 2018), Alzheimer's disease (Vidal et al., 2016) and Parkinson's disease (Pellecchia et al., 2014). Animal models of these disorders have shown a similar association. For instance, IGF-1 down-regulation has been associated with metabolic deficiencies in brain in mouse models of Alzheimer's disease (Trueba-Saiz et al., 2013). And, systemic or peripheral administration of IGF-1 protected hippocampal neurons against amyloid peptide toxicity in aged rats and mouse models of Alzheimer's (Carro et al., 2006, Carro et al., 2002). In diabetic patients, the serum level of IGF-1 was suppressed (Palta et al., 2014). In STZ-induced type 1 diabetic rats, the serum level of IGF-1 was significantly decreased compared to control rats (Ekstrom et al., 1989). The mRNA content of IGF-1 in the liver of spontaneously diabetic obese Zucker rats, and in the liver, adrenal gland and spinal cord of STZ-induced type 1 diabetic rats was markedly reduced (Zhuang et al., 1997, Ishii et al., 1994). Additionally, any

impairment in IGF-1 and IGF-2 levels and signaling can worsen neurodegeneration in diabetes (Ishii, 1995, Rauskolb et al., 2017, Zochodne, 2016b). This is in line with our findings that IGF-1 protein level in the liver and DRG tissues from STZ-diabetic rats were lower than normal. DRG neurons derived from diabetic rats secreted less IGF-1 than DRG neuron cultures from control rats. Depressed IGF-1 mRNA level in DRG tissues or neuron cultures from diabetic rats was consistent with lowered protein levels.

Autocrine/paracrine factors in DRG neurons and peripheral nerves are critical for maintenance and function. In 59 patients with peripheral nerve injury, endogenous nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) were detected in Schwann cells in the sciatic nerve, and the level of both decreased at the time of the injury proximal to the injured site (Anand et al., 1997). In a sciatic nerve crush in rats, the mRNA levels of endogenous NGF, CNTF and brain-derived neurotrophic factor (BDNF) were elevated at the site of crush. Schwann cells were the main source for the production of these neurotrophic factors (Meyer et al., 1992). The level of Schwann cell-derived NGF declined during adulthood and was induced by resident and later by invading macrophages during injury in the sciatic nerve in adult rats (Heumann et al., 1987). There is a reduction in NGF and neurotrophin-3 (NT-3) mRNA levels in sensory neuron target tissues such as foot-skin and soleus muscle in STZ-diabetic rats (Fernyhough et al., 1994, Tomlinson et al., 1997). The level of BDNF mRNA was shown to be elevated in DRG and sciatic nerve tissues correlating with increased NGF mRNA in sciatic nerve over 12 weeks of diabetes in STZ-diabetic rats. Intensive insulin treatment reversed the levels of both neurotrophins to normal levels (Fernyhough et al., 1995a). The level of NGF protein was reduced while its mRNA was elevated after 4 weeks of diabetes in DRGs in STZ-diabetic rats indicating activation of compensatory pathways to increase endogenous NGF in DRG tissues

(Gao et al., 2017, Nori et al., 2013). IGF-1 immunoreactivity was detectable in various tissues including DRGs, sympathetic ganglia, ventral horn, axons and Schwann cells of the sciatic nerve in adult rat (Hansson et al., 1988). Schwann cells derived from postnatal rats expressed their own IGF-1 and prevented apoptosis after serum withdrawal (Syroid et al., 1999). To clarify this matter and determine the main source of endogenous IGF-1 in peripheral nerves, we used RNA-FISH method to detect IGF-1 mRNA. We found that endogenous IGF-1 mRNA was expressed at a higher level in brain cortex and DRG tissue compared with sciatic nerve from normal rats, and a higher level in cultured DRG neurons than DRG non-neuronal cells.

Increased flux of D-glucose through the polyol pathway under diabetic conditions can dysregulate the expression of a wide range of endogenous factors in DRG neurons and peripheral nerve. In a study on rodents, polyol pathway activity raised NGF levels in the iris and substance P in trigeminal ganglia in STZ-diabetic rats, and sorbinil, an aldose reductase inhibitor, could reverse the mRNA levels of both (Fernyhough et al., 1998a). In Schwann cell culture, NT-3-induced production of NGF was suppressed in a medium with high glucose. An aldose reductase inhibitor attenuated the effect of hyperglycemia on NGF gene expression indicating involvement of polyol pathway in suppression of NGF (Suzuki et al., 2004b). In a similar study, sorbitol accumulation promoted IGF-1 gene down-regulation and Schwann cell de-differentiation in hyperglycemic conditions. An aldose reductase inhibitor rescued the IGF-1 suppression along with improvement of nerve conduction and nerve myelination in db/db type 2 diabetic mice or STZ-induced type 1 diabetic mice (Hao et al., 2015). Hyperglycemia exposure of cultured DRG neurons from either control or diabetic rats suppressed endogenous IGF-1 gene expression in our study, and sorbinil or exogenous hIGF-1 could reverse this suppressive effect. Consistent with our findings on exogenous hIGF-1 effect in control culture, endogenous IGF-1 and IGF1R levels

were reduced in response to exogenous IGF-1 treatment for 6 hours in neurons and brain endothelium derived from mice, and returned to baseline level at a longer time point (Trueba-Saiz et al., 2017).

We revealed that CEBP-β binds to *IGF-1* gene promoter with lower affinity in DRG tissues from diabetic rats compared to DRG tissues from control rats suggesting CEBP-β implication in IGF-1 downregulation and pathogenesis of diabetic neuropathy. CCAAT/enhancer-binding protein beta (CEBP-β) is a transcription factor that can bind to DNA as homodimer or heterodimers with related proteins CEBP-α, CEBP-γ and CEBP-δ (Nerlov, 2007). CEBP-β is phosphorylated and activated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Wegner et al., 1992) and mitogen-activated protein kinase (MAPK) kinase kinase 1 (MKK1) (Roy et al., 2002), or inhibited by several upstream enzymes such as protein kinase C (PKC) (Trautwein et al., 1994), protein kinase A (PKA) (Trautwein et al., 1994) and glycogen synthase kinase-3β (GSK-3β) (Shen et al., 2009). Its known target genes are interleukin-6 (IL-6), IL-4, tumor necrosis factor-α (TNFα) and other cytokines involved in immune responses (Greenwel et al., 2000, Natsuka et al., 1992, van Dijk et al., 1999). More recently, the role of CEBP-β in the nervous system has been revealed. CEBP-β increases the expression of neurokinin A and substance P, and has a role in the biosynthesis of acetylcholine (Kovacs et al., 2006, Robert et al., 2002). CEBP-β promotes fatty acid oxidation in mitochondria by binding to PPAR coactivator 1-alpha (PGC-1α) and initiating carnitine palmitoyl transferase 1 A (CPT1A) gene expression in nasopharyngeal carcinoma (NPC) cells (Du et al., 2019). CEBP-β gene ablation in *db/db* mice impaired adipogenesis and lipogenic enzyme activity, and downregulated peroxisome proliferator-activated receptor γ2 (PPARγ2) (Schroeder-Gloeckler et al., 2007). CEBP-β transcription factor accumulated in the islets of diabetic animal models, and its deletion

in β cells of Akita mice and *db/db* mice resulted in amelioration of hyperglycemia and elevated β cell mass (Matsuda et al., 2010). CEBP-β can bind to two regions of *IGF-1* promoter and increase *IGF-1* gene expression in porcine primary hepatocytes (Tang et al., 2015).

