Differential expression of neuregulin-1 isoforms and downregulation of erbin are associated with Erb B2 receptor activation in diabetic peripheral neuropathy

By

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Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Aberrant neuron-glia interactions can contribute to a variety of neurodegenerative diseases. We have previously demonstrated that enhanced activation of Erb B2, which is a member of the epidermal growth factor receptor (EGFR) family, can contribute to the development of diabetic peripheral neuropathy (DPN). In peripheral nerves, Erb B receptors are activated by various members of the neuregulin-1 (NRG1) family including NRG1 Type I, NRG1 Type II and NRG1 Type III to regulate Schwann cell growth, migration, differentiation and dedifferentiation. Alternatively, Erb B2 activity can be negatively regulated by association with the Erb B2-interacting protein, erbin. Since the effect of diabetes on the expression of NRG1 isoforms and erbin in peripheral nerve are unknown, the current study determined whether changes in NRG1 isoforms and erbin may be associated with altered Erb B2 signaling in DPN. Swiss Webster mice were rendered diabetic with streptozotocin (STZ) and after 12 weeks of diabetes, treated with erlotinib, an inhibitor of Erb B2 activation. Inhibition of Erb B2 signaling partially reversed several pathophysiologic aspects of DPN including a pronounced sensory hypoalgesia, nerve conduction velocity deficits and the decrease in epidermal nerve fiber innervation. We also observed a decrease of NRG1 Type III but an increase of NRG1 Type I level in diabetic sural nerves at early stage of diabetes. With disease progression, we detected reduced erbin expression and enhanced MAPK pathway activity in diabetic mice. Pharmacologic inhibition of Erb B2 suppressed MAPK activity in diabetic sural nerves. These results support that hyperglycemia may impair NRG1-Erb B2 signaling by disrupting the balance between NRG1 isoforms, decreasing the expression of erbin and correspondingly activating the MAPK pathway. Together, imbalanced NRG1 isoforms and downregulated erbin may contribute to the dysregulation of Erb B2 signaling in the development of DPN.

Acknowledgements

This work was supported by grants from the Juvenile Diabetes Research Foundation and the National Institutes of Health [NS054847, NS075311 and DK095911]. I also would like to thank OSI Pharmaceuticals for the generous supply of erlotinib.

I would like to thank the opportunity to pursue a graduate degree in the Department of Pharmacology and Toxicology at the University of Kansas, which have a great impact on my life and career. I would like to express my deepest gratitude to my mentor, Dr. Rick T. Dobrowsky. Without his support, guidance, and never-ending asking for data, I would not achieve the work in this project. His "intimidations" have been the motivations to push me forward and I would definitely miss that part of the Ph.D. life. He also wants the best for students, not only in their scientific research, but also their future career development. I am grateful for the freedom that he allowed me to think independently and search for a direction in my project, and the advice of choosing the best career direction.

I would also like to thank my committee members for the great help and support. I will forever remember the talks with Dr. Jeff Staudinger around the fifth floor on Malott Hall about improving my English skills and handling multi-tasks to meet deadlines. Dr. Alexander Moise has always been there to discuss the cumulative exams and qualified defense. Dr. Honglian Shi was a great help to finish the graduate program. Dr. Brian Ackley provided helpful suggestions in my committee meetings.

I am grateful for working with all the former and current members of Dr. Dobrowsky's lab. I learned behavior tests and nerve conduction velocity studies from Dr. James F. McGuire and Michael Urban. Dr. Cuijuan Yu, Dr. Chengyuan Li and Dr. Liang Zhang were so nice and patient to teach me basic biochemical and tissue-culture techniques. Jiacheng Ma always helped me in the project and listened to all my wild thoughts without shutting me out. I would also like to thank everyone in the department. With you guys, the five-year Ph.D. life was full of joy and memory.

Finally, I would like to thank my parents, Jinsheng Pan and Danjiang Su. Thank you for teaching me to be optimistic, be strong and be independent. You have always been there to support me, believe in me and guide me to make every decision in my life. You are my role models that drive me to grow up and stand where I am now and forward in the future.

List of Tables and Figures

List of Abbreviations

AA	Amino Acid
ACCORD	Action to Control Cardiovascular Risk in Diabetes
ACE	Angiotensin-Converting Enzyme
ACR	Albumin-to-Creatinine Ratio
ADA	American Diabetes Association
ADAM	A Disintegrin And Metalloproteinase
ADOA	Autosomal Dominant Optic Atrophy
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation
AGE	Advanced Glycation End-product
ALA	α-Lipoic Acid
Ang	Angiotensin
AR	Aldose Reductase
ARB	Angiotensin Receptor Blocker
ARI	Aldose Reductase Inhibitors
ARIA	Acetylcholine Receptor-Inducing Activity
BACE	β-site Amyloid precursor protein Cleaving Enzyme
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
CAD	Cardiovascular Autonomic Dysfunction
CHF	Congestive Heart Failure
CMT	Charcot-Marie-Tooth
CMV	Cytomegalovirus
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
COX	Cyclooxygenase
CRD	Cysteine-Rich Domain
DAN	Diabetic Autonomic Neuropathy
DCCT	Diabetic Control and Complications Trials
DME	Diabetic Macular Edema
DN-Erb B4	Dominant-Negative Erb B4 receptors
DPN	Diabetic Sensorimotor Polyneuropathy
DPP	Diabetes Prevention Program
DRG	Dorsal Root Ganglia
DRP	Dynamin-Related Protein
EGF	Epidermal Growth Factor
Erbin	Erb B2-interacting protein
ERK	Extracellular signal Regulated Kinases

Erl	Erlotinib
ESRD	End-Stage Renal Disease
ETDRS	Early Treatment of Diabetic Retinopathy Study
FFA	Free Fatty Acid
FPG	Fasting Plasma Glucose
G6P	Glucose-6-Phosphate
GBM	Glomerular Basement Membranes
GFAP	Glial Fibrillary Acidic Protein
GFR	Glomerular Filtration Rate
GGF	Glial Growth Factor
GLUT	Glucose Transporter
GP	Glutathione Peroxidase
GSH	Glutathione
GSIS	Glucose-Stimulated Insulin Secretion
HAPO	Hyperglycemia and Adverse Pregnancy Outcome
HDL	High-Density Lipoprotein
HLA	Human Leukocyte Antigen
HNF	Hepatocyte Nuclear transcription Factor
i.p.	intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IDF	International Diabetes Federation
IENFD	Intra-Epidermal Nerve Fibers Density
IFG	Impaired Fasting Glycemia
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IGF	Insulin Growth Factor
IGFR	IGF receptor
IGT	Impaired Glucose Tolerance
IL-1β	Interleukin-1β
iNOS	inducible Nitric Oxide Synthase
JNK	c-Jun NH2-terminal Kinase
LADA	Latent Autoimmune Diabetes in Adults
LAP	LRR And PDZ
LRR	Leucine-Rich Repeat
MAPK	Mitogen-Activated Protein Kinase
MBP	Myelin Basic Protein
MFN	Mitofusin
MNCV	Motor Nerve Conduction Velocity
MODY	Maturity-Onset Diabetes of the Young
MP	Metalloproteinase

NAG	*N*-Acetyl-beta-D-Glucosaminidase
NF- κB	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NIH	National Institutes of Health
NO	Nitric oxide
NPDR	Non-Proliferative Diabetic Retinopathy
NRG	Neuregulin
NT-3	Neurotrophin-3
OCT	Optical Coherence Tomography
OGTT	Oral Glucose Tolerance Test
OPA1	Optic Atrophy 1
P0	Protein Zero
$p75^{NTR}$	p75 neurotrophin receptors
PDR	Proliferative Diabetic Retinopathy
PDZ	PDS-95/Discslarge/ZO-1
PGP 9.5	Protein-Gene-Product 9.5
PI3K	Phosphatidylinositol-3-Kinase
PKC	Protein Kinase C
PNS	Peripheral Nervous System
PRR	(Pro) Renin Receptor
PTEN	Phosphatase and Tensin homolog
QST	Quantitative Sensory Testing
RAAS	Renin–Angiotensin Aldosterone System
RAGE	AGE receptors
rhNGF	recombinant human NGF
r-metHuNT3	recombinant-methionyl human NT-3
ROS	Reactive Oxygen Species
SBP	Systolic Blood Pressure
SCP	Schwann Cell Precursor
SDG	Sorbitol Dehydrogenase
SDI	Sorbitol Dehydrogenase Inhibitors
SMDF	Sensory and Motor neuron Derived Factor
SNCV	Sensory Nerve Conduction Velocity
SOD	Superoxide Dismutase
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TACE	Tumor-necrosis factor-α-Converting Enzyme
TCA	Tricarboxylic Acid cycle
TGF	Transforming Growth Factor

TNF-α	Tumor Necrosis Factor-α
Trk	Tropomyosin-related kinase
UAE	Urinary Albumin Excretion
UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular Endothelial Growth Factor
Veh	Vechicle
WESDR	Wisconsin Epidemiologic Study of Diabetic Retinopathy
WHO	World Health Organization
ZDF	Zucker Diabetic Fatty
ZLC	Zucker Lean Control

Chapter 1: Introduction

1.1. Overview of Diabetes Mellitus

1.1.1. Diabetes Mellitus

Diabetes Mellitus is a metabolic disorder characterized by chronic hyperglycemia and impaired metabolism of carbohydrate, fat and protein, which result from deficiency in insulin secretion, sensitivity and action. It is the fastest growing non-communicable disease in the world and gives rise to microvascular abnormalities and complications (retinopathy, nephropathy and neuropathy) [1-3].

Diabetes imposes remarkably high human, social and economic impacts worldwide. According to the sixth edition of the International Diabetes Federation (IDF) Diabetes Atlas [4], 382 million people currently are living with diabetes, about 8.3% of the global population. Among them, 175 million cases are undiagnosed. Considering the additional 316 million people with impaired glucose tolerance (IGT), the diabetic population is estimated to reach 592 million in 2035 if the trend continues. Diabetes and its complication make patients as twice as likely to die early compared to non-diabetic individuals. Globally, 5.1 million death cases were caused by diabetes directly or indirectly.

In the United States, 25.8 million people have diabetes including 7.0 million undiagnosed patients in 2011 [5]. 10.9 Million of these individuals, about 26.9%, are 65 years old or older. Every year, diabetes induces more death cases in the U.S. than AIDS and breast cancer combined, making it the 7th leading cause of death.

Unfortunately, 80% of the diabetic population are concentrated in low- and middle-income regions, especially emerging hotspots, such as the Middle East, the Western Pacific archipelago,

sub-Saharan Africa and South-East Asia. Though the Africa region has the smallest population of diabetes (19.8 million) currently, it may reach 41.4 million in 2035, increasing 109.1%. The high increase predicted in this region is mostly attributed to rapid economic development and population growth without adequate healthcare. In 2013, 548 billion USD (increased from 465 billion in 2011) in healthcare expenditures were diabetes-related, accounting for 11% of the total healthcare cost worldwide. However, nearly half of this was accounted by healthcare costs in the United States (263 billion), followed by Europe (147 billion); less than 1% was spent by South-East Asia and Africa combined [4].

1.1.2. Diagnostic Criteria of Diabetes Mellitus

Since 1965, the World Health Organization (WHO) has published guidelines for the diagnosis and classification of diabetes. **Table 1.1.2.1** summarizes the 1999 [1] and 2006 recommendations [6]. Plasma glucose concentration is the basis for diagnosis. However, considering the availability of equipment and cost-efficiency, whole blood samples could also be used in the diagnosis. However, the glucose concentration is 10-15% higher in plasma or serum samples compared to whole blood samples [7].

Table 1.1.2.1: Diagnosis Criteria for Diabetes Mellitus

	Glucose concentration, mmol l^{-1} (mg dl^{-1})		
	Whole Blood		Plasma
	Venous	Capillary	Venous
Diabetes Mellitus			
Fasting *and/or*	≥6.1 (≥110)	≥6.1 (≥110)	≥7.0 (≥126)
2-hr post glucose load	≥10 (≥180)	≥11.1 (≥200)	≥11.1 (≥200)
Impaired Glucose Tolerance (IGT)			
Fasting (if measured) *and*	<6.1 (<110) and	<6.1 (<110) and	<7.0 (<126) and
2-hr post glucose load	≥6.7 (≥120)	≥7.8 (≥140)	≥7.8 (≥140) and <11.1 (<200)
Impaired Fasting Glycemia (IFG)			
Fasting *and*	≥5.6 (≥100) and <6.1 (<110)	≥5.6 (≥100) and <6.1 (<110)	≥6.1 (≥110) and <7.0 (<126)
(if measured) 2-hr post glucose load	<6.7 (<120)	<7.8 (<140)	<7.8 (<140)

The two main methods used to render a diabetes diagnosis are fasting blood glucose (FBG) and the oral glucose tolerance test (OGTT). FBG measures the plasma glucose level in a person who has been fasting for at least 8 hours and is more convenient and cost-efficient compared to an OGTT, although the latter has been shown to be more accurate [8]. An OGTT indicates the action of insulin on promoting glucose uptake and measures the blood glucose level during a 2-hour period after ingesting a 75 g glucose load. The OGTT is always performed after at least an 8-hour fast. The euglycemic hyperinsulinemic clamp and the hyperglycemic clamp are tests for insulin sensitivity and β-cell function, respectively. These tests represent the gold standard for accessing first- and second-phase of glucose-stimulated insulin secretion. Since the first-phase insulin response is absence in all diabetic patients whose FBG levels exceed 115 mg/dL, glucose tolerance test could not quantify the β-cell function in overt diabetes [9]. In 2009, an International Committee [10] and the American Diabetes Association (ADA) recommended glycated hemoglobin (HbA1c) measurement as an additional means to diagnose diabetes. A

measurement of HbA1c higher than 6.5 percent is regarded as diabetes. However, the special equipment requirement and higher cost limits extensive use of this technique by individuals.

An underlying principle of these new criteria is that diagnosis of diabetes should *never* depend on only a single abnormal blood glucose value. At least one additional positive glucose test result is required to confirm diabetes. Thus, periodic re-testing is advised for pre-diabetic patients, who have IGT or IFG. In addition, physicians should take ethnicity, family history, age, and other health issues into consideration [1].

1.1.3. Classification and Clinical Features of Diabetes Mellitus

In 1922, the Canadian scientist Frederick Banting and his medical student Charles Best successfully isolated insulin from dog pancreas. Administration of this pancreatic extract to a 14-year old boy named Leonard Thompson dramatically improved his diabetic condition. Since this breakthrough, insulin therapy has become the definitive treatment for diabetes, especially in the cases of complete loss of β-cell mass. Historically, diabetes was classified based on the dependence of insulin treatment. This gave rise to two sub-types of diabetes (insulin-dependent diabetes and non-insulin-dependent diabetes). However, in 1999, the WHO recommended a new etiological classification based on our improved understandings of the causes of diabetes as non-insulin-dependent diabetes can evolve to an insulin-dependent diabetes [1, 6]. This new classification includes Type 1 (mostly autoimmune diabetic cases), Type 2 (marked by insulin resistance and obesity-related), gestational diabetes and other sub-types related to mutations in genes affecting insulin production and glucose metabolism.

Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM) accounts for less than 5% of total diabetic cases but the population continues to increase. Among those, 90% of T1DM cases are attributable to an autoimmune process, while the remaining cases have no clear evidence of autoimmunity (idiopathic diabetes). The latter situation is common in African and Asian individuals. All Type 1 diabetic patients are prone to ketoacidosis and β-cell destruction [1, 7]. Usually, the destruction of β-cell progresses rapidly, resulting in early and severe onset of diabetes in children. However, this process can manifest in adults, in which cases it is referred as latent autoimmune diabetes in adults (LADA). Since lack of insulin considerably disrupts energy metabolism and glucose homeostasis, polyuria (increased urination), polydipsia (extremely thirsty) and weight loss are three common clinical features of T1DM. Diabetic ketoacidosis may be present acutely or become the first manifestation that leads to a diagnosis of T1DM. Without early diagnosis and proper intervention, diabetic ketoacidosis can cause coma and even death, accounting for 5% mortality [7].

Insulitis and β-cell destruction are pathogenic hallmarks of T1DM. The initial step of T1DM progression (Checkpoint 1) is the infiltration of T lymphocytes and macrophages into islets in response to specific Human leukocyte antigen (HLA) presented on β-cells [11, 12]. Activated T cells ($CD4^+$ and $CD8^+$ T cells) and macrophages then release several pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [13-16]. These cytokines activate their cognate receptors on islet cells and induce several signaling pathways, such as nuclear factor kappa B (NF- κB) and inducible nitric oxide synthase (iNOS), resulting in production of nitric oxide (NO) [17, 18]. The iNOS in human pancreatic islets can only be induced by NF- κB activation via a combination of two or three types of

cytokines, suggesting potential defense mechanisms may exist to protect β-cells if only a single type of cytokine is present. A high concentration of cytokine-induced release of oxygen free radicals and NO production could suppress glucose-stimulated insulin secretion (GSIS) and eventually lead to β-cell apoptosis [19]. In addition, studies have shown that there are other pathways involved in cytokine-induced β-cell apoptosis, such as activation of members of the mitogen-activated protein kinase (MAPK) family [20]. All three sub-types of the MAPK family (extracellular signal regulated kinases (ERK or p42/p44 MAPK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK) were activated in rat islets, correlating with IL-1β-induced NO production. Interestingly, inhibition of the JNK pathway, but not ERK or p38, prevented NO-induced β-cell death [21].

In order to increase β-cell mass for insulin demand in response to inflammation and oxidative stress, β-cells acutely proliferate either by self-replication [22] or by transforming from α-cells [23]. However, with a slow turnover rate, even proliferation of β-cells cannot compensate for the destruction and viability loss induced by long-term exposure to hyperglycemia and oxidative stress. Complete failure of β-cell self-regulation is regarded as the final checkpoint for the onset of T1DM [24].

Given the early onset and rapid progression of T1DM, validated predictive biomarkers are well-studied for diagnosis and risk estimation. However, not all biomarkers reflect the underlying pathogenesis of T1DM, since the analysis from family cases of T1DM does not find a consistent positive prevalence of autoimmunity for $CD4^+$ and $CD8^+$ T cells [24]. Certain HLA molecules presenting on islet β-cells are recognized by T lymphocytes and lead to T cell infiltration into peripheral islets, which is the primary feature of disease progression. Certain HLA class II alleles at the *HLA DR* and *HLA DQ* loci will increase the risk of inherited T1DM

up to 30% in a child with a family history, especially those carrying the *HLA DRB1*03,*04; DQB1*0302* genotype [25]. Interestingly, some protective *HLA DQB*, such as *HLA DQB1*0602* could independently lower the risk estimation. After confirming the genetic risk with or without family history, autoantibody testing is usually performed. Currently, insulin or proinsulin, GAD65 or GAD67, IA-2 (or ICA512 or IA-2β or PHOGRIN), and ZnT8 are four major islet antigens tested for routinely [26]. Islet autoantibodies are rarely detectable before 6 months of age but show a peak incidence at around 1 to 2-year old [27], which correlates with the time-course of β-cell destruction. Thus, early biomarker detection and consideration of the differences in the immune system in infants and older children may provide a preventive strategy before β-cell loss.

Usually, antibodies with specificity to insulin or proinsulin, whose occurrence is associated with the *HLA DRB1*04; DQB1*0302* alleles, appear first, while the detection of antibodies to IA-2 (or ICA512 or IA-2β or PHOGRIN) have the highest risk of diabetes. With disease progression, multiple autoantibodies to islet proteins may be detected. Direct measurement of β-cell function is advised in individuals with multiple positive islet autoantibodies. Unfortunately, no clear triggers have been determined to the onset of T1DM [28]. Indeed, based on the variability in the age of diagnosis and progression rate between individuals, environmental factors such as nutrition, birth weight, birth oxygen level and maternal condition likely influence the onset and progression of T1DM. Detailed genetic analysis may give patients a more accurate assessment of their risk for developing T1DM, but how this information may be used clinically to delay the onset of T1DM is unclear.

Type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus (T2DM) accounts for more than 90% of the diabetic cases. In contrast to the early onset and presence of ketoacidosis in T1DM, T2DM can be asymptomatic for several years before diagnosis. As such, substantial proportion of T2DM cases have already developed microvascular complications. It is often the symptoms of the complications that drive the visit to the physician to yield the diagnosis of T2DM. The WHO and IDF recommend screening for T2DM on a routine basis [29], especially for those with risk factors, such as overweight/obesity, family history, cardiovascular conditions, history of gestational diabetes and drug use [30]. After identifying high risk individuals through Risk Assessment Questionnaires, biochemical tests are performed to measure random blood or urine glucose, HbA1c, or blood pressure and cholesterol levels. Diagnostic tests (FBG and/or OGTT) and intervention are warranted after positive screening tests [29, 30].

T2DM is clinically characterized by insulin resistance due to impaired insulin secretion or action, elevated glucagon and eventual β-cell dysfunction [7]. Glucagon is secreted from islet α-cells and an inability of insulin to suppress glucagon secretion is a hallmark of the overall metabolic stress in T2DM. T2DM is usually caused by a combination of genetic and environmental factors. Though mutations in insulin receptors [31, 32], the insulin-sensitive glucose transporter (GLUT)-4 [33] and a zinc transporter (*SLC30A8* encoded) exclusively expressed on insulin secretory vesicles [34] have been detected in certain sub-groups of Type 2 diabetic patients, no common mutations have been identified across the larger population of Type 2 diabetics. Therefore, unlike T1DM, environmental factors play a predominant role in the development of T2DM.

Aging is a natural process that decreases insulin sensitivity due to changes in body composition and physical activity. The ADA recommends screening every 3 years starting at 45 years of age if no other risk factors are identified [35]. Gestational diabetes and low birth weight also have been linked to IGT and T2DM development later in the lives of both mothers and children [36, 37]. Infants with poor intrauterine nutrition and impaired growth during early life showed higher blood pressure but lower weight at 1 year old, which could strongly affect β-cell development.