Here, we, for the first time, report that NFAT1 (NFATc2) binds to promoter regions of IGF-1 with higher affinity in DRGs from control rats versus diabetic rats emphasizing NFAT1 involvement in pathogenesis of neuropathy through endogenous IGF-1 downregulation. NFATc2 and NFATc4 also play a role in adipogenesis and glucose/insulin homeostasis thus functioning as a marker in metabolic processes (Yang et al., 2006). The nuclear factor of activated T cell (NFAT) family has been first identified as transcription factors regulating the gene expression of cytokines (Durand et al., 1988) and playing a role in immune response (Peng et al., 2001, Xanthoudakis et al., 1996). Upstream enzymes such as serine/threonine phosphatase calcineurin dephosphorylate NFAT transcription factors and induce its translocation to the nucleus, whereas MAPK, PKA and GSK3 re-phosphorylate and inactivate NFATs (Beals et al., 1997, Clipstone and Crabtree, 1992, Yang et al., 2002). Neurotrophins such as NGF and BDNF require NFATc4 activation to promote axonal outgrowth in embryonic DRG neurons derived from mice. Mice lacking NFATc2, c3 and c4 are deficient in axonal outgrowth despite no or little defect in neuronal survival and differentiation (Graef et al., 2003). NFAT and CEBP-β cooperation is required for binding to composite elements on IGF-2 and PPARγ2 regulatory regions (Yang and Chow, 2003).

Hyperglycemia and polyol pathway dysregulate a range of critical kinases and phosphatases in cells. In diabetic patients, the levels of intracellular and endoplasmic reticulum (ER) calcium drops down to a point at which calcium-dependent enzymes such as CaMKII and calcineurin are affected (Ahn et al., 2017). On the other hand, diabetes-induced activation of

polyol pathway and PKC leads to MAPK pathway activation and phosphorylation of several important transcription factors that control intracellular signaling and gene expression (Cheng et al., 2013, Rosse et al., 2010). Therefore, hyperglycemia-induced suppression of CaMKII and calcineurin might be the cause of impaired activity and binding of NFAT1 and CEBP-β to target promoters such as the *IGF-1* gene promoter. Polyol pathway-induced MAPK pathway activation and then inhibitory phosphorylation of these transcription factors might be complementing the aberrant promoter binding.

Paracrine connective tissue growth factor (CTGF) and IGF-1 are upregulated in hyperglycemic condition in human renal fibroblasts and can induce collagen type I and III production (Lam et al., 2003). Rat cardiac fibroblasts but not myocytes generated and secreted IGF-1 in culture and were proposed to function as a paracrine/autocrine factor in cardiac hypertrophy and remodeling (Horio et al., 2005). A gene expression profiling study revealed downregulation of neurotrophin-ERK pathway especially BDNF mRNA in peripheral blood mononuclear cells in patients with diabetic peripheral neuropathy compared to patients with diabetes mellitus (Luo et al., 2017) suggesting a pathogenetic contribution of neurotrophins in development of diabetic neuropathy. Microtubule-dependent transport of IGF-1 along peripheral nerve has been demonstrated (Feng and Von Bartheld, 2010, Payne et al., 2006). A double nerve crush in rat sciatic nerve revealed retrograde transport of IGF-1 to the neuronal cell body (Kanje et al., 1991). However, anterograde transport of IGF-1 was shown to be much higher than its retrograde transport in sciatic nerve in adult rats (Hansson et al., 1987). The IGF-1 synthesized in sensory and motor neurons might be released and exert trophic support for nerve endings as well as innervated target tissues, and consequently prevent distal-dying back denervation seen in diabetes. We propose that suppression of endogenous IGF-1 in DRG cultures impedes

background axonal outgrowth, and its lower levels in diabetic patients and animal models can be a candidate in the pathogenesis of diabetic peripheral neuropathy. Despite accumulating evidence on the value of local or systemic IGF-1 administration for treatment of diabetic neuropathy as well as neurodegenerative disorders such as Alzheimer's, ALS and Rett syndrome, little evidence is available on the role of endogenous IGF-1 in peripheral tissues. Tissue-specific knock-in or knock-out of IGF-1 in animal models will prospectively give an insight into specific roles of this important autocrine/paracrine factor in the nervous system.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Specific details are provided in the Materials and methods section.

Author contributions

M.R.A. designed and performed the in vitro studies and analyzed all tissues from the in vivo study. He performed data generation and analysis and wrote the first draft of the manuscript. V.Y. and D.M. performed nanoparticle assembling of siRNAs. P.A. and V.D. performed bioinformatic screening of IGF-1 promoter. P.F. supervised all in vitro and in vivo work and edited the manuscript.

Supplementary Materials:

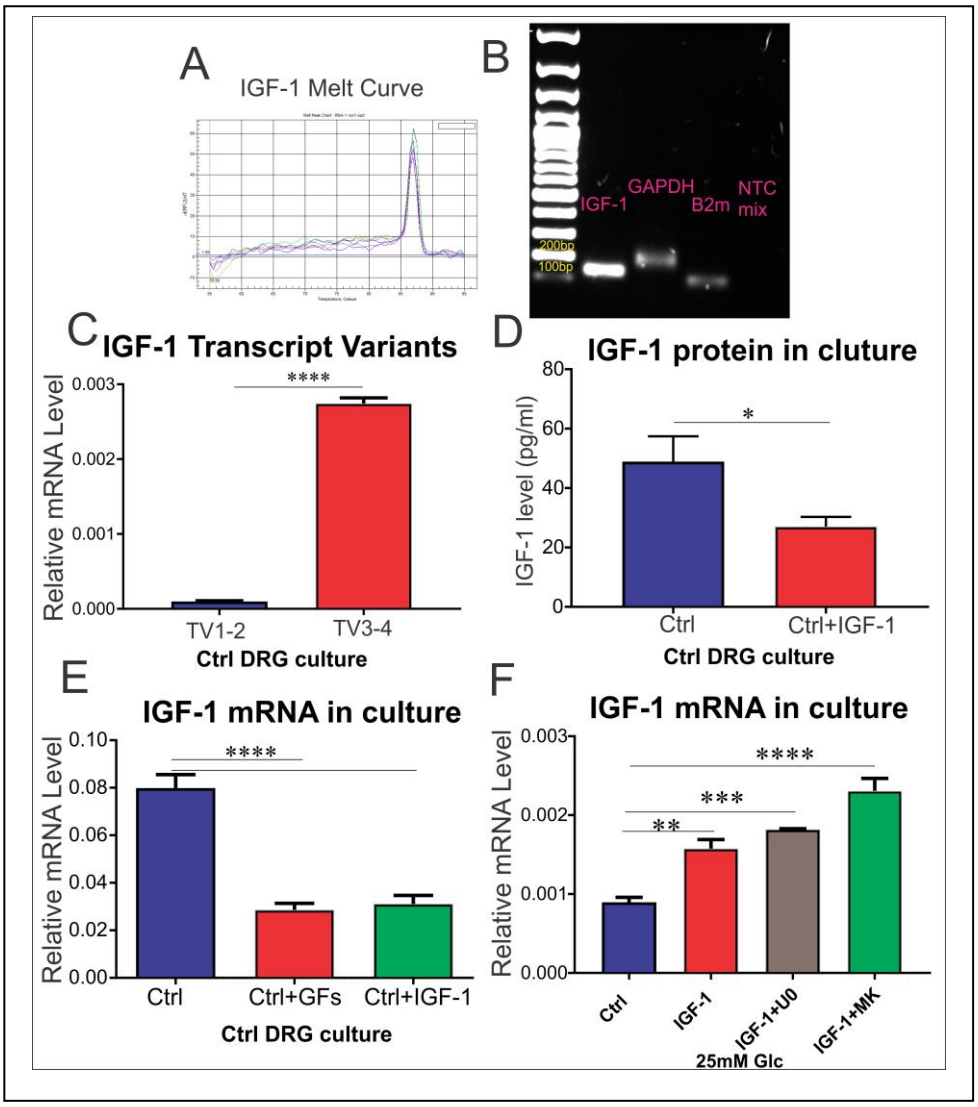

Figure 4.10.Supplemental Figure. IGF-1 transcript variant abundance, and its expression in growth factor-treated DRGs. DRG tissues from control (Ctrl) rats were isolated and subjected to Real-Time PCR assay. In (A and B), melt curve and amplicon bands of IGF-1, GAPDH and B2m PCR products in Real-Time PCR assays are shown. In (C), the level of IGF-1 transcript variants in DRG tissues were compared. In (D and E), growth factors or IGF-1 were treated 6h prior the PCR assay. In (F), the inhibitors (U0126: ERK inhibitor, MK2206: Akt inhibitor) of IGF-1 downstream signaling pathways, Akt and ERK, were used 1h prior hIGF-1

treatment for 2 days. Data are mean ± SEM of N=3-5; *= $p<0.05$ or **= $p<0.01$ or ***=$P<0.001$ or ****=$P<0.0001$; analyzed by Student t-test or one-way ANOVA with Dunnett's *post-hoc* test.