The most important and common risk factor of T2DM is overweight/obesity, which is defined as abnormal or excessive fat accumulation that may harm health. A body mass index (BMI) measurement of more than 25 is regarded as overweight, and a BMI of 30 or greater defines clinically obese. In 2008, more than 1.4 billion adults (20 and older) were overweight, and over 500 million were obese. The onset age of overweight/obesity has moved down into younger adults in recent years and more than 40 million children under the age of five were overweight in 2011 [38].

As elevated glucose levels persist in patients with IGT or T2DM, "glucotoxicity" is considered the primary contributor to the development of insulin resistance [39]. High glucose concentration progressively compromises insulin gene expression, insulin secretion and GSIS in cell lines [40], isolated islets [41] and animals [42]. Due to the tight correlation of obesity and T2DM, the concept of "lipotoxicity" [43] or "glucolipotoxicity" [44] has also been proposed for promoting insulin resistance and T2DM.

Lipotoxicity might be a secondary phenomenon after the onset of hyperglycemia [45]. In obese individuals, plasma levels and turnover rates of free fatty acids (FFAs) are increased compared with lean individuals [7]. Long-term exposure to FFA could increase basal insulin

secretion but inhibit GSIS in both animal models [43, 46, 47] and humans [48]. Meanwhile, high FFA concentrations could not only affect insulin secretion, but also impair insulin action and induce insulin resistance in muscle [49] and liver [50]. Increased FFA oxidation competes with glucose oxidation, resulting in suppressed glycogen synthesis [51] and accelerated gluconeogenesis [52]. FFA can eventually induce β-cell apoptosis in the presence of high glucose [53]. Therefore, the therapeutic indication for "glucolipotoxicity" would be tight control of blood glucose levels in the early stages of diabetes, which might be sufficient to avoid deleterious effects from both hyperglycemia and hyperlipidemia.

Tight glycemic regulation is the primary goal in preventing and managing diabetes clinically. Aiming for an HbA1c level of 7% or less is recommended for most patients based on the UKPDS (United Kingdom Prospective Diabetes Study) [54]. However, obtaining tight glycemic control to further lower HbA1c is a double edge sword due to an increased risk for hypoglycemia. Indeed, one large clinical trial (Action to Control Cardiovascular Risk in Diabetes study, ACCORD) was terminated due to frequent hypoglycemia and higher hypoglycemia-induced mortality (22%) in the aggressive glycemic control cohort (HbA1c level of 6 % or less) [55]. In contrast, aiming at a healthy lifestyle and promoting weight loss is effective in preventing T2DM. Several large intervention studies have demonstrated dietary treatment and physical exercise could reduce diabetes risk, lower mortality rate and even improve metabolic syndromes in American [56], Swedish [57], Finnish [58], Chinese [59], Japanese [60] and Indian [61] racial populations. For instance, the Diabetes Prevention Program (DPP) is one of the largest randomized controlled clinical trials of 3, 234 US adults with impaired glucose tolerance [56]. This study included a larger proportion of women (68%) and ethnic minorities (45%) and compared lifestyle intervention with drug intervention (metformin

850 mg twice daily) over 2.8 years. Although both interventions showed beneficial effects of reducing diabetes risk and improving glucose tolerance, lifestyle intervention also improved fasting triglycerides and high-density lipoprotein (HDL)-cholesterol, and decreased the incidence of hypertension. Interestingly, lifestyle intervention demonstrated more effective reduction in the risk for developing diabetes than metformin intervention, especially in older people. Therefore, a diet of reduced fat intake, increased vegetable consumption with moderate caloric restriction and 30-40 min of moderate physical activity on all or most days of the week is recommended for effective disease intervention.

Gestational Diabetes and Other Sub-types

Gestational diabetes is carbohydrate intolerance resulting in hyperglycemia during pregnancy. It can lead to serious health risks to the mother and infants and increases the risk for developing T2DM later in life [1]. The IDF estimates that 21.4 million or 16.8% of live births to women in 2013 have hyperglycemia in pregnancy [4]. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study showed that a pregnant woman's blood glucose is proportional to her risk for developing pregnancy complications [62]. Formal systemic testing for gestational diabetes is usually performed between 24 and 48 weeks of gestation. The diagnostic criteria are the same as with other types of diabetes. The woman should be re-evaluated as diabetic, IGT, or normal glucose tolerance through an OGTT six weeks or more after delivery.

Other specific sub-types of diabetes are associated with genetic defects of β-cell function and are formally referred to as maturity-onset diabetes of the young (MODY). Common forms of MODY are due to mutations in the genes for hepatocyte nuclear transcription factor (HNF) 4-α (MODY-1), glucokinase (MODY-2), HNF1-α (MODY-3) and insulin promoter factor-1

(MODY-4). In general, all forms of MODY alter insulin production or glucose metabolism in the β-cell. Other genetic defects that alter insulin action include Leprechaunism and Rabson-Mendenhall syndrome, endocrinopathies, diseases of the exocrine pancreas and drugs such as glucocorticoids that can also induce diabetes.

1.1.4. The Action of Insulin

As a central regulator, insulin plays an indispensable role in metabolism of glucose, amino acids (AA) and FFAs, which in turn feedback on insulin synthesis and secretion [63]. Insulin promotes the synthesis of glycogen, lipid and protein. Glucose, AA and FFA stimulate insulin secretion to maintain a certain circulating concentration of insulin, especially after food intake. On the other hand, as glucose levels decrease, glucagon secretion from pancreatic α-cells increases to promote glycogenolysis and gluconeogenesis in the liver, in order to avoid hypoglycemia. Therefore, the balance between insulin and glucagon is the key regulator of proper metabolic homeostasis. Deficits of insulin signaling contribute to the development of both T1DM and T2DM. Without proper interventions, diabetes can cause life-threatening consequences.

In healthy subjects, insulin gene expression, insulin synthesis and release are tightly regulated to meet metabolic demands. Insulin is produced in the pancreatic β-cells that are clustered in structures called islets. Pancreatic islets are surrounded by capillaries with high permeability to aid in sensing changes in plasma glucose concentration and release the corresponding amount of insulin. Glucose enters β-cells via the GLUT-2 transporter that is expressed on β-cells. GLUT-2 is an insulin-independent glucose transporter with low substrate affinity and mediates the facilitated diffusion of glucose into the β-cells. Glucose is phosphorylated to glucose-6-phosphate (G6P) by the rate-limiting enzyme glucokinase, which is

also expressed in hepatic cells and glucose-sensitive neurons. Similar to GLUT-2, glucokinase also has a lower glucose affinity compared to other hexokinases. Due to this low-glucose-affinity feature, insulin secretion quickly declines as glucose levels fall and this relationship helps keep blood glucose above a certain threshold to avoid hypoglycemia.

Oxidation of G6P via glycolysis and the tricarboxylic acid (TCA) cycle in mitochondria lead to the production of ATP. An increase in the intracellular ATP/ADP ratio closes an ATP-sensitive potassium (K^+_{ATP}) channel on the plasma membrane. The closure of the K^+_{ATP} channels depolarizes the plasma membrane and in turn opens the voltage-dependent calcium (Ca^{2+}) channel. Calcium influx triggers granule exocytosis and insulin secretion. This process is called "ATP-sensitive K^+_{ATP} channel-dependent insulin release", which is the basis of GSIS (**Figure 1.1.4.1**) [63].

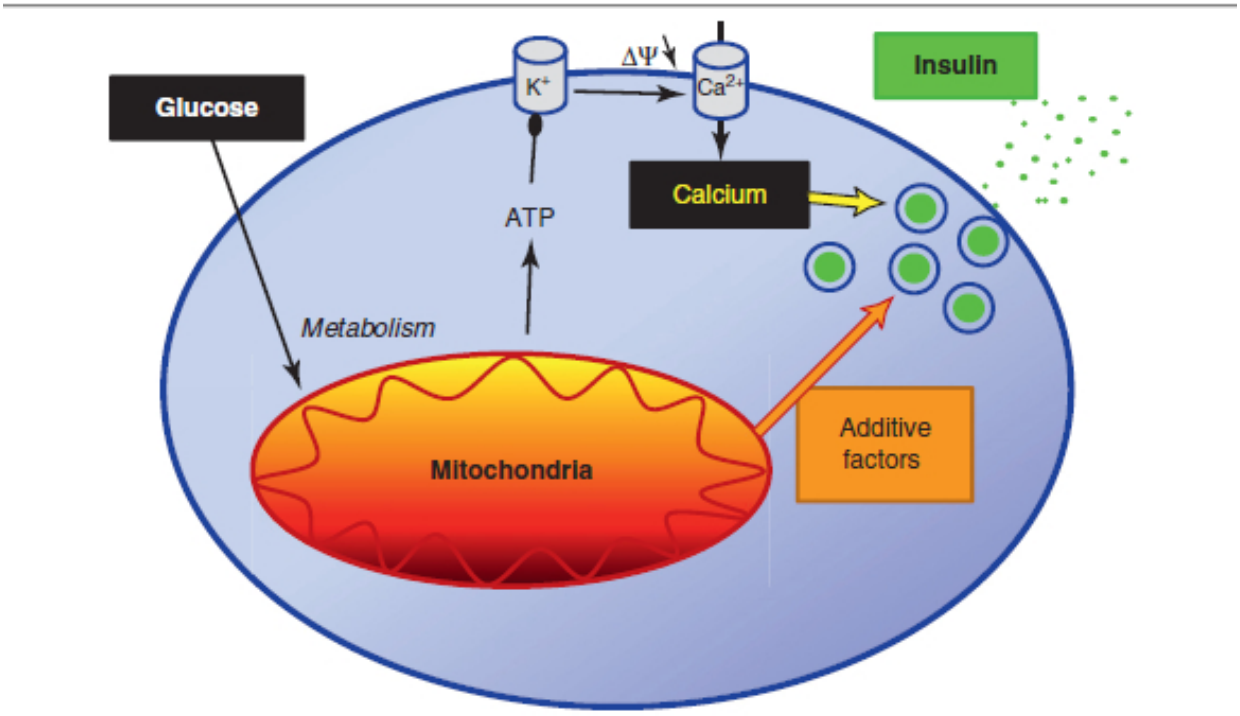


Figure 1.1.4.1: Model for coupling of glucose metabolism to insulin secretion in the β-cell. Glucose enters into β-cell and is phosphorylated by glucokinase. Pyruvate produced by glycolysis enters the mitochondria and serves as the fuel for mitochondrial respiration, resulting

in the generation of ATP and an increased cytosolic ATP/ADP ratio. Subsequently, the closure of K+ATP channels depolarizes the cell membrane, and opens the voltage-dependent Ca^{2+} channels. Calcium influx into cytosol triggers insulin exocytosis [64].

Insulin interacts with insulin receptor, which belongs to a large family of growth factor receptors with an intrinsic tyrosine kinase activity. Insulin receptor is a heterotetramer composed of two extracellular insulin-binding α subunits and two transmembrane tyrosine kinase β subunits. After insulin binding, insulin receptor undergoes trans-phosphorylation on multiple tyrosine residues of the β subunits, leading to the activation of the kinase activity and tyrosine phosphorylation of a number of insulin receptor substrate (IRS) proteins [65]. IRS proteins function as docking proteins to recruit downstream intracellular proteins. These protein-protein interactions are pivotal in transmitting the signal from the receptor to downstream cellular pathways, such as translocation of GLUT4 to plasma membrane, activation of glycogen or protein synthesis and the initiation of certain gene transcription.

1.1.5. Mitochondrial Dysfunction in β-cell Death

In diabetic individuals, β-cells are either damaged completely with little or no insulin secreted in T1DM, or overwhelmed by excessive glucose that can cause impaired insulin release and function in T2DM. Both types of diabetes may eventually result in β-cell apoptosis and loss of β-cell mass. Mitochondrial dysfunction and oxidative stress have been linked to β-cell death in both T1DM and T2DM. Glucotoxicity and/or glucolipotoxicity induce intracellular oxidative stress by compromising mitochondrial electron transport chain activity [63]. Reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\cdot}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are byproducts from normal mitochondrial electron transport chain function and can participate in physiological reactions. For instance, low levels of H_2O_2 induce GSIS in isolated

mouse islets [66]. However, prolonged oxidative stress increases endogenous antioxidant enzymes but inhibits GSIS. These data suggest that oxidative stress may temporarily provide a protective defense but prolonged ROS production can initiate β-cell dysfunction [67]. In this regard, animal studies have shown that increased ROS generation is associated with mitochondrial dysregulation in diabetes [68].

Mitochondrial dysfunction can be reflected by changes in organelle morphology. Mitochondria are highly dynamic organelles that maintain a balance between fusion and fission to ensure organellar integrity (**Figure 1.1.5.1**) [69]. Fission is critical to clear out dysfunctional mitochondria from the mitochondrial network. The mitochondrial network divides asymmetrically to generate a pool of fragments containing functional mitochondria (shown in red), partially defective but reparable mitochondria (shown in yellow), and completely damaged units (shown in blue). Dysfunctional mitochondria are degraded through mitophagy (Step 3), whereas reparable units are stabilized and rescued by fusion with fully functional mitochondria (Steps 2 and 4). Pro-fission proteins, including cytosolic dynamin-related protein (DRP) 1 [70] and the outer-mitochondrial membrane protein FIS1 [71] play important roles in regulating mitochondrial division. Elongated mitochondria undergo fission through recruiting cytosolic DRP1 to FIS1 on the outer mitochondrial membrane. DRP1 oligomerizes and induces constriction of the mitochondrial outer membrane by hydrolyzing GTP [72]. Mitochondrial fusion is mediated by optic atrophy 1 (OPA1) and mitofusin (MFN) 1/2 proteins. The fusion process allows exchange of normal mitochondrial DNA copies and membrane components. Daughter mitochondria re-integrate into the mitochondrial network (Step 5) for the next cycle.

Alterations in the mitochondrial fission-fusion process may be involved in certain disease conditions, since dysfunction of MFN2 is associated with peripheral neuropathy of Charcot-

Marie-Tooth (CMT) Type 2A disease [73]. Similarly, mutations in OPA1 contribute to autosomal dominant optic atrophy (ADOA) disease [74].

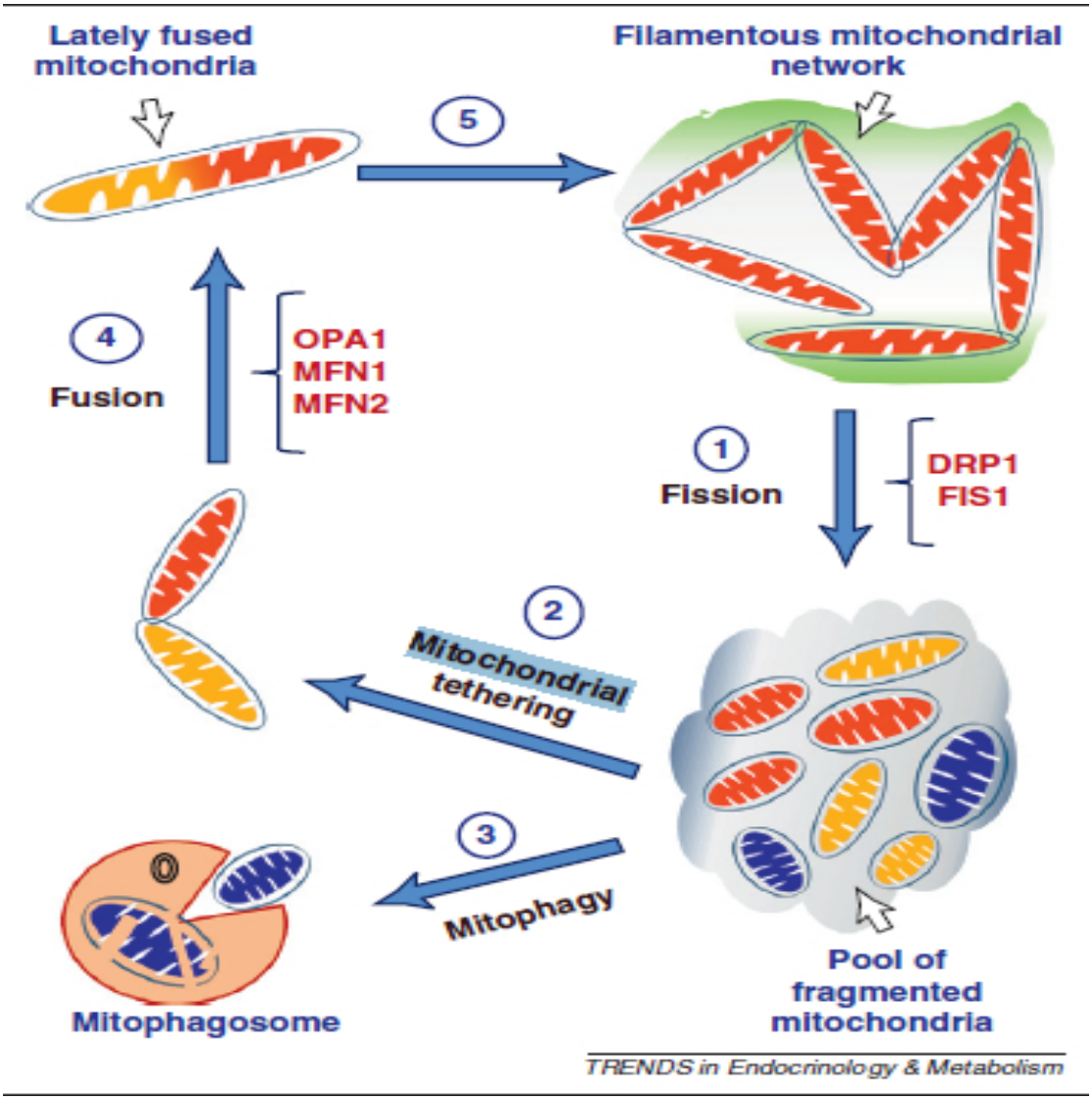


Figure 1.1.5.1: Schematic model for the role of mitochondrial shaping proteins and dynamics of fission-fusion process. Step 1, mitochondria are elongated to form mitochondrial network. With the help of pro-fission protein DRP1 and FIS1, mitochondrial network divides into a pool of fragmented mitochondrial components. Partial defective but reparable unites would fuse with fully functional units (**Step 2** and **Step 4**). However, completed damaged units would go to mitophagosome and be degraded (**Step 3**). After exchanging mitochondrial DNA copies and membrane components, daughter mitochondria could re-enter the next cycle of fusion-fission process (**Step 5**) [75].

Altered mitochondrial morphology in β-cells has been observed in T2DM animal models [64, 76] and Type 2 diabetic patients [77]. The islets from Zucker Diabetic Fatty (ZDF) rats, which develop obesity and hyperglycemia, contain a larger proportion of short swollen mitochondrial fragments (48%) or a combination of long and short fragments (44%), compared to the corresponding Zucker Lean Control (ZLC) rats, which only have 5% fragmented mitochondria [76]. Similarly in T2DM patients, diabetic islets have a similar number of mitochondria, but a disintegrated network and higher mitochondria density volume. This is associated with lower ATP levels, a decrease in the ATP/ADP ratio and an impaired mitochondrial membrane potential [77]. Downregulation of the pro-fission proteins DRP1 or FIS1 decreased mitochondrial autophagy. The subsequent accumulation of damaged mitochondrial components including oxidative mitochondrial proteins reduces mitochondrial respiration and GSIS [78]. In addition, mice with a β-cell specific deficiency in the pro-fusion protein OPA1 developed IGT due to impaired proliferation and insulin release. This was associated with disrupted mitochondrial structure (complex IV) and reduced ATP production [79]. Therefore, the balance between fission and fusion needs to be tightly regulated to maintain normal mitochondrial function but mitochondrial dysfunction might be the cause or the result of aberrant morphology.

1.2. Diabetic Complications

Hyperglycemia causes systemic damage not only to islets but also to other organs to induce diabetic complications such as cardiovascular disease, nephropathy, retinopathy and neuropathy. Large randomized, clinical trials have demonstrated the strong correlation between risks of developing diabetic complications and hyperglycemia in both T1DM [80] and T2DM [81, 82]. The Diabetic Control and Complications Trials (DCCT) compared intensive and conventional therapy in 1, 441 T1DM patients with an average 6.5-year follow-up. Compared to conventional

therapy of one or two daily insulin injections, intensive therapy with three or more daily insulin injections to further lower HbA1c effectively reduced the incidence and slowed the progression of diabetic complications. Similarly in T2DM, the UKPDS recruited 5, 102 newly diagnosed T2DM patients with an average 10 years follow up. Though intensive insulin therapy resulted in a final median HbA1c of 7.0% that was only 0.9% lower than that achieved in the conventional therapy group, the incidence of diabetic complications benefited from lowering chronic blood glucose levels. However, other trials with T2DM patients have not seen a clear benefit between reducing complications with tight glycemic control. Nonetheless, these clinical trials provided remarkably valuable evidence that tight glycemic control is beneficial to overall metabolic improvement and can reduce the risk rate of diabetic complications in both T1DM and possibly some people with T2DM. A caveat is that this benefit comes at an increased risk for recurrent hypoglycemia.

1.2.1. Diabetic Retinopathy

Diabetic retinopathy is a condition that causes progressive damage to the retina. As a serious sight-threatening microvascular complication of diabetes, diabetic retinopathy is the leading cause of visual impairment and even blindness in the working age population and contributes to 50% of total blindness cases [83]. Though retinopathy occurs rarely in the first 3-5 years after the onset of diabetes, nearly all T1DM patients and more than 60% of T2DM patients develop retinopathy within two decades of diabetes [84]. In 2010, 92.6 million adults had diabetic retinopathy globally [85]. 4.2 Million diabetic patients aged 40 years or older have diabetic retinopathy in the US and of those, 655, 000 have advanced diabetic retinopathy [5]. Economically, diabetic retinopathy costs $500 million per year for health care and disease treatment in the US [86]. The DCCT found that intensive glycemic control reduced the risk of

developing retinopathy by 76% in the primary prevention population, and 54% in the secondary prevention population who already had retinopathy. The UKPDS also showed a 25% reduction in the need for retinal laser treatment in T2DM patients with intensive insulin therapy. However, 10 years of follow-up in these studies showed that advanced diabetic retinopathy was not completely prevented, even in the intensive therapy groups [83]. Another large study, the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) in 996 young-onset diabetic patients demonstrated that the incidence and severity of diabetic retinopathy is strongly associated with the duration of diabetes. The prevalence of any form of retinopathy was 8% at 3 years after the onset of diabetes, 25% at 5 years, 60% at 10 years, and 80% at 15 years [87]. Other risk factors of diabetic retinopathy include hypertension, hypercholesterolemia, genetic factors and pregnancy. Both the WESDR and the UKPDS established the relationship between hypertension and worsening of diabetic retinopathy.