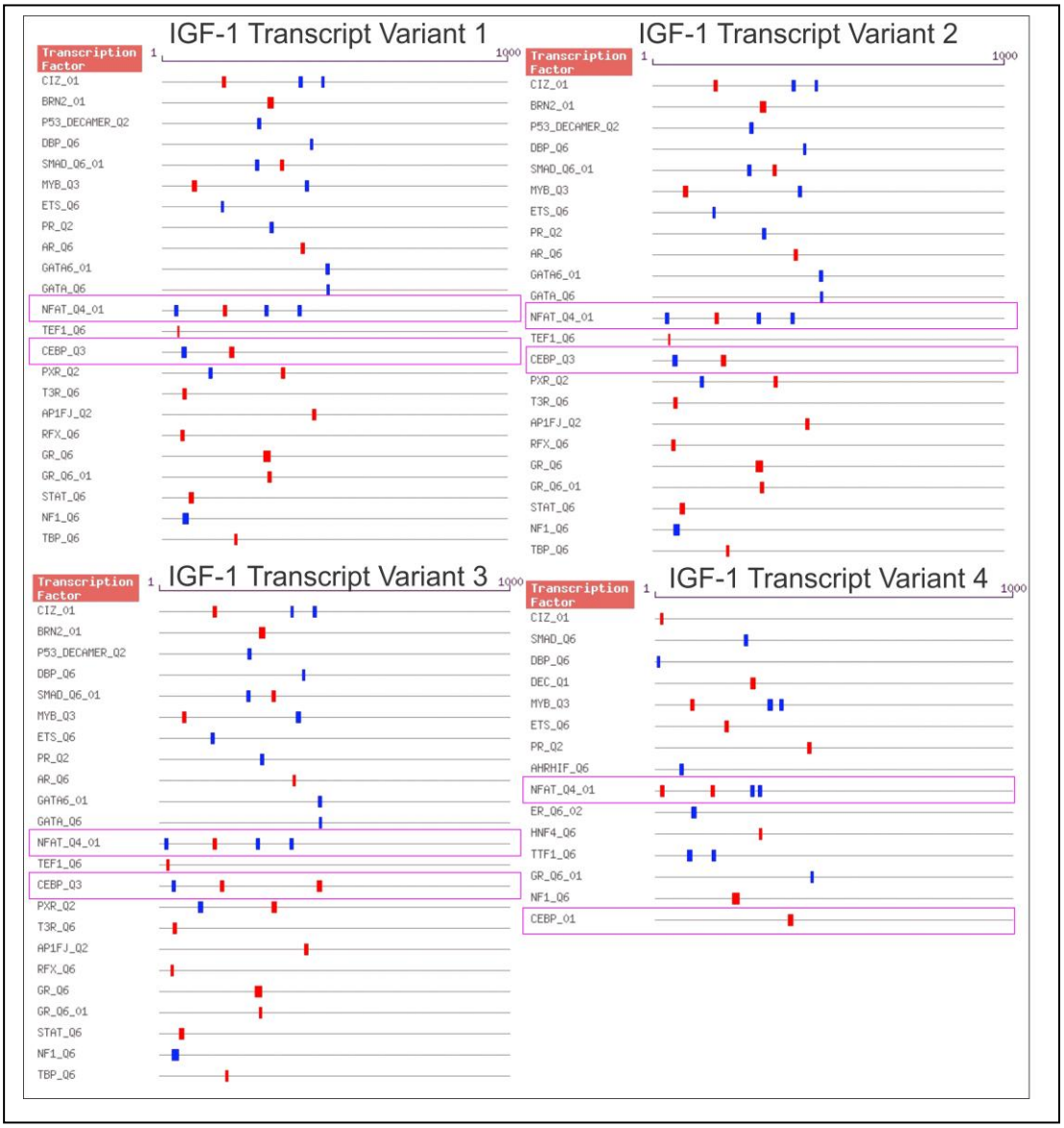

Figure 4.11.Supplemental Figure. Bioinformatic screening of IGF-1 promoter for transcription factor binding. Four transcript variants of rat IGF-1 gene were screened for transcription factor binding on the promoter. The two most probable transcription factors, NFAT1 and CEBP-β, were chosen based on their highest scores for binding and having multiple binding sites on promoter of all IGF-1 transcript variants for further experiments.

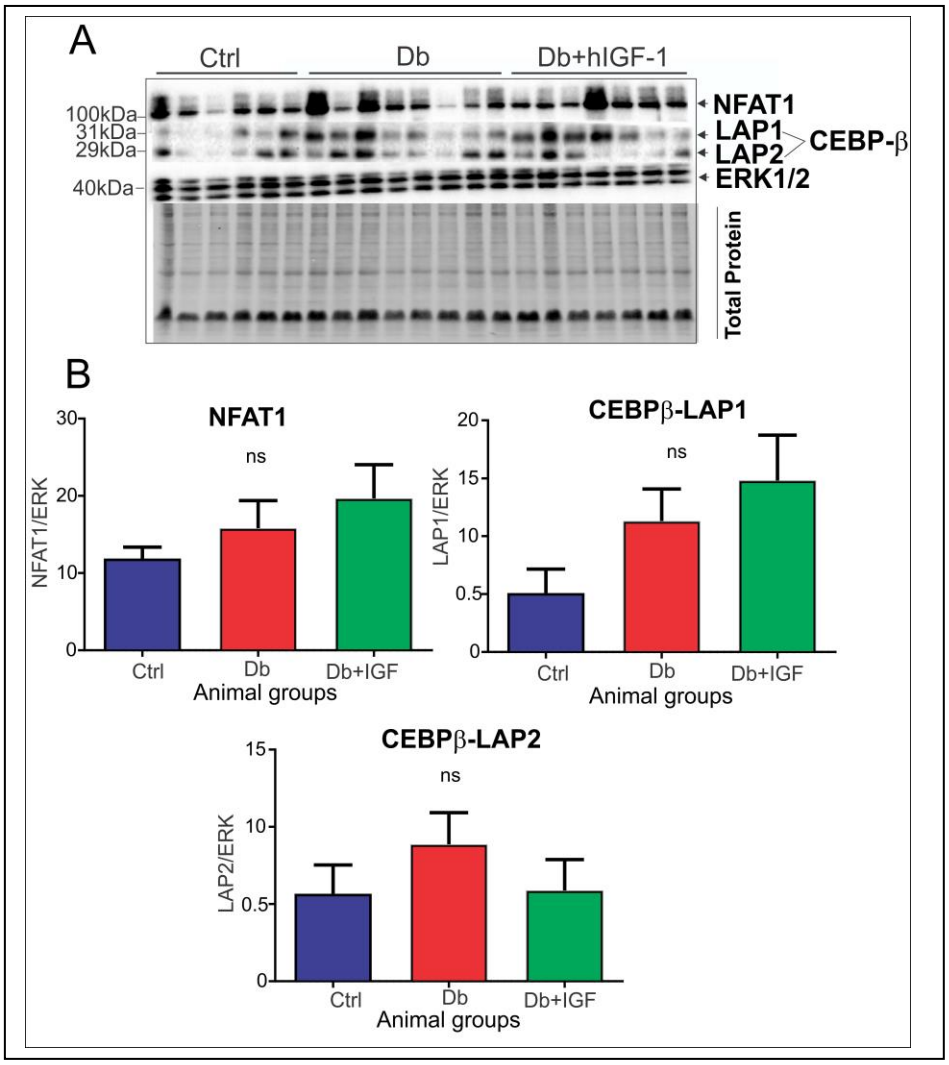

Figure 4.12.Supplemental Figure. The level of NFAT1 and CEBP-β proteins was not changed under diabetic conditions. DRG tissues from control (Ctrl), hIGF-1-treated (Db+hIGF-1) and untreated diabetic (Db) rats were homogenized and underwent Western blotting for NFAT1 and CEBP-β (LAP1 and LAP2 isoforms) proteins. ERK band intensity was used for normalization. Data are mean ± SEM of N=6-8 animals; analyzed by one-way ANOVA with Tukey's *post-hoc* test.

Chapter 5: Discussion

General Discussion:

Summary of findings. In chapter 2 of this thesis, aims 1 and 2 of the hypothesis 1 were addressed and a full discussion why our study of insulin therapy in diabetic neuropathy was important was given at the end of chapter 2. In chapter 2, we have shown that insulin signaling exists in sensory neurons of the adult rat as determined by the detection of Akt and P70S6K activation. Consistent with previous studies, insulin enhanced neurite outgrowth in cultured DRG neurons from rats. We also demonstrated that a physiologically relevant concentration (10 nM) of insulin raised the expression of mitochondrial electron transport system proteins and enhanced respiration in DRG tissue from both control and diabetic rats. Insulin implants in STZ-diabetic rats improved thermal hypoalgesia and IENF profile. Insulin therapy in a subgroup of STZ-diabetic rats also increased the protein expression of subunits of mitochondrial Complexes II, IV and V and activity of Complex IV. This part of the study supports this concept and demonstrates that insulin modulates mitochondrial function and thus may contribute to nerve repair. However, the role of AMPK in insulin signaling and its link to mitochondrial function was not discovered.