The International Clinical Disease Severity Scale [88, 89], based upon the WESDR and Early Treatment of Diabetic Retinopathy Study (ETDRS), divides disease progression into five stages. The first is “no apparent retinopathy”. The second stage is “mild non-proliferative diabetic retinopathy” (NPDR), which is characterized by the presence of a few microaneurysms. The third stage is “moderate NPDR” with the presence of microaneurysms, intra-retinal hemorrhages or venous beading. “Severe NPDR”, the fourth stage, is the key level to determine whether laser treatment is required. The final stage is “proliferative diabetic retinopathy” (PDR), which is marked by neovascularization, vitreous hemorrhage or traction-induced retinal detachment, resulting in vision loss. Co-existing diabetic macular edema (DME) could also contribute to vision loss.

Diabetic retinopathy is mostly asymptomatic. Generally, the symptoms include seeing spots floating in the field of vision, blurred vision, having a dark or empty spot in the center of the visual field and visual deficits at night. By the time impaired vision is observed, pathology might be significantly advanced. Thus, there is an urgent need for screening and early diagnosis. The American Academy of Ophthalmology has recommended screening for diabetic retinopathy 5 years after disease onset in T1DM patients, and at the time of diagnosis in T2DM patients. Dilated fundus examination should be performed annually if no retinopathy symptom has been detected. If mild NPDR is present, exams should be repeated every 9 months; moderate NPDR, every 6 months; severe NPDR, every 3 months. Patients with proliferative diabetic retinopathy should be examined every 2 to 3 months. Diabetic pregnant women should be examined every 3 months, since retinopathy can progress rapidly due to hormone changes [90]. For a definite diagnosis, fluorescein angiography or optical coherence tomography (OCT) should be performed to check the blood vessels and retinal thickness [91, 92].

The major pathophysiology of diabetic retinopathy manifests as thickening and swelling of the macula, microaneurysm formation and neovascularization. Various hyperglycemia-linked biochemical mechanisms have been shown to directly or indirectly contribute to diabetic retinopathy, such as enhanced polyol pathway flux, over activation of the protein kinase C (PKC) pathway, advanced glycation end-product (AGEs) formation, chronic inflammation, oxidative stress and local hypertension [92]. Additional contributing factors include dysregulated growth factors, such as insulin growth factor (IGF) and vascular endothelial growth factor (VEGF) [93, 94]. Clinical studies have found a higher serum concentration of IGF-I [95] and VEGF [96] in diabetic patients, which correlated with higher frequency of PDR or moderate NPDR. Both IGF-I and VEGF can stimulate proliferation, increase blood vessel permeability and promote

neovascularization. Serum levels of IGF can be affected by insulin therapy. Acute increases in circulating IGF after insulin therapy is initiated may explain the early worsening of diabetic retinopathy. Antibody-based targeting of VEGF has become a new strategy to treat retinopathy. Currently, pegaptanib [97], bevacizumab [98], ranibizumab [99] and VEGF-trap [100] are undergoing clinical trials and have showed beneficial effects against diabetic retinopathy and DME. However, blocking IGF-I or VEGF action has been found to cause retinal cell death [92, 93]. Therefore, long-term safety and therapeutic dosage need to be further investigated.

Current treatments of diabetic retinopathy or DME consist of mainly two aspects, controlling hyperglycemia and its related biochemical pathways, or alleviating disease symptoms. Hyperglycemia-linked therapies include insulin to control systemic glucose levels, aldose reductase inhibitors (ARIs), PKC inhibitors, angiotensin receptor blocker (ARB), antioxidant, and intravitreal injection of anti-VEGF agents and corticosteroids. Laser photocoagulation is the widely used therapy to stop vessel leaking and neovascularization. The ETDRS found that laser photocoagulation effectively reduced the risk of moderate visual loss by 50 % in patients with clinically significant macular edema [101]. Early laser photocoagulation treatment could delay the disease progression in NPDR and early PDR.

1.2.2. Diabetic Nephropathy

Diabetic nephropathy is characterized by persistent albuminuria, higher arterial blood pressure and decreased glomerular filtration rate (GFR), which is correlated with a high risk of cardiovascular morbidity and mortality [83]. In the US in 2008, 48, 374 new cases of end-stage renal disease (ESRD) were attributed to diabetic nephropathy, making it the leading known cause of ESRD (accounts for 44%) [5]. Similar to the higher prevalence of diabetic retinopathy in T1DM patients, diabetic nephropathy develops in approximately 20% - 40% of T1DM but in less

than 20% of T2DM patients. The peak time of diabetic nephropathy onset in T1DM patients is between 10 and 15 years after diagnosis of diabetes.

The nephron is the functional and structural unit of the kidney and consists of the glomerulus, Bowman's capsule and various collecting tubules. The glomerulus controls selective substrate permeability through its negative charge and pore size. In the early stages of injury (Stage 1), enhanced cellular proliferation of endothelial cells, epithelial cells or mesangial cells results in widening of the mesangium [102]. Glomeruli may show only hypertrophy without lesions. However, extracellular matrix accumulates within 2 years of diabetes onset (Stage2), progresses with increased duration of diabetes, and eventually contributes to glomerular basement membrane (GBM) thickening [103]. Lesions comprised of thickened GBM, mesangial expansion and the presence of microalbuminuria (Stage 3) are the hallmarks of diabetic glomerulopathy. Diffuse mesangial lesions are progressive. Aggressive extracellular deposition of precipitated plasma protein leads to formation of Kimmelstiel-Wilson nodules, falling GFR and the onset of macroalbuminuria (Stage 4), which typically occur 15 years or more after the start of diabetes. All these changes disrupt the structure of the nephron and causes tissue ischemia and declined GFR. Without intervention, patients with macroalbuminuria will develop ESRD and kidney transplant therapy will be required [83].

Besides hyperglycemia *per se*, hypertension, ethnicity (African American, Mexican American, and Pima Indians), genetic factors, abnormal GFR, increased sodium-lithium and sodium-hydrogen counter-transport, and other renal disease could all enhance the risk of diabetic nephropathy development. The ADA recommends annual screening for all T2DM patients and for T1DM patients 5 years after onset [104]. Currently available tests for screening and diagnosis of diabetic nephropathy include the measurement of albumin-to-creatinine ratio (ACR)

in a random spot collection, a 24-h collection to calculate creatinine clearance, and a timed (4-hour or overnight) urine collection. A Urinary Albumin Excretion (UAE) rate within 30 to 299 mg/24 h (equivalent to 20-199 μg/min on a timed sample or 30-299 mg/g creatinine on a random spot sample) is considered microalbuminuria. UAE that is above the upper threshold is considered macroalbuminuria. Acute hyperglycemia, hypertension, urinary tract infections and exercise could temporarily elevate UAE. Therefore, all abnormal tests must be confirmed in two out of three samples collected over a 3 to 6-month period, before a definite diagnosis and initiation of treatment [105]. Measurement of urinary excretion of *N*-acetyl-beta-D-glucosaminidase (NAG) and NAG-to-creatinine ratio are also predictive parameters for early diagnosis. In addition, downregulation of podocyte-specific proteins including synaptopodin, podocin and nephrin could be critical pre-clinical markers to detect kidney damage prior to microalbuminuria, but are difficult to assess [106].

Similar to diabetic retinopathy, intensive glycemic control can help decrease the incidence of diabetic nephropathy; it reduced the incidence of microalbuminuria by 39% in the DCCT and 30% in the UKPDS. Hypertension is the most common co-morbidity with hyperglycemia. Hypertension accelerates the progression of diabetic nephropathy through the renin-angiotensin aldosterone system (RAAS), which plays an integral role in regulating arterial pressure, tissue perfusion and extracellular volume [107]. Renin and its receptor, (Pro) renin receptor (PRR), could enhance the renal production of inflammatory cytokines such as TNF-α and IL-1β [108]. Angiotensin (Ang) II induces podocyte injury and extracellular matrix accumulation via an increase in ROS production, the expression of mesangial transforming growth factor (TGF)-β [109] and VEGF synthesis [110].

Monotherapy with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARB) is currently recommended as the first line therapy against microalbuminuria or diabetic nephropathy. Captopril is an ACE inhibitor that considerably decreased the creatinine concentration and effectively lowered the risk of death, dialysis, and transplantation by 50% in T1DM patients [111]. Losartan is an ARB that significantly reduced the incidence of elevated serum creatinine concentration by 25%, and ESRD by 28%, but had no effect on altering mortality rate in T2DM patients [112]. More importantly, these protective effects are synergistic with the benefit from blood pressure control. Combined therapy of pharmacological treatments and blood pressure control showed greater protection than either hypertension control or drug monotherapy alone [112]. Therefore, this combined therapy is frequently used in clinical practice for optimal outcomes [113]. As the disease progresses to ESRD, dialysis is needed. Generally, patients have better outcomes with transplantation than dialysis. For T1DM patients, a combined kidney-pancreas transplant leads to better outcomes than kidney transplantation alone [83].

1.2.3. Cardiovascular Disease

Hyperglycemia and co-existing hypertension, insulin resistance and dyslipidemia induce structural damage in the heart, resulting in ventricular dysfunction and ultimately congestive heart failure (CHF). Clinical manifestations include atherosclerosis, diabetic cardiomyopathy, coronary artery disease and peripheral vascular disease [114, 115]. Cardiovascular disease is the most common cause of death in diabetic patients, accounting for 68% among patients aged 65 years or older [5]. Postprandial glucose and HbA1c levels are correlated with the risk of cardiovascular disease and its associated mortality. The UKPDS showed a linear correlation, such that for every percentage point decrease in HbA1c, there was a 25% reduction in diabetes-

related deaths and an 18% reduction in myocardial infarction [81]. Similar protective results were also detected in the DCCT. However, as discussed above, with aggressive glycemic control aiming at an HbA1c level of 6% or less, the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) showed no beneficial effects [116]. Similarly, the ACCORD study found a 22% higher mortality rate, which was persistent after a 5-year follow up. Therefore, lowering HbA1c level to 8% or less is recommended. In addition, controlling blood pressure has been demonstrated effective in lowering the risk of cardiovascular disease in diabetic patients [117]. The ADA recommends controlling blood pressure to less than 130/80 mm Hg in diabetic patients [118]. No further protection is observed in diabetic patients with systolic blood pressure (SBP) less than 120 mm Hg. Other available treatments include metformin, aspirin and lipid-lowering therapy. Healthy lifestyle including low-fat diet, weight loss and exercise is also highly encouraged to prevent cardiovascular disease in diabetic patients.

1.3. Diabetic Neuropathy

Diabetic neuropathy is the nervous system disorder caused by chronic hyperglycemia, and affects about 60% to 70% of diabetic patients. Clinical symptoms include impaired sensation or pain in the feet or hands, slowed digestion of food in the stomach, carpal tunnel syndrome and erectile dysfunction. Severe forms of diabetic nerve damage are the major causes of non-traumatic lower-limb amputations, accounting for 60% of total amputation cases [5].

1.3.1. Classification and Clinical Features

There are two main systems to classify diabetic neuropathy that are based on either the clinical symptoms or the potential underlying pathophysiology. **Table 1.3.1.1** summarizes the

subgroups based on clinical patterns. On the other hand, diabetic neuropathy can be attributed to metabolic-microvascular-hypoxic mechanisms (including DPN and DAN), inflammatory immune reactions, the presence of other diabetic complications (such as ketoacidosis, renal failure and large vessel ischemia) and treatment-induced nerve damage [119, 120]. It is clinically common that diabetic patients develop different forms of diabetic neuropathy simultaneously, since pathological mechanisms co-exist under systemic hyperglycemia.

Table 1.3.1.1: The classification of diabetic neuropathy based on clinical patterns

Symmetry	Type of Neuropathy	Subtype
Symmetrical	Diabetic polyneuropathy (DPN)	
	Diabetic autonomic neuropathy (DAN)	
	Painful distal neuropathy with weight loss, "diabetic cachexia"	
	Insulin neuritis	
	Hypoglycemic neuropathy	
	Polyneuropathy after ketoacidosis	
	Polyneuropathy with glucose impairment	
	Chronic inflammatory demyelinating polyneuropathy (CIDP) in diabetes	
Asymmetrical	Diabetic radiculoplexus neuropathies	Lumbosacral (DLRPN)
		Thoracic (DTRN)
		Cervical (DCRPN)
	Mononeuropathies	Median neuropathy at the wrist (MNW)
		Ulnar neuropathy at the elbow (UNE)
		Peroneal neuropathy at the fibular head
	Cranial neuropathies	Oculomotor palsy
		Abducens palsy

Diabetic Autonomic Neuropathy (DAN)

DAN develops in nearly 50% of diabetic patients and affects many organs resulting in cardiovascular, gastrointestinal, urinary, sweat gland and genitourinary dysfunction [120, 121]. DAN may occur as early as one year after the onset of diabetes and progress with the duration and severity of DPN [119]. **Table 1.3.1.2** summarizes the main clinical manifestations of DAN [122]. Unfortunately, due to the diversity of symptoms, DAN usually goes unnoticed.

Orthostatic hypotension is the most easily recognized symptom. Patients could experience fatigue, visual blurring, weakness, and a failure of blood pressure maintenance after standing or exercise. Gastroparesis might worsen the hyperglycemic condition due to retained food in the stomach. Cardiovascular autonomic dysfunction (CAD), including silent ischemia, arrhythmia and stroke, are the major life-threatening concerns to cause DAN-associated cardiac death and mortality.

Table 1.3.1.2: Clinical manifestations of Diabetic Autonomic Neuropathy

Cardiovascular	Gastrointestinal	Genitourinary	Miscellaneous
Orthostasis	Oesophageal dysfunction	Erectile dysfunction	Hypoglycemia unawareness
Exercise intolerance	Gastroparesis	Retrograde ejaculation	Heat intolerance
Arrhythmia	Diarrhoea	Reduced vaginal lubrication	Argyll Roberson pupil
Silent ischemia	Constipation	Neurogenic bladder	Sweating disturance

Diabetic Sensorimotor Polyneuropathy (DPN)

DPN is the most common form of diabetic neuropathy, characterized by length-dependent lower-limb sensorimotor nerve fiber dysfunction, and loss of myelinated and unmyelinated nerve fibers. Sensory nerve injury occurs earlier and progresses more severely than motor nerve neuropathy. DPN is manifested by a variety of "positive" and "negative" sensory symptoms. Positive symptoms are often worse at night and consist of hyperalgesia and excessive tingling and burning sensations. This painful diabetic neuropathy usually develops in the early stage of diabetes and has been shown to be associated with injury of small unmyelinated fibers [123, 124]. Most DPN patients also present negative symptoms, such as numbness and loss of sensation. Since diabetic patients with negative symptoms might be unaware of the sensory injury, the worst outcome could be foot ulceration and eventual foot/limb amputation.

Electrophysiological tests are the gold standard to diagnose the onset of neuropathy and to predict disease progression. The severity of nerve conduction abnormalities correlates with the severity of hyperglycemia and underlying changes in nerve fiber structure. Nerve conduction deficits have been detected in diabetic patients, even without signs of neuropathy [125, 126]. Decreased amplitudes indicate the pronounced axonal degeneration in DPN, whereas the slow nerve conduction velocities suggest demyelination [119]. However, diabetic patients may present both slow nerve conduction velocity and decreased amplitude when they are diagnosed with DPN.

One limitation of the electrophysiological test is that it provides a more accurate evaluation for myelinated motor nerves (predominantly Aα fibers) than sensory nerves, especially small unmyelinated C-fibers. Thus, Quantitative Sensory Testing (QST) is performed to determine sensory nerve function. QST measures a patient's sensory detection thresholds to heat/cold and vibratory stimulation that is mediated mostly by unmyelinated C-fibers and thinly-myelinated Aδ fibers, respectively. However, QST relies on a patient's psychosensory response and could be subject to bias.

Morphologic studies using teased nerve fibers can accurately evaluate nerve structural changes. Sural nerve biopsy is usually performed due to its relatively distal position and the fact that it consists of predominantly thinly-myelinated Aδ fibers and small unmyelinated C-fibers. Studies have shown that the nerve fiber diameter distribution shifted to more small unmyelinated nerves in DPN patients. This could be attributed to an increased detachment of Schwann cell from axons and a decreased axonal diameter. However, no significant changes of myelinated nerve density and myelin thickness were detected [127-129].

One major problem of nerve biopsy is its invasiveness. In contrast, with minimal and reversible injury, skin biopsy can be used to quantify the small unmyelinated nerve fibers innervating the epidermis with a high sensitivity [130-132]. It has been used to diagnose and stage the disease status of small fiber neuropathy in patients [133] and animal models [134, 135]. A 3 mm diameter and 3 mm depth punch biopsy is collected and undergoes immunohisto-chemistry with antibody against protein-gene-product 9.5 (PGP 9.5), which is an ubiquitin C-terminal hydroxylase expressed in all nerve fibers. Intraepidermal nerve fibers density (IENFD) is counted to determine unmyelinated nerve innervation [136]. Reduced IENFD has been detected in patients suffering from T1DM [137], T2DM [138] and IGT. A strong negative correlation between IENFD and the duration of diabetes has been found in T2DM patients [138, 139].

Therefore, these techniques provide accurate information with the sub-types of the injured nerve fibers, the impaired nerve function and the severity of nerve damage (**Figure 1.3.1.3**). Meanwhile, these methods are critical parameters to evaluate the efficacy of clinical therapies.

Table 1.3.1.3: Nerve Fiber Types in Peripheral Nervous System

<table>
<tr><th colspan="2">Motor</th><th colspan="3">Sensory</th><th colspan="2">Autonomic</th></tr>
<tr><td>Myelinated</td><td>Myelinated</td><td>Thinly-myelinated</td><td>Un-myelinated</td><td>Thinly-myelinated</td><td>Un-myelinated</td></tr>
<tr><td>Aα</td><td>Aα/β</td><td>Aδ</td><td>C</td><td>Aδ</td><td>C</td></tr>
<tr><td rowspan="4">Muscle control</td><td>Touch</td><td>Cold</td><td>Warm</td><td colspan="2">Heat rate</td></tr>
<tr><td>Vibration</td><td>Pain</td><td>Pain</td><td colspan="2">Blood pressure</td></tr>
<tr><td>Position</td><td>Perception</td><td>Perception</td><td colspan="2">Sweating</td></tr>
<tr><td>Peception</td><td></td><td></td><td colspan="2">GIT & GUT function</td></tr>
<tr><td rowspan="3">MNCV</td><td colspan="3">SNCV</td><td colspan="2" rowspan="3"></td></tr>
<tr><td></td><td colspan="2">QST</td></tr>
<tr><td colspan="2"></td><td>IENFD</td></tr>
</table>

1.3.2. Animal Models of Diabetic Peripheral Neuropathy

Diabetic rodents are widely used experimental models to mimic the neuropathy symptoms that are seen in diabetic patients. They are useful tools to investigate the pathophysiology of DPN and evaluate the efficacy of novel therapies. Streptozotocin (STZ) is the most commonly used reagent to induce T1DM in experimental rodents due to its cytotoxic effects on pancreatic β-cells [140]. However, the dose of STZ and the rodent background may influence the onset and the severity of DPN. On the other hand, genetically modified rodents can develop obesity and hyperglycemia, making them well-characterized models for T2DM.

Type 1 Diabetic Models

C57BL/6 is the most common strain of inbred laboratory mice. It is widely used for disease models or gene targeting studies. A single high dose of STZ injection can induce diabetes as well as some neuropathic symptoms in C57BL/6 mice [141, 142]. However, this method is problematic due to its toxicity and high post-injection mortality rate. Instead, several low doses of STZ injections are used to improve the survival rate of diabetic animals. We previously demonstrated that STZ i.p. injections at doses of 75, 60, and 45 mg/kg were sufficient to induce hyperglycemia and certain neuropathic symptoms, such as mechanical and thermal hypoalgesia and the decreased motor nerve conduction velocity (MNCV) without any changes of sensory nerve conduction velocity (SNCV), or IENFD after 12 weeks of diabetes [143], whereas the mice that injected with a regimen of STZ at doses of 85, 70, and 55 mg/kg developed deficits in both decreased MNCV and SNCV after 18 weeks of diabetes [144]. In contrast to the C57BL/6 mice, diabetic Swiss Webster mice develop severe neuropathy including decreased nerve conduction velocities, sensory hypoalgesia and a reduced IENFD, which might be attribute to their outbred background [145-147]. One study reported a significant decrease in axonal areas

and myelin thickness in both sciatic and sural nerves from diabetic Swiss Webster mice after 5 months of diabetes [148]. These results suggest that the Swiss Webster mouse is a better model in this aspect of neuropathy (**Table 1.3.2.1**). The rat is another commonly used laboratory rodent model with a longer lifespan compared to mice. Many groups induced T1DM in Sprague-Dawley rats with a single low dose of STZ and found sensory hypoalgesia, nerve conduction velocity deficits, loss of IENFD and the decreased myelinated fiber areas in diabetic nerves compared to age-matched control rats [149-151]. Another study also showed demyelination in diabetic Long-Evans suckling rats [152].