In chapter 3 of this thesis, aims 1, 2 and 3 of the hypothesis 2 were addressed and a full discussion why our study of IGF-1 therapy in diabetic neuropathy was important and how IGF-1 regulated mitochondrial function and prevented nerve structural and functional loss was given at the end of chapter 3. In chapter 3, we demonstrated for the first time that IGF-1 activated and up-regulated AMPK to augment mitochondrial function, ATP production, mtDNA copy number and expression of ETS proteins in cultured rat DRG neurons. IGF-1 utilized this AMPK pathway and, possibly in parallel, the Akt pathway to drive axonal outgrowth. Further, we showed that

IGF-1 therapy prevented diabetes-induced progressive loss of sensory nerves in the cornea and ameliorated paw thermal hypoalgesia. Correction of these clinically relevant endpoints was associated with suppression of build-up of TCA intermediates in nerve and optimization of mitochondrial phenotype in the DRG of type 1 diabetic rats. The stimulatory effect of IGF-1 on mitochondrial oxygen consumption rate can be explained by signaling via the AMPK α1 isoform. The elevation in mtDNA copy number revealed the involvement of AMPK α2 isoform downstream from IGF-1 signaling.

In chapter 4 of this thesis, aims 1, 2 and 3 of the hypothesis 3 were addressed and a full discussion on the importance of the protein level, source of production and mechanism of regulation of endogenous IGF-1 in sensory neurons in diabetic neuropathy was given at the end of chapter 4. In chapter 4, we showed for the first time that DRG neurons synthesize and secrete their own IGF-1 that can act as an autocrine/paracrine neurotrophic factor to play a key role in neuronal maintenance and axonal outgrowth. We provided evidence that IGF-1 ablation in DRG neuron cultures attenuated this background neurite outgrowth. This endogenous production of IGF-1, detected at both the mRNA and protein levels in DRG tissue and culture, was suppressed under hyperglycemic conditions via effectors mediated via the polyol pathway. We further demonstrated that exogenous hIGF-1 exerted a positive feedback on endogenous IGF-1 transcript levels under hyperglycemic conditions and a negative feedback under normoglycemic conditions in cultured DRG neurons. The endogenous IGF-1 was expressed at a higher level in DRG tissue compared with sciatic nerve, and more specifically higher mRNA expression was detected in neurons compared with non-neuronal cells of the DRG. We also found more than a 2-fold enrichment of the transcription factors NFAT1 and CEBP-β binding to the IGF-1 promoter in DRG tissue from control rats compared with tissue from diabetic rats.

Despite a full discussion of main findings at the end of chapters 2, 3 and 4, some findings were not discussed and strengths, limitations and future direction of the present study as a whole was not given. For that reason, I will now discuss aspects of the data presented in this thesis that were not covered in the previous chapters.

Mitochondrial trafficking proteins. Mitochondrial trafficking is essential for providing energy at multiple sites along myelinated and unmyelinated peripheral nerves. When daughter mitochondria are made from parental mitochondria, they are distributed and trafficked to different regions of the cell via Miro GTPases (Rhot1 and Rhot2), trafficking kinesin-binding protein 1 (TRAK1) and 2 (TRAK2), dynein, kinesin and other adaptor proteins on microtubules or actin filaments (Fransson et al., 2006). Any dysregulation of this process leads to physiological abnormalities particularly in long sensory nerves. For instance, mutations in dynein, an essential protein for mitochondrial retrograde transport, cause Charcot-Marie-Tooth neuropathy and dominant spinal muscular dystrophy (Harms et al., 2012, Weedon et al., 2011). Interestingly, dynein (DYNC1H1) mutant mice displayed abnormal mitochondrial morphology and function in multiple tissues and developed hyperinsulinemia and hyperglycemia (Eschbach et al., 2013). We found that the mRNA levels of kinesin family member 5B (KIF5B), Ras homolog family member T1 (RHOT1 also known as MIRO-1) were downregulated and the mRNA level of dynein intermediate chain 1 (DNAI1) was elevated in DRG tissues from diabetic rats compared to control rats (Chapter 3, figure 3.1 E-G). In other studies, short-term diabetes suppressed the protein expression of KIF5A and KIF5B in DRG tissue in male but not female STZ-diabetic rats (Pesaresi et al., 2018). Cultured hippocampal neurons exposed to high glucose medium showed a reduction in KIF5B and synaptophysin (Baptista et al., 2013). KIF5 superfamily: KIF5A, KIF5B and KIF5C are motor proteins involved in transport of protein

complexes, mRNAs, vesicles and mitochondria. KIF5B is specialized for mitochondrial transport and movement on microtubules in neurons (Hirokawa and Noda, 2008, Shen et al., 2018).

In persons with diabetic neuropathy, mitochondria accumulated in axonal swellings of distal IENF (Ebenezer et al., 2007, Lauria et al., 2003). In animal models of diabetic neuropathy including STZ-diabetic mice, NOD mice and Ins2Akita mice accumulation of mitochondria in dendrites and axonal terminals of autonomic ganglia has been reported (Schmidt et al., 2003, Schmidt et al., 2009, Schmidt et al., 2008). The cause of these aberrant accumulations of mitochondria remain a mystery, however, in humans with sensory neuropathy this process may precede the eventual loss of mitochondria in the distal axons (Casanova-Molla et al., 2012). Therefore, the down-regulation of kinesin, a key protein for mitochondrial anterograde transport, in DRGs in diabetic rodents might reflect aberrant mitochondrial trafficking along the nerves and its abnormal accumulation and eventual depletion in cell bodies or in distal axons (Hamid et al., 2014). Although the general axonal transport process is not impaired in diabetic neuropathy based upon measurements of protein complexes and cargos such as NGF and substance P at mid sciatic nerve (Delcroix et al., 1997, Macioce et al., 1988, Tomlinson et al., 1982, Calcutt et al., 1990b), the reduction and aberrant distribution of KIF5B and/or RHOT1 adaptor protein in DRG tissue and along the axons could lead to a distal loss of mitochondrial transport via aberrant anterograde and retrograde trafficking. Elevation of dynein, a key protein for mitochondrial retrograde transport (Hafezparast et al., 2003), might be an intrinsic mechanism of neurons to counteract early signs of mitochondrial accumulation in distal axons even though its abundance in DRG cell bodies does not reflect its presence in distal axons since the dynein mRNA expressed in the cell body has to be translated and trafficked along the long axons to reach the terminals. The effect of insulin/IGF-1 signaling on mitochondrial trafficking and related genes

needs to be further investigated to reveal if their loss in diabetes is a contributing factor to these abnormalities.