Table 1.3.2.1: Mouse model comparison C57BL/6 vs. Swiss Webster

Model	Duration (Weeks)	STZ (mg/kg /days)	Sensory Testing	NCV	Anatomy	Citation
C57BL/6	9	150	NA	Decreased SNCV/MNCV	NA	[141]
C57BL/6	6	180	No changes in mechanical sensitivity	NA	Decreased DRG neuron size	[142]
C57BL/6	12	75, 60, 45	Mechanical/ Thermal Hypoalgesia	Decreased MNCV No changes in SNCV	No changes in IENFD	[143]
C57BL/6	18	85, 70, 55	Mechanical/ Thermal Hypoalgesia	Decreased SNCV/MNCV	NA	[144]
Swiss Webster	24	100/2	Thermal Hypoalgesia	Decreased SNCV/MNCV	NA	[145]
Swiss Webster	26	100/2	Mechanical/ Thermal Hypoalgesia	Decreased SNCV/MNCV	Decreased IENF	[147]
Swiss Webster	36	85, 70, 55	NA	Decreased SNCV/MNCV	Decreased IENF, DRG neurons loss	[146]

Swiss Webster	20	60, 50, 40	NA	Decreased SNCV/MNCV	Decreased axonal areas and myelination thickness	[148]

Type 2 Diabetic Models

Spontaneous genetic mouse models, such as the BKS.Cg-m+/+*Lepr*^{db}/J (BKS-*db*/*db*) mouse and the B6.Cg-*Lep*^{ob}/J (B6-*ob*/*ob*) mouse develop diabetic symptoms similar to Type 2 diabetic patients. The BKS-*db*/*db* mice express a mutant in the gene encoding the leptin receptor, resulting in deficient leptin receptor activity. On the other hand, the B6-*ob*/*ob* mice express a *leptin* mutant and cannot produce leptin. Both models develop severe obesity, hyperinsulinemia and hyperglycemia beginning at approximately 4 weeks of age. These mice also develop neuropathic symptoms including increased sensory latencies, decreased nerve conduction velocities and a reduced IENFD [153, 154]. High-fat diet can induce hyperglycemia and insulin resistance in C57BL/6 mice to mimic T2DM. This mouse model presents peripheral nerve dysfunction but no structural abnormalities [155]. Type 2 diabetic rat models include the WBN/Kob rat and the Zucker diabetic fatty (ZDF) rat. Similar to Type 1 diabetic rat models, these Type 2 diabetic rats develop distal degeneration [156] and segmental demyelination [157, 158], nerve dysfunction and sensory impairment in long-term diabetes. These results suggest that diabetic rat models may be more vulnerable to diabetes-induced demyelination.

1.3.3. Pathology, Current Management and Treatments

Diabetic rodent models provide useful tools to investigate the pathophysiology in diabetic patients and to evaluate the efficacy of clinical therapies. Several metabolic pathways have been shown to be perturbed in diabetic patients and animal models due to systemic hyperglycemia,

such as the enhanced formation of AGEs, increased polyol pathway flux, abnormal PKC pathway activation, chronic inflammation and mitochondrial dysfunction. Dysregulation of these pathways contribute to the development of various diabetic complications including diabetic neuropathy.

Due to the high prevalence and severity of diabetic complications, great efforts have been put forth to identify effective treatments and prevention of diabetic complications. Currently, tight glycemic control remains to be the first line strategy to prevent and/or delay the onset of diabetic complications. Strategies targeting secondary metabolic pathways are also gaining attention and a broad range of agents, such as receptor antagonists and enzyme inhibitors, can be potentially used to alleviate symptoms of diabetic complications.

Advanced Glycation End-products Pathway

Glucose undergoes non-enzymic reaction with amino groups of proteins to form stable advanced glycation end-products (AGEs) [159]. The rate of this reaction is directly proportional to glucose concentration [160, 161]. Accumulated AGEs directly alter protein function, gene expression, and subcellular signal transduction pathways in target tissues. For instance, AGEs have been shown to modify peripheral nerve myelin protein and contribute to segmental demyelination [162]. In addition, AGE modified plasma proteins are recruited to AGE receptors (RAGE), which stimulate downstream signaling pathways, such as MAPK and NF-κB, and induce the production of ROS [163]. In a long-term STZ-induced Type 1 diabetic mouse model, RAGE deletion decreased diabetes-induced activation of NF-κB and PKC in peripheral nerves, particularly in Schwann cells [148]. These diabetic RAGE-deficient mice showed improved nerve conduction velocity and nerve structural parameters, suggesting that blocking AGE

formation and RAGE activation could be potential therapeutic strategies against diabetic neuropathy.

Several clinical strategies have been developed to prevent or modulate glycation formation and its associated tissue damage. Aminoguanidine was the first compound designed to inhibit AGE synthesis and effectively reversed abnormalities of peripheral nerve function and improved peripheral nerve blood flow in diabetic animal models [164]. However, the human clinical trial of aminoguanidine (the ACTION II in Type 2 diabetic patients) was terminated due to safety concerns, such as gastrointestinal disturbance, liver function abnormalities and an apparent lack of efficacy [165]. Another approach to block AGE signaling is to use the AGE cross-link breakers, such as ALT-711, to release the covalently bound AGE from proteins. However, no agents are clinically approved to block AGE/RAGE signaling in diabetic patients.

Polyol Pathway

The polyol pathway converts glucose to fructose through a two-step reaction: First, aldose reductase (AR) reduces glucose to sorbitol with the cofactor nicotinamide adenine dinucleotide phosphate (reduced form, NADPH). Sorbitol dehydrogenase (SDH) then oxidizes sorbitol to fructose using the cofactor nicotinamide adenine dinucleotide (oxidized form, NAD^+) [161, 166].

AR has a low affinity for glucose that ensures the activity of polyol pathway is minimal in non-diabetics. However, hyperglycemia shuttles excessive glucose into this pathway, resulting in increased consumption of NADPH and accumulation of sorbitol. This process depletes the available NADPH for regenerating antioxidant glutathione (GSH). Thus, the cells are more susceptible to oxidative stress [167]. A strong correlation has been reported between AR gene expression and the risk of developing diabetic complications [168, 169]. In addition, the elevated fructose production could promote the AGE formation and the activation of PKC

pathway [170]. Therefore, the polyol pathway has become a target of drug intervention for diabetic neuropathy.

In a Type 1 diabetic mouse model, AR deficiency reversed diabetes-induced reduction of nerve conduction velocity and the accumulation of sorbitol. Aldose reductase inhibitors (ARI) are designed to block the formation of sorbitol and prevent NADPH depletion. Fidarestat effectively reversed nerve conduction velocity defects and small nerve fiber damage in diabetic animals [171]. Epalrestat is a clinically approved ARI for use in Japan since 1992. A 3-year clinical trial in Japanese patients found that Epalrestat moderately delayed the progression of diabetic neuropathy and ameliorated the associated symptoms [172, 173], suggesting that polyol pathway partially contributes to the development of diabetic neuropathy. However, results with ARIs have been generally disappointing in humans and none are approved in the US. Similarly, the efficacy of sorbitol dehydrogenase inhibitors (SDIs) is controversial due to inconsistent results across studies [174-176].

Protein Kinase C Pathway

PKC pathway contributes to the pathophysiology of diabetic neuropathy mostly through its effects on vascular blood flow. Activated PKC increases the expression of the angiogenic proteins VEGF and TGF-β [177]. These proteins could alter vasoconstriction and capillary permeability to cause hypoxia, angiogenesis, basement membrane thickening, and endothelial proliferation [178, 179]. The PKC inhibitor Ruboxistaurin completely reversed diabetes-induced nerve conduction velocity defects, thermal hyperalgesia and reduced neurovascular blood flow in a Type 1 diabetic rat model [179]. Based on these promising results, Ruboxistaurin showed significant improvement in neuropathy symptoms with no serious side effects in phase I-II human clinical trials [180-182]. Similarly, diabetic retinopathy and diabetic nephropathy also

benefited from Ruboxistaurin therapy [181]. Given that microvascular dysfunction is a common fundamental contributor to these diabetic complications, regulating blood flow and vasoconstriction through inhibiting PKC pathway may be beneficial to different diabetic complications. Indeed, the manufacturer, Eli Lilly Co., is developing Ruboxistaurin for the prevention of vision loss in patients with diabetic retinopathy and is waiting for the approval notification from the Food and Drug Administration (FDA). However, Ruboxistaurin for diabetic peripheral neuropathy has not been successfully demonstrated in phase III clinical trials, whereas further development is needed to determine the effects of Ruboxistaurin on diabetic nephropathy [183].

Inflammation Pathway

Hyperglycemia-induced activation of various signaling cascades stimulates NF-κB and the activity of iNOS. Increased production of NO contributes to microvascular damage by diminishing the blood supply to nerves, which could lead to tissue ischemia, axon and myelin degeneration, and the development of neuropathic pain [184]. iNOS deficiency preserved normal nerve conduction velocities and prevented the loss of IENFD in a STZ-induced Type 1 diabetic mouse model [185]. The NF-κB-mediated extensive infiltration of macrophages and the release of cytokines from endothelial cells exacerbated cellular oxidative stress and nerve damage [186].

Cyclooxygenase (COX) is the rate-limiting enzyme in prostaglandin (PG) synthesis, which is involved in inflammatory response. The expression of COX-2 (the inducible form of COX) was upregulated in peripheral nerves and dorsal root ganglia (DRG) neurons in diabetic animal models [187]. A selective COX-2 inhibitor and COX-2 gene inactivation prevented the diabetes-

induced peripheral nerve dysfunction, oxidative stress and inflammation [187]. These results indicate that COX-2 could be a potential therapeutic target in DPN.

Oxidative Stress and Mitochondrial Dysfunction

Mitochondrial oxidative phosphorylation is the major pathway for ATP synthesis. In this process, electrons from reducing substrates, such as nicotinamide adenine dinucleotide (reduced form, NADH) and flavin adenine dinucleotide (reduced form, $FADH_2$), are transferred to molecular oxygen, producing H_2O. Through respiratory chain complexes I-IV embedded on the inner mitochondrial membrane, a proton gradient across the inner mitochondrial membrane is established, driving ATP synthesis by ATP synthase.

Though the electron transport system is precisely organized and regulated, 1-4% of the oxygen is incompletely reduced, forming ROS such as superoxide anion. Low levels of ROS are reduced by antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GP). However, hyperglycemic cells metabolize excessive glucose through glycolysis, the TCA cycle and oxidative phosphorylation, producing an over-abundance of ROS. In addition, hyperglycemia-driven over activation of cellular signaling pathways (discussed above) could further contribute to ROS generation. Accumulated ROS can impair mitochondrial function by damaging mitochondrial DNA, proteins and membrane integrity, and also by disrupting the fusion-fission process that controls mitochondrial morphology and mitochondrial number [188].

As a major antioxidant, Mn-SOD or SOD-2 (mitochondrial form of SOD) is critical for cellular development and survival, since deletion of the SOD-2 gene is prenatally lethal [189]. Decreased expression of Mn-SOD has been found in the *db*/*db* Type 2 diabetic mouse model and is associated with worse neuropathy symptoms [190]. In addition, our lab previously

demonstrated that hyperglycemic treatment induced oxidative stress and decreased Mn-SOD expression in primary DRG neuron cultures [191]. Overexpression of Mn-SOD prevented the formation of mitochondrial ROS *in vitro* [190], suggesting that Mn-SOD is a critical regulator against oxidative stress in mitochondria. Therefore, antioxidant agents have become a potential strategy to increase the reducing potential against cellular oxidative stress.

α-Lipoic acid (ALA) is a natural cofactor in enzymatic reactions, which could directly terminate free radicals, chelate transition metal ions, and increase cytosolic GSH levels [192]. One promising phase III clinical trial, the SYDNEY trial, demonstrated that intravenous administration of ALA significantly reversed positive neuropathy symptoms and improved nerve function in Type 2 diabetic patients [193]. Similarly, oral administration of ALA also demonstrated protective effects on diabetic patients in the SYDNEY 2 trial [194]. Though ALA treatment benefited patients by both administration routs, the limitation of these clinical trials is the short duration in the investigation (3-5 weeks). To this end, the Neurological Assessment of Thioctic Acid in Neuropathy (NATHAN) 1 trial was a 4 year study of oral treatment with 600 mg/d of ALA in 460 diabetic patients with mild to moderate DPN [195]. ALA did not improve nerve conduction but modestly improved some scores of small fiber neuropathy. Part of this lack of efficacy in the NATHAN 1 trial was due to the lack of worsening of nerve conduction deficits in the placebo-treated group [195].

In summary, multiple metabolic pathways interact and induce oxidative stress in diabetes. Currently, other than intensive insulin intervention, no pharmacologic therapy could prevent or delay the onset and progression of diabetes-induced complications. Moderate improvement from targeting a specific pathway indicates that multiple mechanisms are involved in the disease development, and novel targets need to be investigated.

1.4. Growth Factors in Peripheral Nervous System

Growth factors are critical regulators in the nervous system both during the development and after nerve injury. In the peripheral nervous system (PNS), certain neural crest cells migrate from the neural plates form melanocytes in the skin, or generate Schwann cell precursors (SCPs). SCPs then differentiate to immature Schwann cells, which can develop into myelinating and non-myelinating Schwann cells that ensheath large and small diameter axons, respectively. Myelination is selectively activated in myelinating Schwann cells that envelop single large diameter axons. The development and myelination of nerve fibers is a dynamic and tightly regulated process, which is dependent on survival factors, mitogens and differentiation signals from axons. Meanwhile, Schwann cells provide essential trophic support for sensory and motor neurons. Another important feature of this process is plasticity. Mature myelinating and non-myelinating Schwann cells can revert to immature Schwann cell by dedifferentiation in response to nerve injury and re-entering the cell cycle (**Figure 1.4.1**) [196]. Given the essential function of growth factors in the developing PNS and the plasticity of the Schwann cell lineage, impaired expression levels of growth factors may render neurons and Schwann cells more vulnerable to hyperglycemic stress.

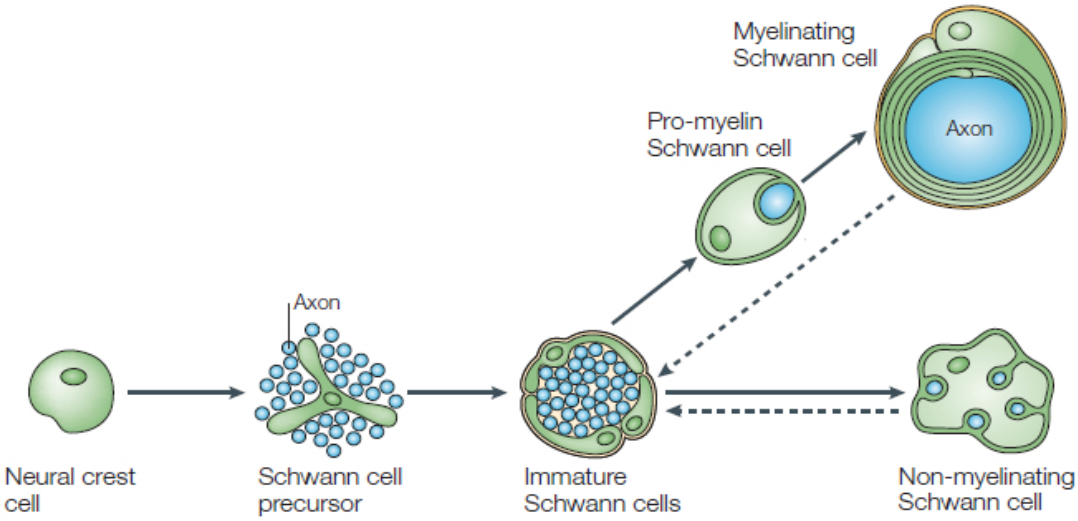


Figure 1.4.1: Schematic illustration of the main cell types and developmental transitions involved in Schwann cell development. Dashed arrows indicate the reversibility of the transition from mature myelinating and non-myelinating cells to immature Schwann cell. The embryonic phase of Schwann cell development involves three transition processes: the migration of neural crest cells, the differentiation of SCPs into immature Schwann cells, and the maturation into myelinating and non-myelinating Schwann cells. Schwann cells enveloping single large diameter axons differentiate into mature myelinating cells, whereas Schwann cells that ensheath small diameter axons become mature non-myelinating cells [196].

1.4.1. Neurotrophin Family

Neurotrophins play specific roles in the development, maintenance and regeneration of sub-populations of peripheral nerve fibers [197, 198]. The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6. They are initially synthesized as proneurotrophins, which are subsequently cleaved and released as mature proteins. Neurotrophins fulfill their functions through activating their receptors including high affinity tropomyosin-related kinase (Trk) receptors and low affinity p75 neurotrophin receptors ($p75^{NTR}$) [199]. NGF activates Trk A receptor and/or $p75^{NTR}$ on small sensory and autonomic nerve fibers. NT-3 stimulates Trk C signaling on large nerve fibers,

whereas BDNF and NT-4/5 may exert their effects on medium-sized fibers through the Trk B receptor [200].

Nerve Growth Factor

NGF was discovered in the 1950s and is the first member of the neurotrophin family. It is synthesized in a variety of cell types, including muscle cells, keratinocytes, sympathetic neurons, DRG neurons and Schwann cells [200, 201]. Blocking NGF-Trk A signaling is devastating to the development of nervous system, since both NGF [202] and Trk A receptor [203].

In STZ-induced Type 1 diabetic rodent models, NGF levels are decreased in sympathetic neuron-innervated target organs, such as muscle, skin [204], sensory neurons and sciatic nerves [205]. Allogeneic pancreatic islet transplantation that restored euglycemia reversed the decreased NGF levels and improved nerve function [206]. Similarly, reduced NGF levels were detected in the submandibular gland in the *db*/*db* Type 2 diabetic mouse model [207], suggesting that the synthesis of NGF might be disturbed by hyperglycemia.

As a target-derived neurotrophin, NGF activates Trk A receptor and forms a "signaling endosome" in distal nerve terminals. The endosome complex is retrogradely transported up long axons to the proximal cell bodies in order to promote neuronal survival [208]. Retrograde axonal transport of NGF is decreased in diabetic rats [209]. Decreased synthesis in distal axonal termini and reduced retrograde transport induce a lack of NGF-mediated support for neuronal survival. Therefore, these results triggered a number of studies to investigate the efficacy of treating diabetic rodents with exogenous NGF as a replacement therapy. Intrathecal or subcutaneous administration of recombinant human NGF (rhNGF) reversed multiple indices of diabetic neuropathy, including impaired C-fiber function [210], mechanical [211] and thermal hypoalgesia [212], declined myelin thickness [213] and the loss of IENFD [211].

In contrast, little is known about the NGF profile in diabetic patients. DPN patients have low serum NGF levels, which correlated with decreased motor nerve conduction velocity [214]. One group detected increased expression of $p75^{NTR}$ along the whole degenerating fibers in DPN patients, suggesting a potential compensating mechanism of nerve tissue repair [215]. However, studies from human skin biopsies showed controversial results. An increase in NGF mRNA [216] and Trk A mRNA levels [217], but a decrease in protein levels of NGF and its precursor [218, 219] were found in skin biopsies from DPN patients. These inconsistent results indicate that translational/post-translational modifications may affect the NGF profile in diabetic patients.

Given the protective effects of rhNGF in diabetic animal models, a phase I-II human clinical trial was conducted in 250 patients who were sub-grouped to receive placebo, 0.1 μg/kg rhNGF or 0.3 μg/kg rhNGF subcutaneously [220]. Though injection site hyperalgesia was reported, 6-month rhNGF treatments significantly improved the neurological examination parameters and QST scores. However, disappointing outcomes were found in a 12-month phase III trial in 505 patients [221]. In this trial, a dose of 0.1 μg/kg was chosen to avoid injection site pain, which was still severe compared to the placebo treatment. Other than a moderate improvement of the global symptom assessment, no significant therapeutic benefits were demonstrated. Several potential reasons may have caused the failure in the phase III trial. For instance, 0.1 μg/kg of dose could be at the edge of its effective threshold. With a different manufacturing method in the phase III trial, the bioactive concentration could be below the efficacy window. More importantly, NGF affects mainly small sensory nerves and autonomic nerves consisting of thinly-myelinated Aδ fibers and unmyelinated C-fibers. Nerve conduction studies and QST performed in clinical trials may not reflect any improvement in this specific sub-population of nerve fibers affected by the NGF treatment. Therefore, structural and functional tests of C-fiber

integrity, such as IENFD and measures of skin blood flow, might overcome the end point limitations. On the other hand, these negative results could be attributed to the inefficient drug delivery system. Cytomegalovirus (CMV) immediate-early promoter and herpes simplex latency active promoter 2 were used to deliver NGF into the DRG, adipose tissue and the footpad of diabetic mice. In these NGF-treated diabetic mice, decreased sensory nerve amplitude was prevented [222]. In summary, NGF is indeed a potential therapeutic agent against diabetic neuropathy. However, further human studies and a modified drug delivery system are needed.

Brain-derived Neurotrophic Factor and Neurotrophin-3

BDNF and NT-3 are detected in a variety of cell types, such as sensory neurons, sympathetic neurons, motor neurons and their targeted organs (skin and muscle) [223]. STZ-induced Type 1 diabetic rats showed an increase in BDNF mRNA levels in muscle, DRG and sciatic nerve [224, 225], but a transient decrease in Trk B receptor mRNA levels in sciatic nerve [226]. The retrograde accumulation of BDNF was decreased in diabetic rats, but not due to impaired receptor-mediated uptake [227]. The inconsistency between increased BDNF mRNA levels but decreased protein accumulation suggests that translational/post-translational modifications could play an essential role to regulate the function and expression of neurotrophins. Exogenous BDNF treatment of diabetic rats protected against MNCV deficits and myelin damage in motor nerve fibers. However, no improvement has been observed on sensory nerve-related function [228]. This suggests that BDNF may have more effects on myelinated motor nerves than small unmyelinated fibers, which may explain the negative results in human clinical trials using sural nerve conduction and cutaneous axon-reflexes as end point parameters [229].

The third member of neurotrophin family is NT-3, which was also decreased in sciatic nerve, hindlimb skeletal muscle and DRG neurons in STZ-induced Type 1 diabetic rats [230]. These

diabetic rats also showed reduced axonal transport of NT-3 in sciatic nerves. Exogenous NT-3 delivery by adenovirus-based gene therapy prevented the motor and sensory nerve conduction velocity deficits in diabetic rats [231]. In contrast, skin biopsies from diabetic patients showed increased NT-3 protein expression levels [232] and Trk C mRNA levels [217]. Unlike the promising efficacy in diabetic animal models, a phase I trial of recombinant-methionyl human NT-3 (r-metHuNT3) was discontinued due to no significant improvement in neurologic function, though the dosage was tolerated and the most reported side effect was mild injection site pain [233].

1.4.2. Insulin-like Growth Factor Family

Due to the sequence homology to proinsulin, the Insulin-like Growth Factor (IGF) family is well-studied. The IGF family consists of IGF-I and IGF-II and mediate their function by activating the IGF receptor (IGFR). IGF-I is broadly expressed throughout the nervous system such as brain, spinal cord, sensory DRG neurons, and sciatic nerves, whereas IGF-II is more enriched in the brain and motor neurons. IGF signaling is critical to normal development and the maintenance of the nervous system since deleting the IGFR is embryonic lethal [93].

STZ-induced Type 1 diabetic rats showed reduced serum levels of IGF-I compared to control rats [234]. mRNA levels of both IGF-I and IGF II were also decreased in sciatic nerves from these diabetic rats [235]. Subcutaneous infusion of either IGF-I or IGF-II effectively reversed the hyperalgesia in this Type 1 diabetic rat model [236]. Similarly, in ZDF Type 2 diabetic rats, IGF-II mRNA levels were decreased in brains, spinal cord, sciatic nerves and liver, whereas IGF-I mRNA levels were only reduced in liver [237]. Thus, IGF-II, but not IGF-I, was injected subcutaneously to diabetic rats and restored the normal sensory thresholds.

Though few studies have been conducted to investigate the changes of IGFs in diabetic patients, one group detected decreased serum levels of IGF-I and IGFR in Type 2 diabetic patients with neuropathy. Interestingly, a clinical trial in Type 1 diabetic patients demonstrated a transient but significant reduction of HbA1c levels with daily subcutaneous injections of 40 μg/kg recombinant human IGF-I (rhIGF-I) [238]. These results suggest that IGF could serve as a supplementary therapy to conventional insulin therapy. However, whether IGFs could protect against diabetic neuropathy needs further study.

In summary, many diabetic animal studies and human clinical trials have been conducted to investigate the efficacy of growth factors as replacement therapies against diabetic neuropathy. Unfortunately, no agent has been approved clinically to treat diabetic neuropathy. Injection site pain is widely reported, suggesting that high exogenous amounts of growth factor at the injection sites could be harmful to the surrounding skin tissues. However, the concentration that actually reaches nerve tissues might be below the effective threshold. Tissue-targeted delivery may specifically restore the growth factor concentration in nerve fibers. Meanwhile, end point parameters of clinical trials might affect the outcome results. Different assessments should be performed to distinguish the response from various nerve fibers.

However, the major problems with the "growth factor replacement" hypothesis are the inconsistency across animal studies and little understanding in diabetic patients. For instance, controversial results have been reported between different laboratories, and/or between protein concentrations and mRNA levels within the same group [216, 218, 219]. Thus, multiple factors, such as translational/post-translational modifications, diabetic models, duration of diabetes, and the tissue types need to be taken into account when interpreting data. Since no deficiency of NT-3 has been detected in diabetic patients, the failure of the NT-3 clinical trial is less surprising

[233]. This human clinical trial raised a question of whether exogenous injection of any growth factor, regardless of the type, would benefit diabetic patients. More importantly, interactions among different types of growth factors play critical stage- and spatial-dependent roles during nervous system development. For instance, BDNF signaling promotes the release of soluble Neuregulin-1 (NRG1) from axons to Schwann cells, which is critical to initiate the differentiation of SCPs into immature Schwann cells. Otherwise, SCPs would undergo programmed cell death [239]. The interactions between various growth factors maintain a balanced network and compensate for dysfunction of one growth factor. The exogenous increase of one growth factor may disrupt the balance and lead to a rather destructive result. Therefore, more understanding of different types of growth factors under diabetic conditions could provide broader and more comprehensive insights into the growth factor replacement therapy.

1.5. Neuregulin-Erb B Signaling in Peripheral Nervous System

Axonal neuregulins (NRGs) that activate Erb B receptors, which localize primarily to Schwann cells, are the key molecules regulating different stages of Schwann cell development and myelination. They also contribute to Schwann cell dedifferentiation and degeneration after nerve injury. However, little is known about how diabetes may affect gliotrophic signaling by altering NRG-Erb B signaling in PNS.

1.5.1. Ligand Neuregulin-1 Family and Erb B Receptors

The NRG family has four members: NRG1, NRG2, NRG3, and NRG4. Other than NRG1, little is known about the biological functions of NRG2, NRG3, and NRG4 proteins. The *NRG1* gene is about 1.4 megabases, however, less than 0.3% of this span encodes for protein. Through multiple promoter usage and extensive alternative RNA splicing, more than 30 different isoforms

are generated from the single *NRG1* gene. NRG1 isoforms are divided into sub-types based on structural features: the type of epidermal growth factor (EGF)-like domain (α or β), the N-terminal sequence (Type I-VI), and whether the isoform is synthesized as a membrane-associated or secreted protein. All NRG1 isoforms contain the EGF-like domain, which is necessary and sufficient to activate Erb B receptors. NRG1 isoforms with a β-type EGF-like domain are 10 to 100 times more potent than isoforms with an α-type EGF-like domain. On the basis of N-terminal sequence, NRG1 Type I (also known as heregulin, neu differentiation factor, or acetylcholine receptor-inducing activity (ARIA)) and NRG1 Type II (also known as glial growth factor (GGF)) have N-terminal immunoglobulin (Ig)-like domains. NRG1 Type III (also known as sensory and motor neuron derived factor (SMDF)) is defined by its cysteine-rich domain (CRD), which functions as a second transmembrane domain. Membrane-associated NRG1 isoforms undergo proteolytic cleavage by metalloproteinases (MP), including α-secretase tumor-necrosis factor-α-converting enzyme (TACE) and β-secretase β-site amyloid precursor protein cleaving enzyme (BACE). As a consequence, NRG1 Type I and NRG1 Type II are released from the cell surface and function as paracrine signaling molecules. In contrast, due to the hydrophobic domain that contains CRD, NRG1 Type III remains tethered to the cell surface after cleavage and functions as a juxtacrine signal. Cytoplasmic domains of NRG1 can be further cleaved by γ-secretase and translocate to nucleus (**Figure 1.5.1.1**). Sub-types IV-VI with shorter N-terminal domains have not been well-characterized. Within the nervous system, NRG1 Type I

and NRG1 Type III are the most abundant isoforms that are detected in many neurons, such as spinal motor neurons and DRG neurons, and also in glia [240, 241].

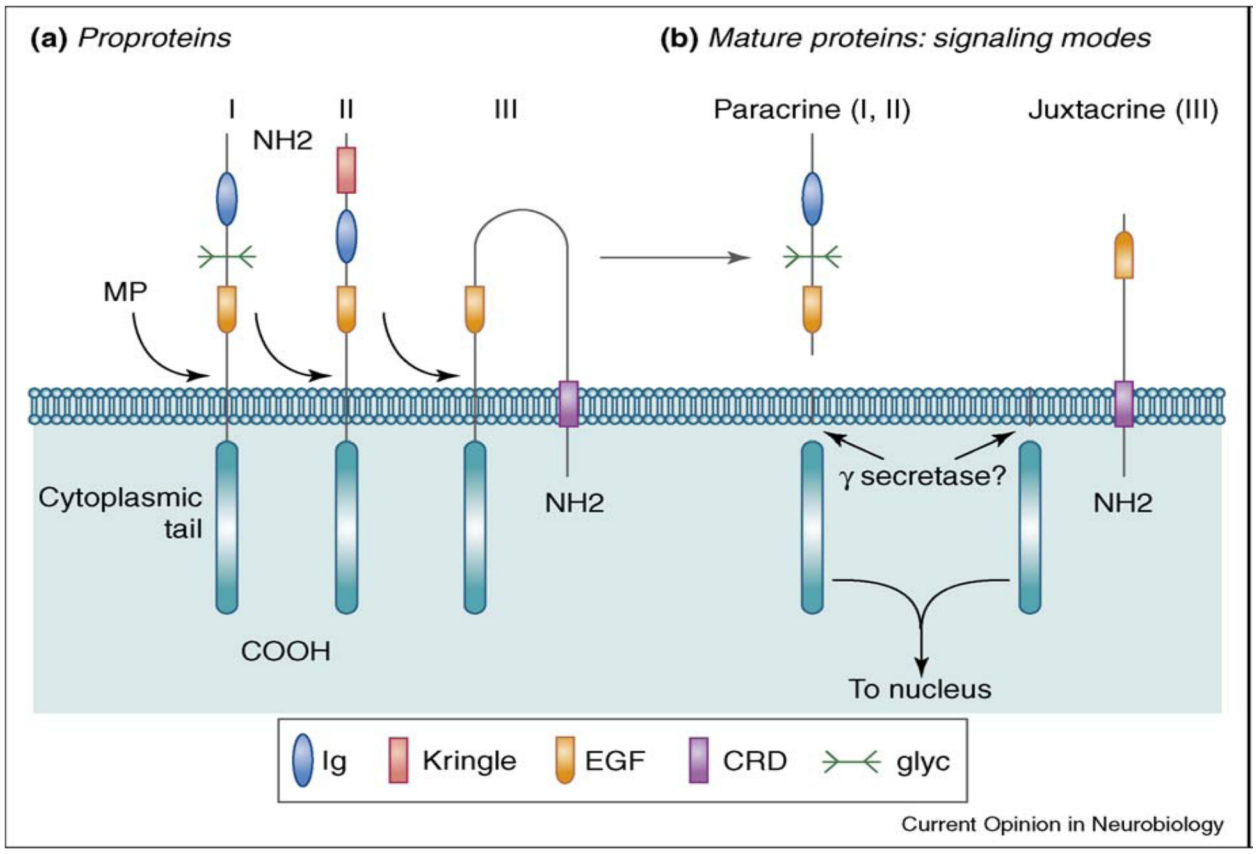


Figure 1.5.1.1: Schematic illustration of different proteolytic cleavages of NRG1 isoforms. **(a):** NRG1 Type I and NRG1 Type II are synthesized as single transmembrane proteins, whereas NRG1 Type III has two transmembrane domains. **(b):** After metalloproteinase (MP) cleavage, NRG1 Type I and NRG1 Type II are released from axonal membrane and act as paracrine molecules. However, NRG1 Type III remains tethered to the membrane due to its cysteine-rich domain (CRD) and functions as a juxtacrine signal [240].

NRG1 isoforms mediate their effects by interacting with Erb B receptors (Erb B2, Erb B3 and Erb B4 receptor), which are members of the EGF receptor superfamily. NRG1 binds to Erb B receptors and induce conformational changes that trigger dimerization of Erb B receptors. The formation of receptor dimers activates their intrinsic tyrosine kinase activity, which leads to cross-phosphorylation on the receptors, recruitment of adaptor molecules and activation of

downstream signaling pathways, such as phosphatidylinositol-3-kinase (PI3K) and MAPK pathways. Erb B receptor dimerization is mediated by a unique dimerization arm, which is exposed only after a conformational rearrangement promoted by ligand binding. Though Erb B2 is a ligand-less receptor, it constitutively exposes a dimerization loop required for dimerization. However, due to the lack of shape and/or electrostatic self-complementarity interface, the ability to form Erb B2-Erb B2 or Erb B3-Erb B3 homodimers is poor [242]. In addition, Erb B3 receptor lacks an active kinase domain and Erb B4 receptor is minimally expressed by Schwann cells. The heterodimer Erb B2-Erb B3 is the relevant NRG1 receptor pair in peripheral nerves [240].

1.5.2. Neuregulin-Erb B Signaling in Peripheral Nerve Development

Genetic animal models with targeted mutations in the *NRG1* gene are valuable tools to elucidate the functions of NRG1-Erb B signaling. Unexpectedly, homozygous *pan-NRG1*$^{-/-}$ mice and *Ig-NRG1*$^{-/-}$ mice that have both NRG1 Type I and NRG1 Type II sub-types inactivated died during embryogenesis (E10.5) due to cardiac malformations [243, 244]. *CRD-NRG1*$^{-/-}$ mice with mutated NRG1 Type III isoforms died at birth (P0) since lung alveoli fail to expand [245]. The number of cranial sensory neurons and sympathetic neurons are considerably decreased in all three genetic models. However, only *Ig-NRG1*$^{-/-}$ mice have normal SCP development [244, 246]. Though these results demonstrate the importance of NRG1 proteins in embryogenesis, early death of these homozygous mice fails to reveal *in vivo* functions of NRG1 proteins in nervous system development and myelination.

In contrast, heterozygous mice and mice that conditionally express targeted mutations in NRG1 can survive to adulthood. These transgenic mice are useful tools to investigate the role of NRG1-Erb B signaling in developing peripheral nerves. Heterozygous *NRG1*$^{+/-}$ mice showed

hypomyelinated sciatic nerves, which have not been observed in heterozygous *Erb B2*$^{+/-}$ mice [247]. However, transgenic mice that have an inducible complete ablation of Erb B2 perinatally in peripheral nerves survived, but lost motor axons and displayed abnormally thin myelin sheaths due to a decreased number of myelin wraps [248]. These results indicate that NRG1 might be the rate-limiting factor in NRG1-Erb B signaling, whereas Erb B receptors are expressed at saturating level. In addition, the blockade of Erb B receptor activity could lead to aberrant development of both myelinated and unmyelinated nerve fibers. Postnatally, overexpression of dominant-negative Erb B4 receptors (DN-Erb B4) under the control of myelinating Schwann cell specific 2', 3'-cyclic nucleotide 3'-phosphodiesterase (*CNPase*) promoter specifically disrupted NRG1-Erb B signaling in myelinating Schwann cells. These transgenic mice developed hypomyelination, shorter internode length and associated mechanical hyperalgesia [249]. Under control of the non-myelinating Schwann cell specific promoter for glial fibrillary acidic protein (GFAP), overexpression of DN-Erb B4 disturbed the NRG1-Erb B signaling in non-myelinating Schwann cells, which resulted in abnormal structure of Remak bundles, loss of unmyelinated fibers, extensive Schwann cell death and associated thermal insensitivity [250].

Due to the large diversity in the NRG1 family, a series of carefully designed studies differentiated the functions between NRG1 isoforms. All three types are critical to the survival and differentiation of SCPs [251, 252] (**Figure 1.5.2.1**). NRG1 Type I is the first NRG1 isoform expressed in many cell linages at E6.5, followed by NRG1 Type II and a strong expression of NRG1 Type III at E10 [246]. Axon-derived NRG1 Type I/II translocated from axon to Schwann cell surfaces in the embryonic chick during a critical period of Schwann cell survival. Indeed, downregulation of NRG1 Type I/II resulted in an inhibition of SCP differentiation and an increase in programmed cell death [239]. On the other hand, NRG1 Type III isoform has major

effects on later stage in the development of motor and sensory neurons, including migration [253] and myelination [254]. Heterozygous *NRG1 Type III*$^{+/-}$ mice have thinner myelin sheaths, and mice that overexpress NRG1 Type III, but not NRG1 Type I, develop hypermyelination [247]. Indeed, only axonal NRG1 Type III rescued the hypomyelination seen in primary DRG/Schwann cell co-cultures prepared from heterozygous *NRG1 Type III*$^{+/-}$ and homozygous *NRG1 Type III*$^{-/-}$ mice [255].

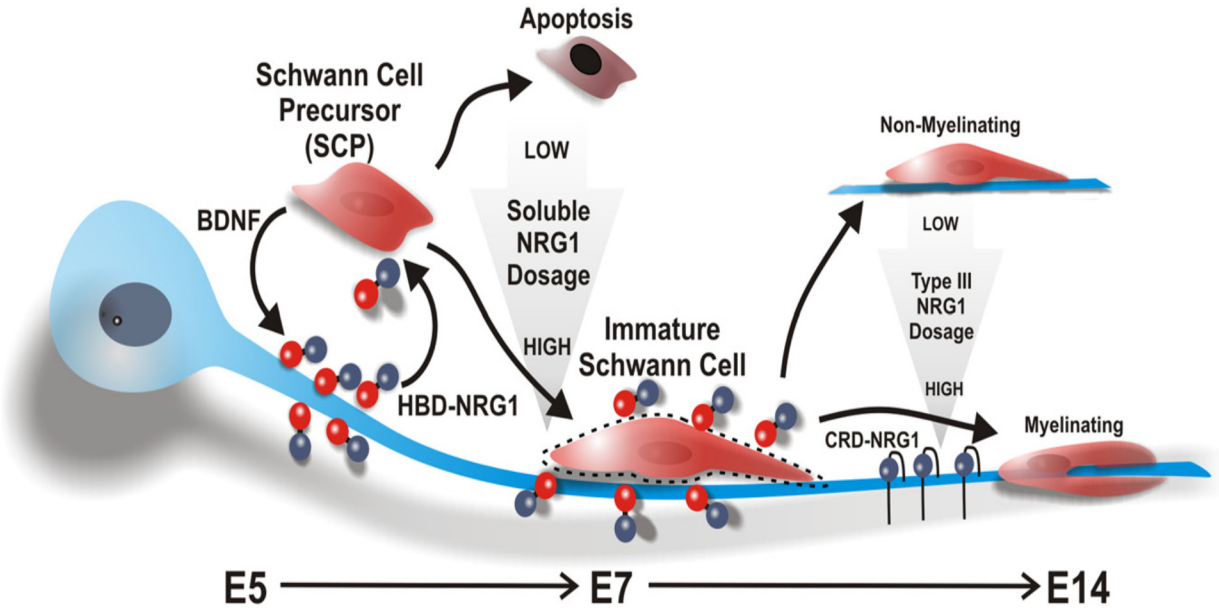


Figure 1.5.2.1: A stage-dependent model for NRG1 signaling in developing peripheral nerves. From E5 to E7 in chick embryos, SCP survival is dependent on soluble HBD-NRG1 (NRG1 Type I and NRG1 Type II) that can be increased by local BDNF signaling. Those SCPs that do not receive sufficient levels of NRG1 undergo apoptosis. Those that survive require additional NRG1 signaling to differentiate into immature Schwann cells. Later, the myelination of axons at E14 depends on the concentration of axonal CRD-NRG1 (NRG1 Type III). HBD, heparin-binding domain; BDNF, brain-derived neurotrophic factor; CRD, cysteine-rick domain. The red spheres represent the heparin-binding domain, and the blue spheres are the EGF-like domain of NRG1 Type I/II [239].

Activation of the PI3K pathway has been shown to be crucial for Schwann cell survival [256] and the initiation of myelination [257]. However, the overall effects of NRG1 isoforms primarily depend on the balance between MAPK and PI3K activation [257]. In myelinated

DRG/Schwann cell co-cultures, a high-concentration of NRG1 isoforms, regardless of the sub-type, induced subsequent Schwann cell dedifferentiation [258] and demyelination [259, 260] that was linked to activation of ERK pathway [260]. In addition, the JNK pathway could negatively regulate myelination through the demyelinating transcription factor c-jun [261, 262]. However, the role of p38 pathway in myelination is controversial, since one group found that p38 pathway induced demyelination [263], whereas other studies demonstrated that p38 activation is required for Schwann cell differentiation and myelination through the pro-myelinating transcription factor Krox-20 [264, 265]. Other signaling pathways, such as NF-κB and phosphatase, calcineurin/NFAT and tensin homolog (PTEN), are also involved in regulating myelination. NRG activates calcineurin and its downstream NFAT and regulates myelination through Krox-20 [266]. PTEN is a negative regulator of the PI3K pathway and is activated by NRG1 signaling to prevent over-myelination [267]. Additionally, NF-κB may serve as a convergent pathway downstream of PI3K and ERK activation stimulated specifically by NRG1 Type III to promote myelination [268]. Thus, Schwann cell differentiation and myelination is a dynamic process regulated by the balance between various NRG1 isoforms and different downstream pathways.

Since all membrane-associated NRG1 isoforms need to undergo proteolytic cleavage in order to be released as bioactive molecules, different secretases could affect the sequence and the function of NRG1 isoforms. BACE1 performs the most important proteolytic cleavage in the stalk region of NRG1 Type III and produces the active N-terminal fragment [269]. Deficiency of BACE1 resulted in the accumulation of uncleaved NRG1 Type III proteins and hypomyelination of peripheral nerves [270]. Interestingly, neuronal overexpression of full-length NRG1 Type III still successfully induced hypermyelination in BACE1 null mutant mice, indicating that other secretases are also involved in the cleavage and secretion of NRG1 isoforms [271]. α-Secretases

include disintegrin and metalloproteinase (ADAM)10 and ADAM17 (also known as TACE). Though ADAM10 has minor effects on NRG1 Type III-mediated myelination [272, 273], TACE inhibits myelination in peripheral nerves. This inhibitory effect might be due to its cleavage site within the EGF-like domain of NRG1 Type III, which inactivates the function of NRG1 Type III and the downstream PI3K pathway [274]. In contrast, γ-secretases may not be directly related to myelination. The C-terminal domains cleaved by γ-secretases translocate to nucleus and represses apoptosis [275], indicating a pro-survival role in neurons.