Gene expression of mitochondrial dynamics components. The size and number of mitochondria changes dynamically to adapt to physiological conditions and energy demands in cells. Mitochondria undergo fission (one mitochondrion divides into two smaller mitochondria) and fusion (fusing mitochondria into a long interconnected structure) processes to keep a balance in their morphology and function. The key GTP-dependent proteins involved in fusion are mitofusin-1 (MFN-1) and MFN-2 for outer mitochondrial membrane, and optic atrophy 1 (OPA1) for inner mitochondrial membrane fusion (Galloway and Yoon, 2013, Otera and Mihara, 2011). MFN-1 has higher activity than MFN-2 and is the main player in mitochondrial membrane tethering, and OPA1 could not fuse mitochondria in the absence of MFN-1 but not MFN-2 (Cipolat et al., 2004, Ishihara et al., 2004). Studies have shown the link between axonal degeneration in neuropathies and defected mitochondrial fusion/fission or dysregulation of the key proteins that regulate these processes. For example, an inherited MFN-2 mutation is the cause of Charcot-Marie-Tooth neuropathy type 2A associated with abnormal axonal variants in patients, although a small proportion of patients do not display mitochondrial fragmentation (Zuchner et al., 2004). Persons with obesity and type 2 diabetes exhibited smaller mitochondria exhibiting some degree of vacuolization in their skeletal muscle (Kelley et al., 2002).

A variety of studies have demonstrated increased number of small mitochondria, as a result of increased fission or defected fusion, predominantly in axonal terminals and dendrites of autonomic ganglia in persons with diabetic neuropathy as well as in STZ-diabetic mice, NOD mice and Ins2Akita mice (Schmidt et al., 2003, Schmidt et al., 2009, Schmidt et al., 2008, Schroer et al., 1992). Similarly, a greater number of mitochondria of a smaller size were

observed in dorsal root axons in db/db mice (Vincent et al., 2010). We found that the mRNA level of MFN-1 was reduced (despite no change in DRP1 mRNA levels) in DRG tissues from STZ-diabetic rats compared to control rats (Chapter 3, figure 3.1 E-G). Elevation of DRP1 protein expression was observed in DRG tissues from STZ-diabetic rats (Leinninger et al., 2006a). Hyperglycemia induced mitochondrial fragmentation in cultured DRG neurons derived from E15 Sprague-Dawley rat embryos (Leinninger et al., 2006a). Placed together these studies suggest that DRP1 is upregulated in the diabetic condition leading to accelerated fission, and MFN-1 is downregulated resulting in defected fusion which might combine to trigger accumulation of small mitochondria in DRG tissue similar to observations in human pathological studies (Ebenezer et al., 2007, Lauria et al., 2003). However, the protein level of both MFN-1 and DRP1 were diminished in the cortex of STZ-diabetic rats (Roy Chowdhury et al., 2018). The reduction of the DRP1 protein but not mRNA might be the result of post-transcriptional downregulation which could be specific to the cortex, or indicates suppression of both fission and fusion in the cortex. However, there is no data available on mitochondria morphology in the cortex of diabetic rodent to support the latter statement. Another possibility is that the phosphorylated and active form of DRP1, which was not measured in this study, was not reduced or was most likely sufficiently active to catalyze mitochondrial fission in the cortex of STZ-diabetic rats. We showed that IGF-1 upregulated MFN-1 mRNA levels in cultured DRG neurons from control rats, and upregulated OPA1, MFN-1 and DRP1 mRNA levels in cultured DRG neurons from diabetic rats (Chapter 3, figure 3.1 E-G). The regulation of these genes by IGF-1 might be due to the extreme deficit in mitochondrial dynamics caused by dysregulation and malfunction of fission/fusion-related genes in the diabetic condition. Downregulation of endogenous IGF-1 and loss of insulin signaling in DRG tissue could possibly be the trigger for

dysregulation of fission/fusion-related genes in diabetic animals. Taken together, MFN-1 downregulation in the nerve and DRG tissues may contribute to mitochondrial fragmentation and energy deficits causing nerve dysfunction in diabetes.

Effect of IGF-1 on GLUT1/GLUT3 expression. In peripheral nerve, glucose transport 1 (GLUT1) is expressed in endoneurial capillaries, perineurium, myelinating Schwann cells in the Schmidt-Lanterman incisures and nodes of Ranvier, and GLUT3, is expressed in adulthood at low levels in paranodal Schwann cells and nodal axons (Magnani et al., 1996, Takebe et al., 2008). In DRG tissues from frogs and rats, both GLUT1 and GLUT3 are expressed (Rigon et al., 2013, Aghanoori et al., 2019). Cardiac and skeletal muscle cells and adipocytes express an insulin-dependent glucose transporter, GLUT4, which is absent in nervous system except in the hypothalamus (Piroli et al., 2007). We showed that the mRNA levels of GLUT1 increased, despite no change in GLUT3, in DRG tissues from STZ-diabetic rats (Chapter 3, figure 3.1 E-G). Enhanced GLUT1 expression must have occurred in non-neuronal satellite cells, endoneurial capillaries or perineurium in DRG tissues in STZ-diabetic rats since DRG neurons do not express GLUT1 although the possibility of induction of GLUT1 gene expression in neurons cannot be dismissed. This induction of mRNA level may be reflective of increased turnover of the GLUT1 transporter protein due to hyperglycemia. Schwann cells exposed to high glucose (25mM) showed a reduction in GLUT1 mRNA level after 7 days (Muona et al., 1992). There might be a different mechanism of response in terms of GLUT1 gene expression by each cell type to hyperglycemia as Schwann cells constitute the majority of the cells present in the nerve and the downregulation could be a defense mechanism by which Schwann cells serve to protect the peripheral nerve against hyperglycemic stress. Another explanation for this discrepancy could simply be that one process is an acute hyperglycemia-induced reduction observed in vitro while

the other was a chronic hyperglycemia-induced elevation of GLUT1 *in vivo* under diabetic conditions. IGF-1 treatment of cultured DRG neurons from diabetic or control rats acutely upregulated GLUT1 mRNA expression (Chapter 3, figure 3.1 E-G). This seems counter-intuitive, but may reflect increased mRNA production to overcome increased turn over of the GLUT1 transporter due to increased glucose uptake. In control neurons this may be due to increased need for ATP and in diabetic cultures due to hyperglycemia. Therefore, tissue-specific knock-down of GLUT1 as a therapeutic/prevention tool can be a control mechanism for glucose import into nerve cells and part of the resolution for the pathogenesis of diabetic neuropathy. However, there might be some issues such as risk of glucose deprivation in the target tissue as well as off-target hypoglycemia in surrounding tissues particularly in which GLUT1 is the dominant glucose transporter. These limitations should be addressed and taken into account before designing such a knock-down study.