1.5.3. Neuregulin-Erb B Signaling in Nerve Injury

In contrast to the critical role of NRG1 signaling in the development of peripheral nerves, NRG1-Erb B signaling is dispensable for maintenance of myelinated peripheral nerves [276, 277]. However, nerve injury induced a rapid change of NRG1-Erb B signaling. An acute and transient increase in the activity of Erb B2-Erb B3 has been found in the distal stump of damaged nerves within the first hour after axotomy [278]. The increased activities of Erb B2-Erb B3 receptors are associated with Schwann cell demyelination during Wallerian degeneration [279], which is the process to remove the transected axons and myelin debris. In addition, *Mycobacterium leprae* that binds to and activates Erb B2 receptors without dimerization strongly activates the ERK pathway and promotes subsequent demyelination [280]. A pharmacological inhibitor of the Erb B2 receptor, PKI-166, abrogated axotomy-induced [278] and *Mycobacterium leprae*-induced demyelination [280]. Furthermore, our lab previously demonstrated that over-activation of Erb B2-Erb B3 receptors in Schwann cells is sufficient to induce peripheral neuropathy since the Erb B2 receptor inhibitors, erlotinib and PKI-166, reversed the nerve conduction velocity deficits and sensory hypoalgesia [143]. In contrast, one group using transgenic mice that overexpress Erb B2 receptors observed improved nerve regeneration after

median nerve crush [281], which might be attributed to different nerve injury (nerve crush vs. surgical transection).

Consistent with the increase in Erb B receptor activity after sciatic nerve transection, NRG1 Type II was increased in the distal nerve stump [282]. More importantly, sural nerve biopsies from patients with axonal neuropathy showed an increase in NRG1 Type I in the early stage of axonal degeneration [283]. However, NRG1 Type III was dramatically downregulated in axotomized motoneurons [284]. A neuropathic pain model (L5 spinal nerve ligation) showed increased mRNA levels of NRG1 Type I and Type II, but a decrease in NRG1 Type III mRNA levels [285]. Though NRG1 Type II has been found increased after nerve transection, exogenous NRG1 Type II may promote nerve repair. One study demonstrated that NRG1 Type II treated rats have less severe degeneration and earlier robust remyelination after sciatic nerve crush, which was associated with improved motor nerve function and nerve morphology [286]. Similar results with NRG1 Type II treatment have also been observed in a facial nerve axotomy model. Interestingly, the beneficial effects of NRG1 Type II treatment are even greater than NGF treatment [287]. It remains unclear whether the change of NRG1 isoforms is the primary cause to induce Schwann cell degeneration and demyelination, or it is the result from self-regulatory response secondary to nerve injury.

1.5.4. Adapter Protein Erb B2-Interacting Protein

Erb B2-interacting protein (erbin) is an adapter protein that belongs to leucine-rich repeat (LRR) and PDS-95/Discslarge/ZO-1 (PDZ) domain (LAP) family. The LAP family shares a common structural feature of 16 LRRs at the N-terminus and one to four PDZ domains at the C-terminus. LAP proteins serve as scaffolding molecules and regulate signal transduction and protein-protein interactions in polarized cells such as epithelial cells and neurons.

The N-terminal LRRs of erbin can bind to Ras and block its interaction with Raf-1, thereby abolishing Ras activation of Raf-1 and inhibiting ERK activation [288, 289]. Erbin may also regulate other signaling pathways such as the PI3K [290] and NF-κB pathways [291]. X-ray crystallographic studies reveal that erbin contains one PDZ domain, which recognizes the peptide sequence EYLGLDVPV corresponding to the C-terminal residues 1247-1255 of Erb B2 receptor [292, 293]. This interaction is specific to Erb B2, but not Erb B3 or Erb B4 receptor. In addition, erbin is a necessary component of NRG1-Erb B2 signaling pathway. Erbin could reduce NRG1-stimulated Erb B2 receptor endocytosis through binding to non-phosphorylated Erb B2 and stabilizing it on the plasma membrane. More importantly, the sciatic nerves of erbin null mice are hypomyelinated [290] and have a decreased number of myelinated fibers after nerve injury [294]. These data suggest that erbin has a critical role in both Schwann cell myelination and regeneration.

1.6. Dissertation Hypothesis

Although it has been established that NRG1-Erb B signaling plays an important role in the development of peripheral nerves and regeneration after nerve injury, the changes and function of this pathway in diabetic neuropathy remain unclear. Our lab previously demonstrated that a transient increase in Erb B2-Erb B3 receptor activity contributed to the development of diabetic neuropathy [143]. Therefore, the aim of the current study is to determine how diabetes affects positive and negative regulators Erb B2-Erb B3 receptors. The NRG1 family is the primary ligand for Erb B2-Erb B3 receptors in the PNS and function as positive regulators. However, different sub-types of NRG1 family have distinguished functions. In contrast, the adapter protein erbin negatively regulates Erb B2 activation of the MAPK pathway. Therefore, the overall hypothesis of the current study is that differential expression of NRG1 isoforms and

downregulation of erbin are associated with Erb B2 receptor activation in diabetic peripheral neuropathy. The specific aims for the current study are:

1. To determine whether changes of NRG1 isoforms and erbin expression are associated with Erb B2 receptor activation in DPN.
2. To determine whether the activation of MAPK pathway is involved in DPN.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Animals

Wild-type C57BL/6 and Swiss Webster mice were obtained from Harlan Laboratories (Indianapolis, IN). All animals were housed at the Animal Care Unit at the University of Kansas on a 12-h light/dark cycle at 70°F and 70% humidity with *ad libitum* access to water and Purina diet 5001 chow. All animal procedures (e.g. handling, drug administration, euthanasia, tissue collection and breeding colony management) were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) and regulations set by the National Institutes of Health (NIH).

2.1.2. Antibodies

Table 2.1.2.1: List of Primary and Secondary antibodies Utilized in the studies

Antibody	**Provider**	**Catalog No.**
β-actin	MP Biomedicals, Solon, OH	691002
NRG1 Type I	Abcam, Cambridge, MA	ab27303
NRG1 Type III (SMDF c-16)	Santa Cruz Biotechnology, Santa Cruz, CA	sc-33271
ErbB2 clone 24B5	Millipore Corporation, Billerica, MA	04-291
Erbin	Abcam, Cambridge, MA	ab55930
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling, Boston, MA	9101
p44/42 MAPK (Erk1/2)	Cell Signaling, Boston, MA	9102
P0	Chemicon, Temecula, CA	AB9352
PGP 9.5	AbD Serotec, Oxford, UK	7863-0504

A NRG1 Type III polyclonal antibody was produced in rabbits by Chi Scientific (Maynard, MA) using the CIAGLKWVFVDKIFEYDSPTHL peptide sequence near the N-terminal CRD of NRG1 Type III as the immunizing antigen.

2.1.3. Peptide Competition Assay

Sciatic nerves from untreated wild-type adult mice were homogenized in mRIPA (modified Radio Immuno Precipitation Assay) lysis buffer (50 mmol/L Tris–HCl, pH 7.5, 1 mmol/L EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1 mmol/L Na_3VO_4, 150 mmol/L NaCl, 0.5 mmol/L sodium molybdate, 40 mmol/L NaF, 10 mmol/L β-glycerophosphate, and 1x Complete Protease inhibitors (Roche Diagnostics)), protein lysates were separated by SDS-PAGE and transferred onto nitrocellulose. The various NRG1 antibodies were pre-incubated to buffer or the immunizing peptide at a 1:50, 1:100 or 1:200 mass ratio for 1 hour at 25°C prior to their use for detecting NRG1 isoforms in the sciatic nerve lysates by immunoblot analysis.

Immunoblot of sciatic nerve extracts with the custom NRG1 Type III antibody detected a prominent band at about 75 kDa (**Figure 2.1.3.1**), which represents the N-terminal fragment after cleavage and is consistent with the protein size previously reported [295]. As a complementary approach, we also used a commercial NRG1 Type III antibody that was raised against a region within the C-terminal domain of NRG1 Type III (SMDF) and detects a single band at about 65 kDa. The immunizing peptide specifically decreased the detection of the 75 kDa NRG1 Type III band. However, the peptide had no effect on detecting NRG1 Type I or the 65 kDa C-terminal NRG1 Type III fragment by SMDF c-16 antibody, indicating the 75 kDa band represents the N-terminal fragment of NRG1 Type III containing the distinct CRD.

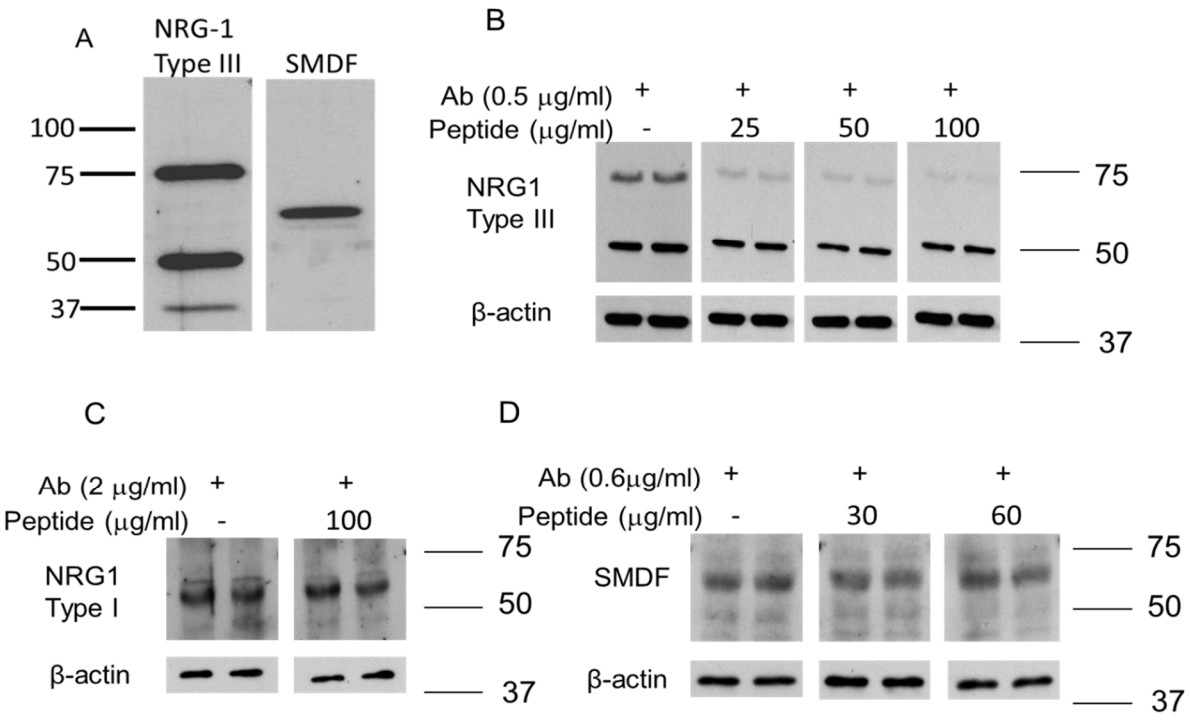


Figure 2.1.3.1: Validation of specificity of the custom NRG1 Type III antibody. (A): Comparison of NRG1 Type III detection using the custom N-terminal NRG1 Type III polyclonal antibody **(left)** and a commercial C-terminal NRG1 Type III polyclonal antibody (SMDF, **right**). **(B-D)** Demonstration of the specificity of the N-terminal NRG1 Type III polyclonal antibody. The immunizing peptide was preabsorbed in a 1:50 to 1:200 ratio with the indicated amounts of the N-terminal NRG1 Type III antibody **(B)**, a polyclonal antibody against NRG1 Type I **(C)** or the C-terminal NRG1 Type III antibody (SMDF, **D**) for 1 hour at 25°C and then used for immunoblot analysis of a sciatic nerve sample.

2.1.4. Reagents

Streptozotocin (STZ) was obtained from Sigma-Aldrich (St. Louis, MO).

N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine (erlotinib) was provided by OSI Pharmaceuticals (Melville, NY).

2.2. Methods

2.2.1. Induction of Diabetes and Measurement of Fasting Blood Glucose (FBG)

Eight-week old C57BL/6 and Swiss Webster mice were rendered diabetic through a series of intraperitoneal (i.p.) injections of freshly dissolved STZ in 200 µL of sterile sodium citrate/sodium chloride solution (10 mmol/L sodium citrate, 154 mmol/L NaCl, pH 4.5). C57BL/6 mice were fasted overnight prior to receiving i.p. injections of STZ at doses of 85, 70, and 55 mg/kg over three days. Swiss Webster were fasted 6 hrs prior to receiving i.p. injections of STZ at 100 mg/kg for two consecutive days. Mice were fasted an additional two hrs before re-feeding but had *ad libitum* access to water. One week after the last injection, mice were fasted for 6 hrs and blood was obtained from the tail vein. FBG was measured with a One-Touch Ultra glucometer (Lifescan, Milpitas, CA). Animals with a FBG concentration ≥ 290 mg/dl (16 mmol/L) were deemed diabetic. Mice with FBG < 250 mg/dl were re-administered up to two additional STZ injections at 85 mg/kg. In addition, FBG was measured the day before euthanasia to confirm their diabetic status.

2.2.2. Measurement of Glycated Hemoglobin

Percent glycated hemoglobin levels were measured using the A1C NOW+ multi test A1C kit (Bayer Healthcare, Sunnyvale, CA) immediately before euthanizing the animals. HbA1C levels ≥6.5% were consistent with a prolonged diabetic state.

2.2.3. Assessments of Sensory Sensitivity

Thermal Sensitivity

Sensitivity to thermal stimulation was assessed using the Hargreaves Analgesiometer (Ugo Basile, Comerio, Italy). The Hargreaves apparatus was calibrated using a heat flux radiometer.

Mice were placed on the glass surface of the apparatus and allowed to acclimate for 30 minutes before tests. Activation of the heat source produced a focal, radiant heat that increased in intensity at a rate of approximately 0.3°C/s, which predominantly activates small unmyelinated C-fibers and thinly-myelinated Aδ fibers [296]. The heat source was stimulated under the plantar surface of alternating hind paws with approximately 5 minutes intervals to avoid hyperalgesia. The length of time required to induce paw withdrawal (in seconds) from three to five trials per animal were recorded and averaged.

Mechanical Sensitivity

Sensitivity to mechanical stimulation mediated by myelinated Aδ fibers was evaluated using the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy). A 0.5 mm diameter steel monofilament attached to a force actuator controlled the amount of force produced. The force actuator was calibrated using a 50 g weight. Mice were placed on the wire mesh platform of the apparatus and allowed to acclimate for 30 minutes before testing. The monofilament was activated under the plantar surface of alternate hind paws at an upward force of up to 8 g for C57BL/6 mice or 10 g for Swiss Webster mice at a ramping speed of 2 seconds with about 5 minutes intervals between tests. The amount of force that induced paw withdrawal (in grams) from three to five trials per mouse were recorded and averaged.

2.2.4. Nerve Conduction Velocity

Mice were anesthetized with an i.p. injection of 100 mg/kg ketamine and 10 mg/kg xylazine in sterile normal saline solution. Anesthesia was confirmed by evaluating the eye blink reflex before initiating the tests. Body temperature was monitored with a rectal probe connected to

Physitemp TCAT-2DF Controller (Physitemp Instruments, Clifton, NJ) and maintained at 37°C using a heat lamp.

Motor and sensory nerve conduction velocities were measured using a TECA™ Synergy N2 (Carefusion, San Diego, CA) system with 12 mm subdermal disposable platinum/iridium bipolar needle electrodes (Cardinal Health Neurocare, Madison, WI).

Motor Nerve Conduction Velocity

The sciatic nerve was stimulated proximally at the sciatic notch and distally at the ankle via bipolar electrodes with a supramaximal stimulus (9.9 mA) of 0.05 ms duration square wave pulse. The resulting waveforms were filtered with low and high settings of 3 and 10 kHz. Latencies were defined as the time between stimulus artifact (**Figure 2.2.4.1**, position 1) and the onset of negative M-wave deflection (position 2). MNCV (m/s) was calculated by dividing the difference between proximal and distal latencies by the distance between stimulating and recording electrodes.

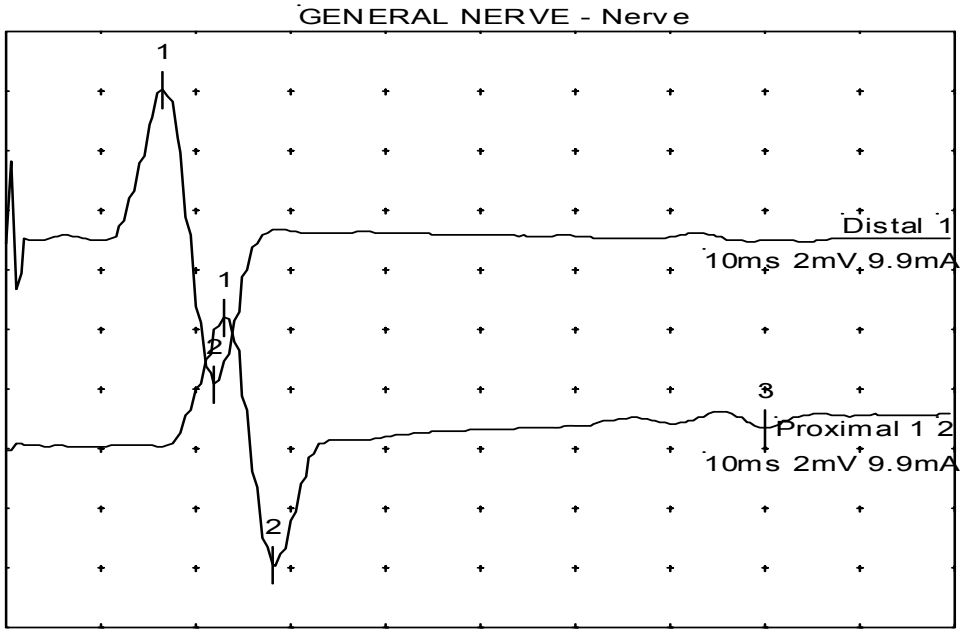


Figure 2.2.4.1: Representative trace of motor nerve conduction. A supramaximal stimulus (9.9 mA) were induced proximally at the sciatic notch and distally at the ankle. Latencies were defined as the time between stimulus artifact (position 1) and the onset of negative M-wave deflection (position 2).

Sensory Nerve Conduction Velocity

For hindlimb sensory nerve conduction velocity (SNCV), the digital nerve to the second toe was stimulated with a square-wave pulse of 0.05 ms duration using the smallest intensity current that resulted in a maximal amplitude response, typically 2.4 mA. The resulting wavelengths were filtered with low and high settings of 3 and 10 kHz, respectively. Ten sensory nerve action potentials were recorded behind the medial malleolus and averaged to generate a single waveform. Latency was determined by the time from stimulus artifact (**Figure 2.2.4.2**, position 1) to the onset of peak negative deflection (position 2). The maximal SNCV was calculated by dividing the latency by the distance between stimulating and recording electrodes.

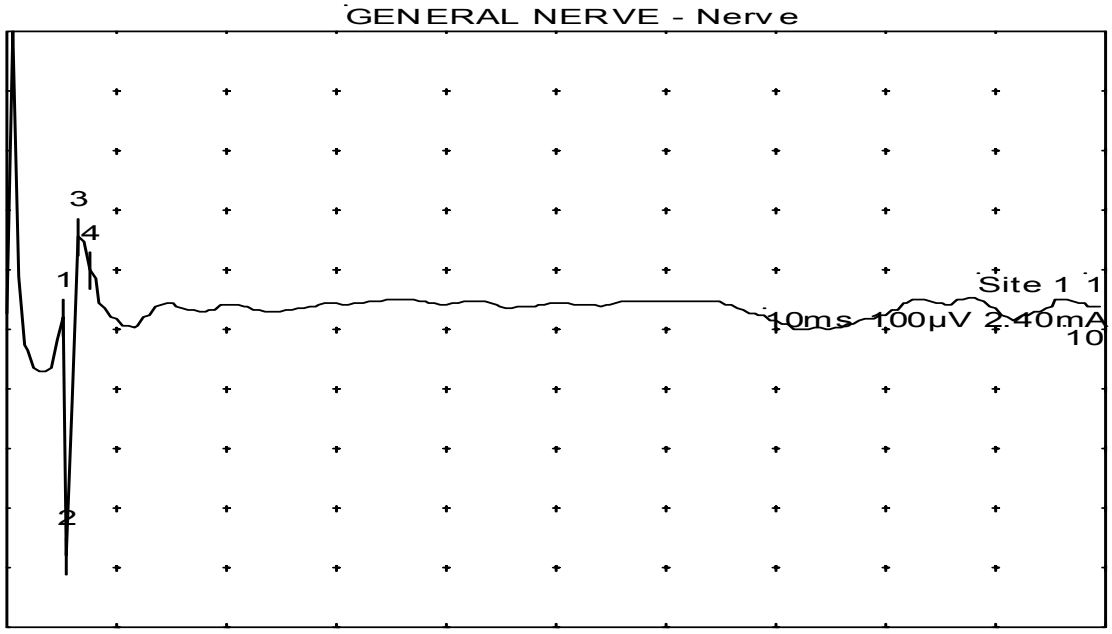


Figure 2.2.4.2: Representative trace of sensory nerve conduction. The digital nerve to the second toe was stimulated with a square-wave pulse and sensory nerve action potentials were recorded behind the medial malleolus. Latency was determined by the time from stimulus artifact (position 1) to the onset of peak negative deflection (position 2).

2.2.5. Erlotinib Administration

Erlotinib was dissolved in 0.1 M Captisol (CyDex Pharmaceuticals, Lenexa, KS) and administered via an i.p. injection at the indicated dose either weekly or twice per week.