Krebs cycle intermediate build-up in nerve. Interestingly, IGF-1 therapy prevented the build-up of Krebs cycle intermediates in tibial nerve in STZ-diabetic rats, which was associated with IGF-1 upregulation of mitochondrial function and ETS proteins (Chapter 3, results section). However, how hyperglycemia causes the build-up of Krebs cycle metabolites and what drives the effect of IGF-1 on glucose catabolism in cultured DRG neurons and in animal models remains to be studied. Glucose flux analysis in cerebellar neurons isolated from Wistar rats, indicated 52±6 % glucose metabolism by glycolysis, 19±2 % by oxidative/non-oxidative pentose cycle for hexose phosphate recycling, and 29±8 % by oxidative pentose phosphate pathway/de novo nucleotide synthesis. In a different approach, 16±1 % of glucose was oxidized by mitochondria from 62±6 % of glucose, which was converted to pyruvate. The remaining pyruvate, 46±6 %, was exported as lactate (Gebril et al., 2016). Consistent with our results, metabolic dysfunction in terms of

glucose metabolism and fatty acid β-oxidation occurred in sciatic nerve but not in DRG tissues in STZ-diabetic rats after 14 weeks of diabetes (Freeman et al., 2016). The activity of a rate-limiting enzyme, hexokinase I, in glycolysis was diminished in DRG tissue in STZ-diabetic Wistar rats after 6 or 12 weeks of diabetes (Gardiner et al., 2007). *In vivo* metabolic flux analysis in db/db mice revealed mitochondrial dysfunction and upregulation of Krebs cycle metabolites in kidney cortex but not in sciatic nerve and retina. The possible explanation for build-up of Krebs cycle intermediates in the kidney cortex, but not sciatic nerve is that Krebs cycle defects could be the result of mitochondrial dysfunction, which occurred in the kidney cortex but not in sciatic nerve and retina. Most of the intermediates of the fatty acid β-oxidation were upregulated in kidney cortex, sciatic nerve and retina in these 24-week-old diabetic mice (Sas et al., 2016). The metabolic flux in db/db mice, which is a type 2 diabetic animal model, does not reflect metabolic status in type 1 diabetic animal models such as STZ-diabetic rats. To date, no data from metabolic flux analysis of the DRG tissues and nerves of type 1 diabetic rodents is available. And also, the effect of IGF-1 on glucose flux in cultured DRG neurons needs to be investigated.

AMPK isoforms. We demonstrated that catalytic isoforms of AMPK (α1 and α2) had differential functions in regulating mitochondrial respiration, mtDNA replication and neurite outgrowth when DRG neurons were treated with IGF-1 (Chapter 3, results section). The transcriptional level of PGC-1α was reduced in skeletal muscle in AMPKα2-deficient but not AMPKα1-deficient mice fed a high fat diet for 12 weeks (Um et al., 2010). Muscle-specific AMPKβ1β2 knock-out in mice led to reductions in mitochondrial content and impaired glucose uptake (O'Neill et al., 2011). AMPKα1-deficient mice displayed low fertility associated with low mitochondrial content in their sperm (Tartarin et al., 2012). Any definitive function of these two AMPK isoforms in the pathogenesis and treatment of diabetic neuropathy has not been

demonstrated. DRG-specific knock-out mice for AMPKα1 and AMPKα2 would reveal their specific roles in glucose uptake and mitochondrial function *in vivo* opening new windows to future therapeutic options.

In our second study, there was downregulation of AMPK α2 mRNA level in DRG tissues from diabetic rats, and IGF-1 upregulated its mRNA expression in cultured DRG neurons from both control and diabetic rats (Chapter 3, figure 1.2 E-G). DRG-specific AMPK α2-overexpression might have the potential to prevent/correct mitochondrial dysfunction and neuropathy indices in diabetic animal models. Activation of AMPK in skeletal muscle in mice and non-human primates promoted glucose uptake in skeletal muscle and lowered glucose levels in plasma (Cokorinos et al., 2017). Liver-specific activation of AMPK (constitutively active: mutated AMPKγ1) in mice protected against triglyceride accumulation by inhibiting lipogenesis (Woods et al., 2017). Glucose uptake was stimulated by AICAR, an AMPK activator, in skeletal muscle in AMPK α1-knock-out mice but not in AMPK α2-knock-out mice. Contraction-induced glucose uptake was induced in both knock-out mice (Jorgensen et al., 2004b). AICAR promoted AMPK inhibition of glycogen synthase and stimulation of glucose transport in skeletal muscle tissue (Jorgensen et al., 2004a). Therefore, if we suppose AMPK has similar function in neurons, then the downside of AMPK α2-overexpression in DRG tissue would be excessive glucose uptake although it could improve mitochondrial function, glucose metabolism and triglyceride metabolism.

IGF-1 therapy. We and others have shown that IGF-1 therapy improves neuropathy indices in experimental diabetic neuropathy (Toth et al., 2006, Brussee et al., 2004, Homs et al., 2014, Chu et al., 2008, Aghanoori et al., 2019, Fernyhough et al., 1993, Ishii and Lupien, 2003, Zhuang et al., 1997), and yet, no clinical trial study has been established for IGF-1 therapy in

human diabetic neuropathy. Given the critical role of IGF-1 in neurite outgrowth, differentiation and development, a plethora of nervous system-related diseases including Parkinson's disease, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, Fragile X syndrome and Rett syndrome have become candidates for IGF-1 therapy (Costales and Kolevzon, 2016). The IGF-1 peptide crosses the blood brain barrier and recombinant human IGF-1 (rhIGF-1) has been approved by FDA in children with growth deficiencies (Backeljauw and Chernausek, 2012, Reinhardt and Bondy, 1994). Long-term (12 years) treatment of rhIGF-1 (60-120 μg/kg twice daily subcutaneous injection) stimulated linear growth in children with IGF-1 deficiency with no serious adverse effects (Chernausek et al., 2007).

IGF-1 therapy (subcutaneous injection of 100 μg/kg twice daily for six months) on six Rett patients was safe and well-tolerated, and improved motor skills, breathing and cognition abilities. Predicted side effects such as tonsillar hypertrophy, hypoglycemia, seizures and hyperplasia were assessed, and except for mammary hyperplasia in one patient which disappeared after administration interruption, other side effects were not reported (Pini et al., 2012). Subcutaneous injection of IGF-1 (4-week multiple ascending dose at 40-120 μg/kg twice daily for 4 weeks followed by open-label extension at maximum dose for 20 weeks) improved measures of anxiety, mood and apnea and ameliorated behavioral and breathing abnormalities without any evidence of hypoglycemia or adverse effects in 10 girls with Rett syndrome (Khwaja et al., 2014). A follow-up phase II clinical trial on 30 girls Rett syndrome was not successful with minor improvement in stereotypic behavior (O'Leary et al., 2018). In ALS, nine patients received low dose (0.5 μg/kg every two weeks for 40 weeks) or high dose (3 μg/kg every two weeks for 40 weeks) of IGF-1. Intrathecal delivery of high-dose IGF-1 slowed the limb dysfunction, but did not improve forced vital capacity (FVC, a lung function test), with no

serious adverse effects (Nagano et al., 2005). Nevertheless, subcutaneous injection of rhIGF-1 (50 μg/kg twice daily for 2 years) in a double-blind randomized phase III clinical trial did not have any beneficial effects for patients with ALS (Sorenson et al., 2008). IGF-1 could be the therapy of choice in type 1 and 2 diabetes where there is insulin resistance (Weber et al., 2014, Bondy et al., 1994). Thus, conventional IGF-1 therapy remains a potential therapeutic option in diabetic neuropathy but has been hindered by other potential systemic actions of the peptide such as induction of hyperplasia and hypoglycemia seen in related clinical trials.