2.2.6. Immunohistochemistry of Footpad Tissue

The integument of the plantar surface of both hind paws was dissected and fixed in Zamboni's fixative overnight. Tissues were then rinsed in PBS with sodium azide at 4°C, cryoprotected in 30% sucrose overnight, embedded in OCT (Sakura USA, Torrence, CA), frozen on dry ice, and stored at -80°C. Frozen tissues were sectioned at 30 µm, placed on Fisherbrand Superfrost Plus microscope slides and stored at -80°C. Immunohistochemistry was performed using the Vectastain Elite ABC-Peroxidase kit for rabbit IgG (Vector Laboratories, Burlingame,

CA) and an anti-PGP 9.5 antibody (AbD Serotec, Oxford, UK). In detail, tissue samples were surrounded using a PAP pen (Newcomer Supply, Middleton, WI), incubated in blocking buffer containing 10% normal goat serum for 30 minutes followed by incubation in a 1:1000 dilution of anti-PGP 9.5 antibody or blocking buffer (negative control) for 3 hours at room temperature. Samples were then incubated with secondary antibody for 1 hour at room temperature and subsequently treated with an avidin-biotin solution (ABC solution) for 1 hour at room temperature. After incubation with NovaRED peroxidase (Vector Laboratories, Burlingame, CA) for 2-3 minutes, the sections were counterstained with hematoxylin for 45 seconds and a coverslip applied. Two slides from each animal were stained and a minimum of twelve images per animal were captured using a Zeiss light microscope (Carl Zeiss microimaging, Thornwood, NY) with a color CCD digital camera (Diagnostic for Instruments Inc. Sterling Heights, MI) for quantification. Single nerve fibers crossing the dermal/epidermal junction were counted from each image. The IENF density was calculated by the number of fibers counted divided by the length of the dermal/epidermal junction (number of fibers/mm).

2.2.7. Immunoblot Analysis

Tissues were collected immediately upon euthanasia. Sciatic, tibial, and sural nerves were dissected from both hind limbs and placed in 0.1 to 0.2 ml of mRIPA buffer on ice. The tissue was homogenized with a Polytron fitted with a micro-tissue tearor and the lysate centrifuged at 10, 000 × *g* at 4°C for 10 minutes. The supernatant was collected and stored at -20°C. Protein concentration was determined using the Bio-Rad protein assay. Ten µg of protein was separated by SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with 5% non-fat dry milk in PBS-T (PBS buffer with 1% Tween) for 1 hour at room temperature with gentle rocking and probed with primary antibodies at 4ºC overnight. For detection of phosphorylated

protein, 3% bovine serum albumin was used to avoid non-specific blocking of the phosphoepitope. After primary antibody incubation, membranes were washed and further incubated with HRP-conjugated secondary antibodies for 2 hours at room temperature. β-actin was probed as a loading control. Immunoreactivity for each protein was visualized using HRP-conjugated chemiluminescence detection (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, U.K.) and exposed to autoradiography film. Immunoblots were quantified by densitometry with the aid of ImageJ (NIH) software and the level of the proteins of interest was normalized to β-actin unless otherwise stated. Changes in protein expression are expressed as a percent of the control values.

2.2.8. Erbin-shRNA Lentivirus Preparation and Infection

The nucleotide sequence of erbin shRNA was 5'-UAG ACU GAC CCA GCU GGA AdTdT-3' [288]. The pENTR™/U6 entry constructs containing erbin shRNA or lacZ-shRNA were generous gifts from Dr. Lin Mei, Georgia Health Science University. Lentiviral vectors were generated using BLOCK-iT™ Lentiviral RNAi Expression System according to the manufacturer's manual (Invitrogen, Carlsbad, CA). Ligation reactions were performed between pENTR™/U6 and pLenti6/ BLOCK-iT™-DEST vectors to generate an expression clone containing the shRNA sequence. 293FT cells were co-transfected by pLenti6/ BLOCK-iT™-DEST expression contrast and ViraPower™ Packing Mix to produce a lentiviral stock. The lentiviral stock was stored in -80°C. To examine the effect of erbin on myelination, the cells were infected with lenti-virus expressing erbin-shRNA or control lacZ-shRNA for 16 hours. Myelin protein expression levels were assessed by immunoblot analysis 48 hours after infection.

2.2.9. Preparation of Myelinated Mouse DRG/Schwann Cell Co-cultures

DRG neurons were isolated from C57BL/6 pups born at day 0 (P0) into L15 medium as previously described [297]. Following dissociation of tissues using 0.25% trypsin and 0.25% collagenase at 37ºC for 30 minutes, cells were collected by centrifugation for 5 minutes at 1, 000 × g and resuspended in DMEM containing 25 mM glucose and 10% fetal calf serum (Atlas Biologicals, Fort Collins, CO, U.S.A.). Cells were triturated, counted with a hemocytometer and 6-7×10^4 cells were seeded onto collagen-coated glass coverslips. The cultures were maintained in DMEM maintenance medium containing 100 U/mL penicillin, 100 µg/mL streptomycin (Thermoscientific, Logan, UT, U.S.A.), 50 µM gentamycin (MP Biologicals, Solon, OH, U.S.A.) and 50 ng/mL nerve growth factor (Harlan Biosciences, Indianapolis, IN, U.S.A.). Fast-growing fibroblasts were removed by treating the cells with 10 µM cytosine β-D-arabinoside for 2 days and cultures were maintained in regular medium for one week to allow Schwann cell to proliferate and associate with axons. Myelination was then initiated by addition of 50 µg/ml ascorbic acid for 18-21 days in culture with the medium replenished every 2-3 days.

2.2.10. Preparation of Schwann Cell Cultures

Sciatic nerves were dissected from postnatal day 2 or day 3 Sprague-Dawley rat pups as previously described [298, 299]. The nerves were rinsed with Leibovitz's L15 medium (L15) and incubated with 0.25% collagenase in L15 medium for 30 minutes at 37 °C. The cells were collected by centrifugation for 10 minutes at 1, 000 × g and resuspended in L15 medium containing 0.25% trypsin plus 0.25% collagenase for an additional 30 minutes, and protease activity was inactivated with DMEM containing 10% fetal calf serum (FCS). The cells were triturated through a fire-polished glass pipette and plated onto poly-D-lysine-coated plates in low glucose (5.5 mmol/L) DMEM containing 10% FCS and 2 µM forskolin (complete medium).

The cells were grown in the presence of 10 μM cytosine arabinoside for 3 days to remove the faster growing fibroblasts and passaged upon confluency after approximately 1 week in culture. The Schwann cells were subcultured upon confluency and used for no more than 5 passages. To examine the effects of hyperglycemia and NRGs on Schwann cell mitochondrial function, cultures were treated with low glucose or high glucose (25 mmol/L) DMEM medium for 6 days and sub-groups were treated with 10 ng/ml NRG1 Type I (recombinant human NRG-1-β1 epidermal growth factor domain, aa 176-246, R&D Systems, Minneapolis, MN) or 10 ng/ml NRG1 Type III (recombinant human NRG1 SMDF isoform, R&D Systems, Minneapolis, MN) for 24 hours. Mitochondrial bioenergetics were measured by Seahorse Biosciences XF96 Extracellular Flux Analyzer.

2.2.11. Measurement of Mitochondrial Respiration

Oxygen consumption rate (OCR) was assessed in intact mouse lumbar DRG sensory neurons or primary rat Schwann cells using an XF96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). The maintenance medium was changed to unbuffered Dulbecco's modified Eagle's medium supplemented with 1 mmol/L pyruvate and 5.5 mmol/L D-glucose 1 hr prior to the assay and the cells were incubated at 37°C. The plate was introduced into the XF96 analyzer, a 3-minute mix cycle used to oxygenate the medium, and respiration was assessed in a 4-minute measurement cycle. As a general description of a mitochondrial stress assay, the initial rates provide a measure of the basal OCR prior to assessing mitochondrial dysfunction using respiratory chain poisons. The portion of basal OCR that is coupled to ATP synthesis was estimated by the decrease in OCR following addition of the ATP synthase inhibitor, oligomycin (1 μg/ml). The residual OCR that persists after oligomycin treatment is from uncoupled respiration (proton leak). Next, maximal respiratory capacity (MRC) was

assessed following dissipation of the proton gradient across the inner mitochondrial membrane with the protonophore FCCP (1μM). Non-mitochondrial respiration was then assessed by coinjection of 1 μM each of rotenone and antimycin A. After the respiratory measures, the cells were harvested and OCR values were normalized to the total protein content of each well. ATP-linked respiration, proton leak, maximal respiratory capacity, spare respiratory capacity, and respiratory control ratio were determined as described previously [150, 300].

2.2.12. Mitochondrial Fraction

Schwann cells were washed with ice-cold PBS and resuspended in 0.5 ml of cell homogenization medium containing 150 mmol/L magnesium chloride, 10 mmol/L potassium chloride, 10 mmol/L Tris-HCl, pH 7.4, 0.25 mol/L sucrose and 1x Complete® protease inhibitors. The lysates were centrifuged at 800 × g for 10 minutes at 4°C to get rid of the nuclear fraction. The supernatant was recovered and centrifugation at 8, 000 × g for 10 minutes at 4°C. The supernatant was collected as the cytosolic fraction. The pellet was washed twice with ice-cold sucrose/Mg^{2+} medium (150 mmol/L magnesium chloride, 0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4 and 1x Complete® protease inhibitors) and resuspended in mitochondrial suspension buffer (0.25 mol/L sucrose, 10 mmol/L Tris-Base and 1x Complete® protease inhibitors). Immunoblot analysis was performed to detect the Erb B2 levels in the mitochondrial fraction.

2.2.13. Statistical Analysis

Data are presented as arithmetic means ± SEM. After verifying equality of variance, differences between treatments were determined using a one-way or two-way ANOVA by

SigmaStat 3.5. Differences between group means were ascertained using Tukey's Honestly-Significant-Difference *post hoc* test.

Chapter 3: Dysfunction of Neuregulin-Erb B2 Ligand-Receptor Signaling Pathway in Diabetic Peripheral Neuropathy

3.1. Mouse Models of Diabetic Peripheral Neuropathy

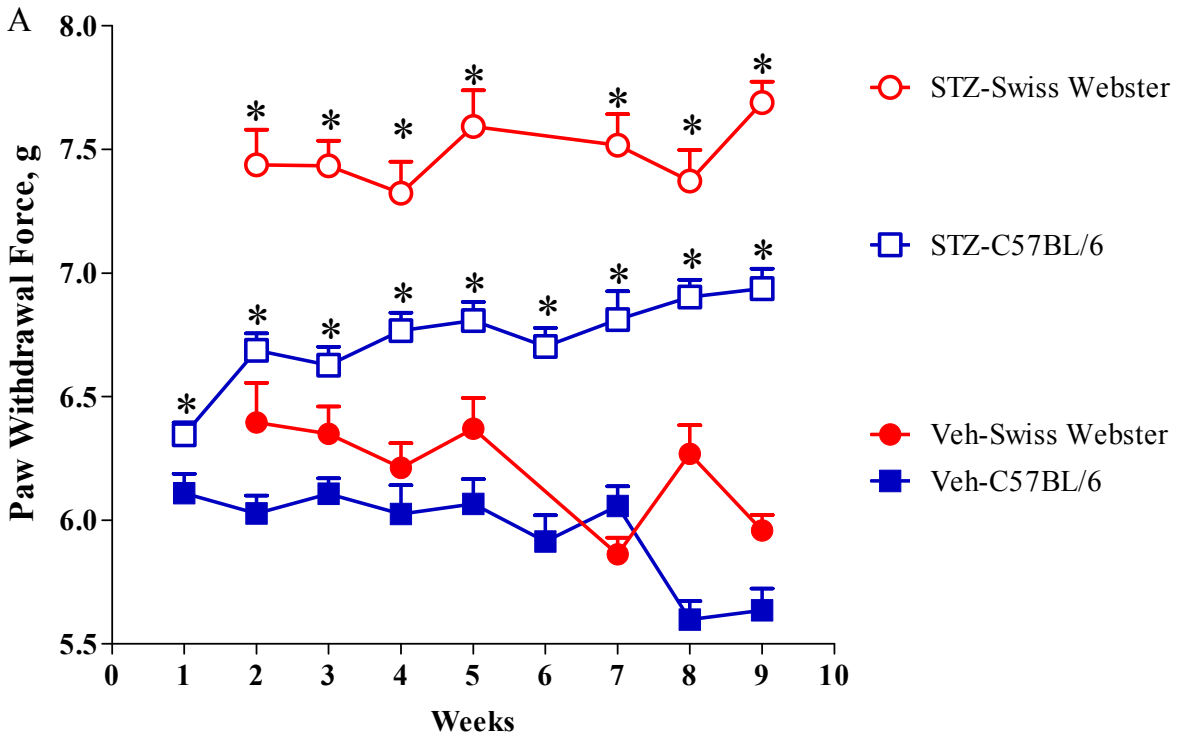
We have previously demonstrated that a transient increase in Erb B2 receptor activation contributed to diabetes-induced sensory neuropathy since inhibiting Erb B2 signaling reversed some of the pathophysiologic features of DPN [143]. Therefore, disrupted Erb B2 signaling might contribute to the axonal degeneration and Schwann cell demyelination in diabetic nerves. However, it remains unknown whether the increase in Erb B2 activity in diabetic nerves is associated with changes in NRG1 isoforms and erbin that may serve as positive and negative regulators of Erb B2 signaling, respectively.

Experimental mice provide a useful model to investigate the pathophysiology of diabetes and its complications. Since T1DM is the focus in the current study, we used STZ to induce T1DM in mice. However, the dose of STZ injection and the mouse background may influence the onset and the severity of DPN. Consistent with other groups using C57BL/6 mice, we did not observe a significant decrease in SNCV and loss of fiber innervation [143]. In contrast, other studies have reported that outbred Swiss Webster mice develop severe diabetic neuropathy and are a more robust model that better tolerate long-term untreated diabetes [146]. Therefore, we compared inbred C57BL/6 and outbred Swiss Webster mice to determine if the later would be useful for our study.

Eight-week old wild type C57BL/6 mice were rendered diabetic with STZ i.p. injections daily at doses of 85, 70, and 55 mg/kg, which have been determined to be effective to induce diabetes in C57BL/6 mice [144]. Eight-week old Swiss Webster mice were induced diabetic

with STZ i.p. injections at 100 mg/kg for two consecutive days. Mechanical and thermal sensitivity were measured as the parameters to compare the severity of neuropathy between mouse models.

In both models, diabetes induced significant mechanical and thermal hypoalgesia as early as two weeks after the onset of hyperglycemia, compared to corresponding non-diabetic controls (**Figure 3.1.1 A** and **B**). In addition, diabetic Swiss Webster mice required 5% - 15% more force to induce paw withdrawal than diabetic C57BL/6 mice did (**Figure 3.1.1 C**), whereas thermal latencies were comparable between two models. These result suggest that diabetic Swiss Webster mice could develop more robust diabetic neuropathy, especially in myelinated nerve fibers that mainly contribute to the mechanical sensation.



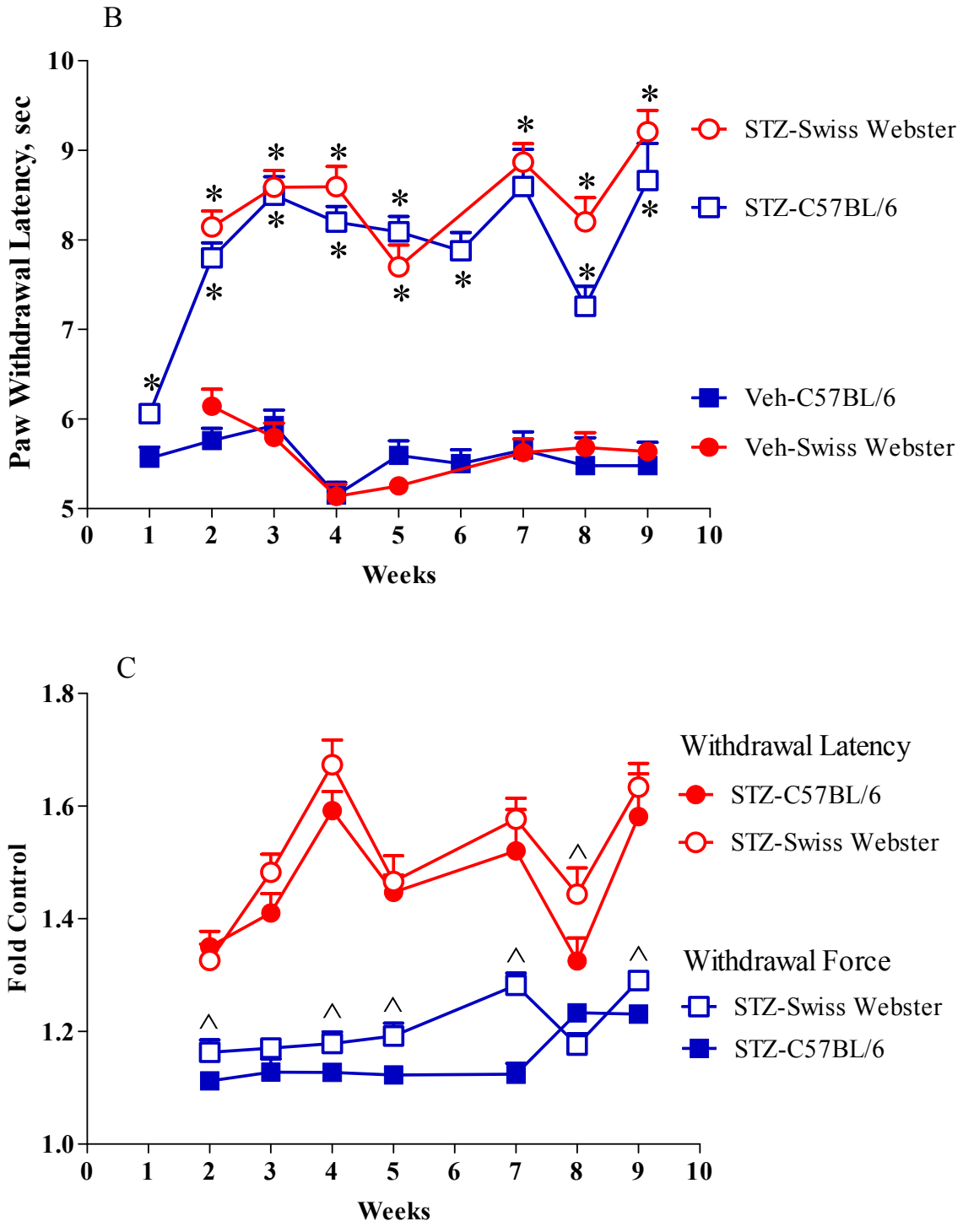
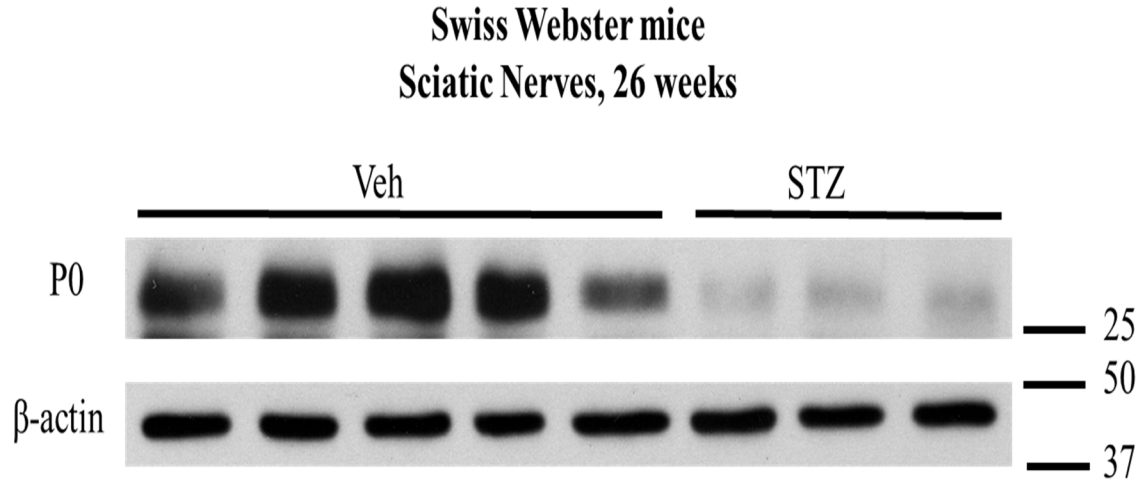


Figure 3.1.1: Mechanical and thermal hypoalgesia in C57BL/6 and Swiss Webster mice over 9 weeks of diabetes. Diabetes induced significant mechanical **(A)** and thermal **(B)** hypoalgesia in both C57BL/6 and Swiss Webster mice compared to corresponding time-matched non-diabetic control mice. **(C):** The ratio of withdrawal threshold in diabetic mice compared to

control ones within each mouse model. Diabetic Swiss Webster mice demonstrated more severe mechanical insensitivity (**blue squires**) than diabetic C57BL/6 mice, whereas thermal withdrawal latencies (**red circles**) were comparable between mouse models (*, $p < 0.05$ for STZ vs. time-matched Veh; ^, $p < 0.05$ for STZ-Swiss Webster vs. time-matched STZ-C57BL/6).

In order to determine the effects of longer-term hyperglycemia on myelin proteins in Swiss Webster mice, sciatic nerves were collected from 26-week diabetic mice and time-matched control mice. Protein lysates were separated by SDS-PAGE and probed with an antibody against myelin protein zero (P0). Immunoblot analysis (**Figure 3.1.2**) showed a remarkable reduction in P0 levels in diabetic sciatic nerves, which was not been observed in our previous study in C57BL/6 mice [143]. Although morphological studies such as g-ratio measurement and teased nerve fibers are needed to determine demyelination, a decrease in compact myelin protein P0 levels suggest that diabetes might induce worse nerve damage and sensory deficits in Swiss Webster mice, especially in myelinated nerve fibers, through compromising the myelin sheath components.