Strengths and limitations:

Strengths

- Solid evidence of insulin effect on mitochondrial function in sensory neurons in chapter 2: It clarified that insulin is important for bioenergetics in sensory neurons and could be used therapeutically to optimize mitochondrial function in neurodegeneration in diabetes
- Specific and highly efficient knock-down of AMPK isoforms in chapter 3: This provided confidence for implementing mechanistic studies on IGF-1 regulation of mitochondrial function
- Providing mechanism of action of IGF-1 on mitochondrial function, mtDNA copy number and neurite outgrowth in chapter 3: This data afforded insight on mechanism of IGF-1 effects on neuronal structure and function in normal and diabetic conditions
- Validation of IGF-1 gene expression using a variety of techniques such as RNA-FISH, Northern blotting and Real-time PCR in chapter 4: We carefully demonstrated that IGF-1 was produced and secreted by sensory neurons but not glial cells

- Revealing evidence on the mechanism of IGF-1 suppression in diabetic conditions in chapter 4: We characterized that IGF-1 gene expression was affected by high glucose condition
- Introducing two new transcription factors that regulate endogenous IGF-1 in sensory neurons in chapter 4: This novel work provides future direction for focusing on these transcription factors for potential therapeutic studies in diabetic neuropathy

Limitations

- Lack of evidence fully describing the insulin effect on mitochondrial function in chapter 2: This area could be enhanced by using a variety of inhibitors blocking different signaling pathways to clarify how insulin enhanced mitochondrial function in an acute manner
- The focus on use of only STZ-induced type 1 diabetic rats in chapters 2 and 3: We could have compared the effect of insulin and IGF-1 in other animal models of type 1 diabetes as well as type 2 diabetes; however, it would be extremely costly, time consuming and ideally would be the focus of another follow-up project
- The need for stronger *in vivo* evidence of involvement of AMPK in IGF-1 regulation of mitochondrial function and nerve repair in chapter 3: Employment of transgenic animals deficient in specific AMPK isoforms would have significantly enhanced the project
- More evidence needed for neuron-specific involvement in the DRG in chapters 2, 3 and 4: Cell sorting and functional analysis of each cell type derived from the DRG would have revealed the effect of each treatment on each neuronal sub-type; although, the number of neurons feasibly isolated from DRGs would limit the applicability of a cell sorting approach

- Absence of transgenic animal models overexpressing or deficient in endogenous IGF-1 in DRG neurons in order to study the function of endogenous IGF-1 *in vivo* in control and diabetic conditions in chapter 4: Limitations in time and cost were the obstacles to implement this more mechanistic approach

Future Directions:

In chapter 4, we demonstrated that endogenous IGF-1 is downregulated in the liver and DRG tissues in experimental diabetic neuropathy, and its basal level is essential for neurite outgrowth in DRG neurons in vitro (Chapter 4, results section). However, further investigations are required to manipulate endogenous IGF-1 in vivo in diabetic neuropathy and other neurodegenerative disorders, and measure beneficial outcomes. DRG tissue-specific IGF-1 knock-in and knock-out animals are experimentally logical models to study the role of endogenous IGF-1 in DRG tissue in vivo. Advillin-Cre driver on C57B6:129S7 mouse strain would be a proper option for IGF-1 knock-out, with the possibility of a tamoxifen-inducible approach, as this mouse strain develops diabetic neuropathy after STZ injection and the knock-out will be specific to sensory neurons (Hasegawa et al., 2007, Lau et al., 2011, Zurborg et al., 2011). DRG tissue-specific IGF-1 knock-in can also be developed. The level of endogenous IGF-1 in these transgene animals may not be conveniently manageable, and also not translatable to clinical studies. Muscle-specific overexpression of IGF-1 in spinal and bulbar muscular atrophy (SBMA) mice delayed disease onset and survival, and prevented motor neuron loss in transgenic animals (Palazzolo et al., 2009). IGF-1 was upregulated more than 80 fold in skeletal muscle of these animals. To translate this tissue-specific gene overexpression or down-regulation approach into human treatment studies, a variety of techniques have been proposed. Transplantation of human adipose stem cells (ASC) carrying the IGF1 gene into the lumbar

region 2 (L2) of the spine, extended life span and delayed onset of clinical symptoms in both rat and mouse models of ALS (Sung et al., 2014). Transplantation of stem cells overexpressing IGF-1 into DRG/nerve tissues might have the potential for human trials although the efficacy of this therapeutic approach is still under development.

Recombinant adeno-associated virus 9 (rAAV9) constructs, which can stably express target genes, have been developed to specifically target neurons in experimental models and can cross the blood-nerve barrier in neonatal and adult animals (Duque et al., 2009). For example, intravenous delivery of rAAV9/IGF-1 induced 27-week expression of IGF-1 in the nervous system, but not in other organs, and prevented deficits in nerve function in spontaneous autoimmune peripheral polyneuropathy (SAPP) mice (Gao et al., 2018). AAV9 is currently being considered as a delivery method in two phase I clinical trials for neurological disorders (Spinal Muscular Atrophy type 1, ClinicalTrials.gov Identifier: NCT02122952, and Glycogen Storage Disorder, ClinicalTrials.gov Identifier: NCT02240407). The outcomes will reveal the safety and efficacy of the use of rAAV9 in human studies. A wide range of phase I-IV clinical trials for cancer, diabetes, cystic fibrosis, cardiovascular disorders, hepatitis B and HIV infections involving delivery of plasmids or oligonucleotides (naked or in nanoparticles) are in the pipeline and are reviewed elsewhere (Hardee et al., 2017). Despite an improved safety profile, some challenges such as target specificity, the level of gene expression and duration of target gene expression still need to be optimized.

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