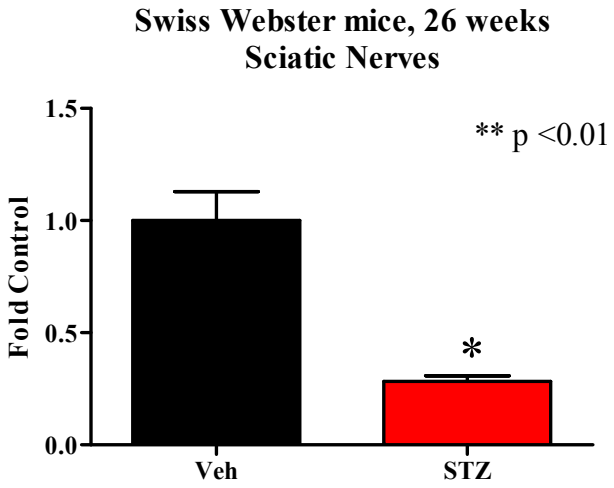


Figure 3.1.2: Decreased P0 expression levels in sciatic nerves of Swiss Webster mice after 26-week diabetes. Diabetic Swiss Webster mice (STZ) demonstrated a significant reduction of P0 expression levels in sciatic nerves after 26-week diabetes compared to non-diabetic control mice (Veh). Levels of P0 are normalized to β-actin levels (**, $p < 0.01$ for STZ vs. Veh).

3.2. Dysfunction of NRG1-Erb B2 Signaling in Diabetic Swiss Webster Mice

To elucidate the role of altered NRG1-Erb B2 signaling in DPN, we used diabetic Swiss Webster mice, which after prolonged diabetes develop severe pathophysiologic symptoms of DPN [146]. **Figure 3.2.1** shows the time-course of the current study. Swiss Webster mice were rendered diabetic with STZ injections and after 12 weeks of diabetes, treated with erlotinib once a week for 4 weeks and twice a week for an additional 4 weeks. Sensory tests (Von Frey and Hargreaves) and nerve conduction velocity studies (MNCV and SNCV) were conducted to measure sensory thresholds and nerve function. IENFD was performed to detect changes in small fiber innervation. Peripheral nerve samples (sciatic, tibial and sural nerves) were collected at weeks 9, 12, 16 and week 21 and immunoblot analysis was performed to detect changes in NRG1 isoforms and erbin expression.

Inhibition of Erb B2 signaling partially reversed several pathophysiologic aspects of DPN including a pronounced sensory hypoalgesia, nerve conduction velocity deficits and the decrease in epidermal nerve fiber innervation (**Chapter 3.3**) In addition, we observed that diabetes altered the expression of NRG1 Type III and NRG1 Type I in distal nerve fibers with increasing duration of diabetes. In particular, a decrease in NRG1 Type III expression was accompanied by an increased expression of NRG1 Type I level in diabetic sural nerves and tibial nerves (**Chapter 3.4**). Furthermore, the expression of erbin was decreased in sciatic nerves of diabetic mice and this corresponded with an increase in p42/p44 MAPK pathway activity (**Chapter 3.5**). These data are the first to characterize that diabetes can alter the expression of proteins that serve as positive and negative regulators of Erb B2 activity and suggest that an altered neuregulinism may contribute to Schwann cell pathology in DPN [301].

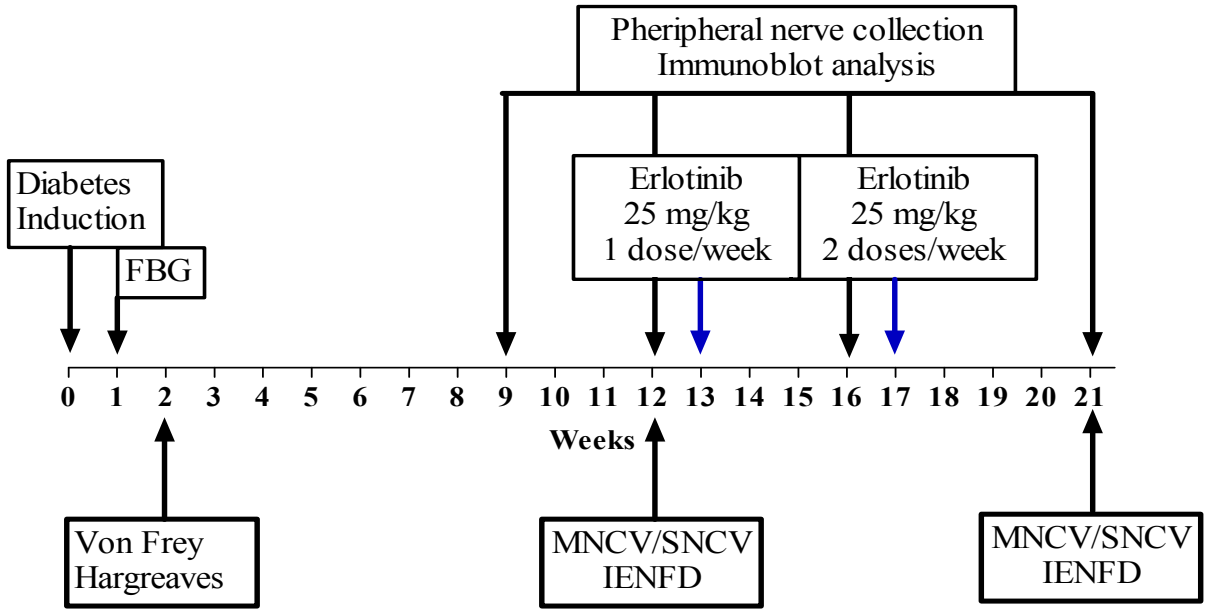


Figure 3.2.1: Time-course of the current study. Swiss Webster mice were rendered diabetic with STZ injections, at the time that considered week 0. FBG measurements at week 1 were performed to confirm the status of hyperglycemia. After 12 weeks of diabetes, mice were sub-

grouped and received 25 mg/kg erlotinib or vehicle once a week for 4 weeks and twice a week for additional 4 weeks. Starting at week 2, behavior tests (Von Frey and Hargreaves) were initiated. Nerve conduction velocity studies (MNCV and SNCV) and IENFD study were performed between (at week 12) and after (at week 21) the erlotinib treatments. Peripheral nerve samples were collected at week 9, 12, 16 and week 21.

3.3. Erb B2 Receptor Inhibitor Attenuates Pathophysiological Indices of Diabetic Peripheral Neuropathy

Swiss Webster mice were rendered diabetic with STZ and changes in mechanical and thermal sensitivity were monitored for 12 weeks to assess the onset of hypoalgesia in both myelinated (mechanical) and unmyelinated (thermal) nerve fibers. Diabetic mice showed a significant increase in both paw withdrawal force and thermal latency, indicating mechanical (**Figure 3.3.1 A**) and thermal (**Figure 3.3.1 B**) hypoalgesia. To address whether Erb B2 signaling contributed to the sensory deficits, mice were treated at week 13 with vehicle or 25 mg/kg erlotinib, which is a clinically approved inhibitor of the EGFR that can also inhibit Erb B2 receptors [143]. The mice were initially given one treatment per week for 4 weeks and this modestly improved mechanical and thermal sensitivity. Increasing the frequency of dosing to twice per week (**Figure 3.3.1 A** and **B, dashed arrows)** yielded a greater improvement in mechanical sensitivity, but thermal sensitivity did not change beyond that seen with a single dose per week.

In order to directly assess the nerve function, we measured the motor (MNCV) and sensory (SNCV) nerve conduction velocity. Consistent with the sensory hypoalgesia, both MNCV and SNCV were significantly decreased after 12 weeks of diabetes compared to non-diabetic animals (**Figure 3.3.2 A** and **B**). After 8-weeks of erlotinib treatment, the pre-existing deficits in both MNCV and SNCV were partially reversed. Interestingly, the improvement in SNCV and thermal hypoalgesia in erlotinib-treated diabetic Swiss Webster mice was not observed in our

previous study using C57BL/6 mice [143]. Whether this observation is attributable to differences between the mouse strains is unclear. Nonetheless, these data support that Erb B2 signaling may contribute to glial cell dysfunction in DPN.

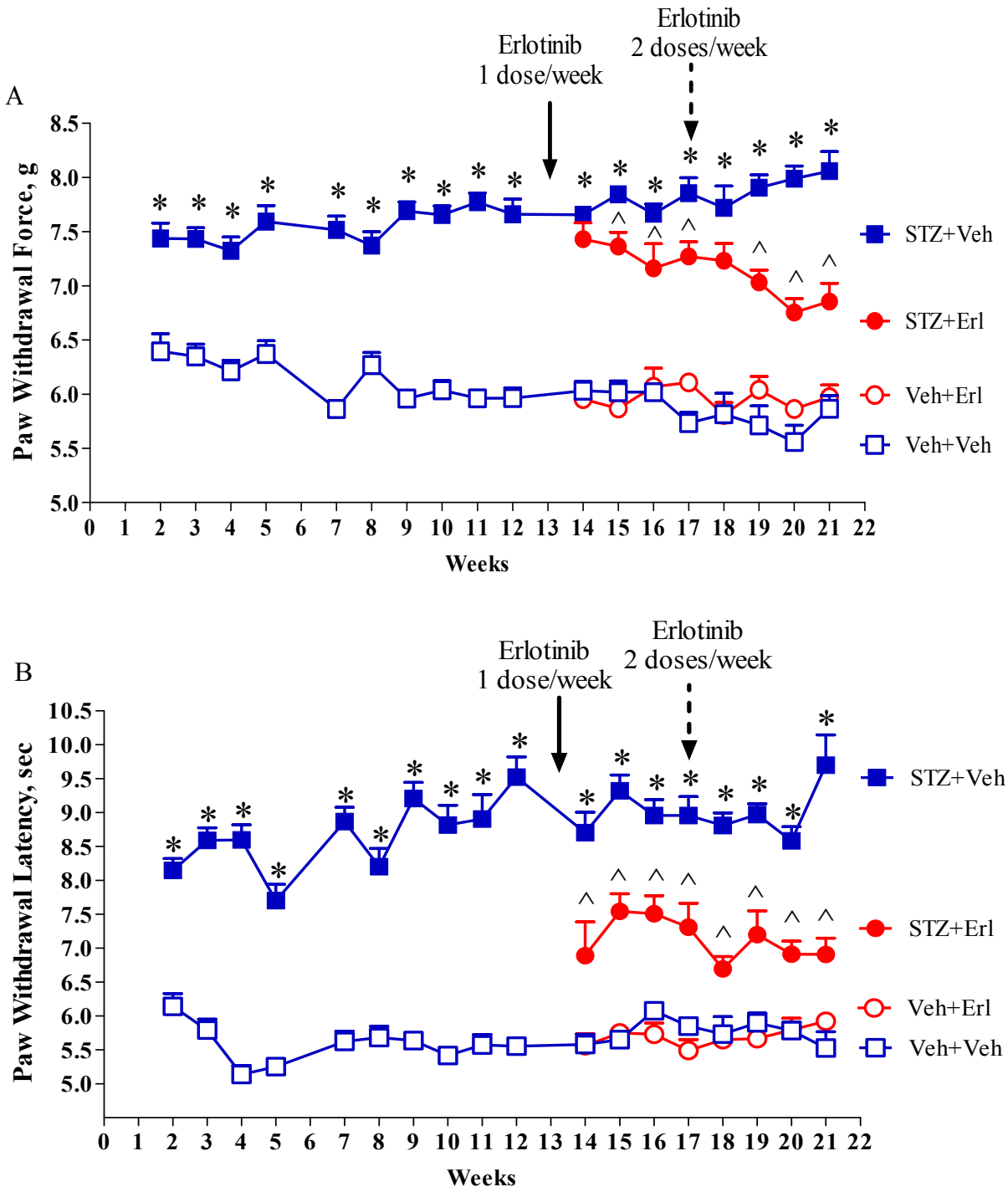
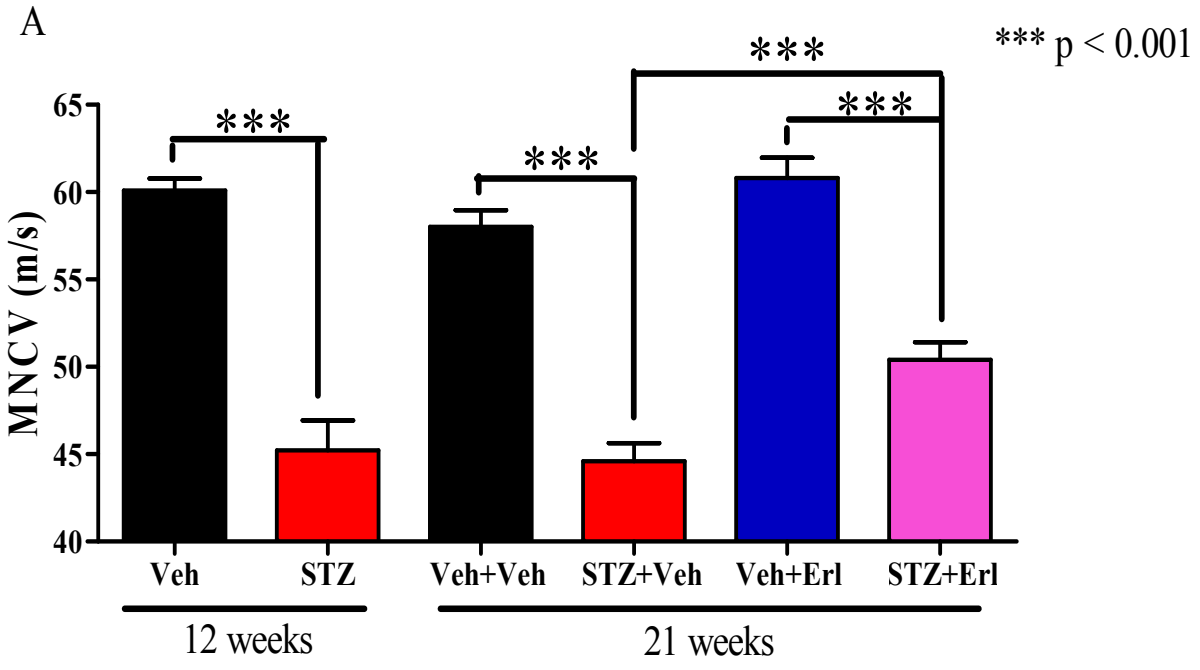


Figure 3.3.1: Inhibition of Erb B2 receptors partially attenuated sensory deficits in diabetic Swiss Webster mice. Swiss Webster mice were rendered diabetic and after two weeks of diabetes, mechanical **(A)** and thermal sensitivity **(B)** were assessed weekly (*, $p < 0.05$ for STZ+Veh vs. time-matched Veh+Veh; ^, $p < 0.05$ for STZ+Erl vs. time-matched STZ+Veh). At 13 weeks of diabetes, animals were given vehicle or 25 mg/kg erlotinib once per week for 4 weeks (**solid arrow**) and then twice per week (**dashed arrow**) for the final four weeks (n = 8-12 per group). Erlotinib significantly improved mechanical and thermal sensitivity compared to vehicle treated diabetic mice.



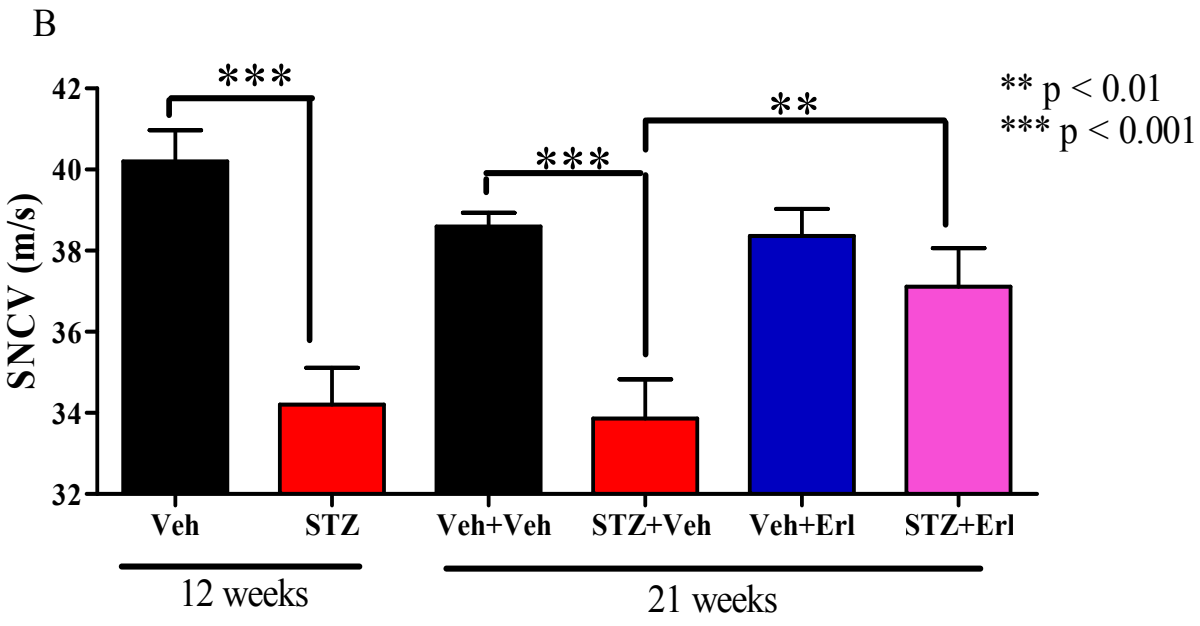
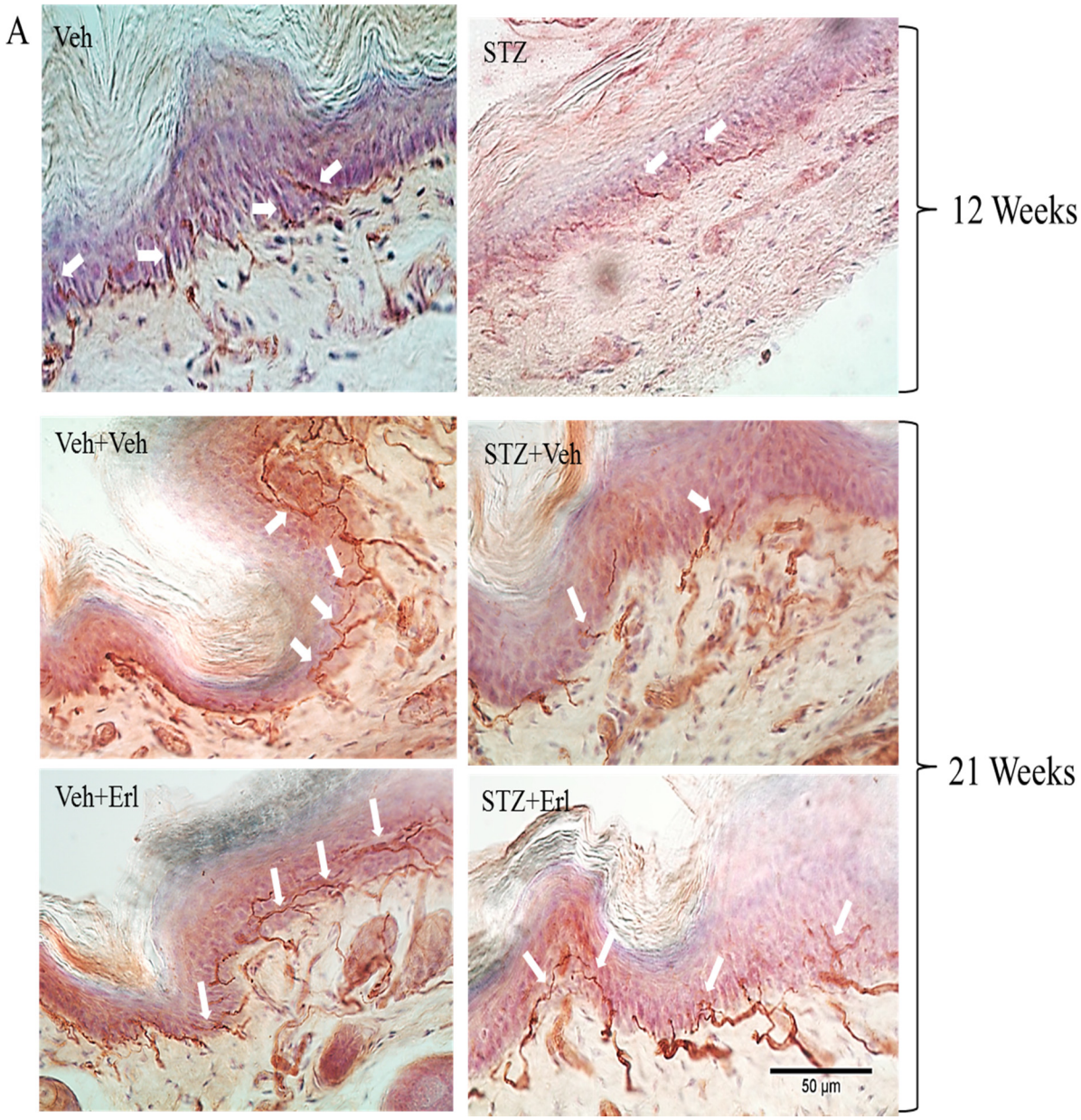


Figure 3.3.2: Inhibition of Erb B2 receptors partially restored decreased nerve conduction velocity in diabetic Swiss Webster mice. Swiss Webster mice were rendered diabetic and assessment of MNCV **(A)** and SNCV **(B)** in a subgroup of animals (n = 4 per group) after 12 weeks of diabetes confirmed the onset of nerve dysfunction prior to drug treatment. At 13 weeks of diabetes, animals were given vehicle or 25 mg/kg erlotinib once per week for 4 weeks and then twice per week for the final four weeks (n = 8-12 per group). Erlotinib significantly restored the nerve function in both MNCV and SNCV compared to vehicle treated diabetic mice.

Measurements of IENFD is emerging as a highly sensitive technique for diagnosing and staging the disease status of small fiber neuropathy in patients [133] and animal models [134, 135]. We collected the plantar surface of the hind paws before and after erlotinib treatments and stained the nerve fibers with an antibody against PGP 9.5, which is a cytosolic ubiquitin C-terminal hydroxylase specifically expressed in neurons. The PGP 9.5-positive nerve fibers that crossed the epidermal/dermal junction were counted to quantify distal nerve innervation (**Figure 3.3.3**). As anticipated, 12 weeks of diabetes significantly decreased IENFD (20%) but fiber loss was not significantly greater after 21 weeks of diabetes (27%). However, erlotinib treatment partially reversed the diabetes-induced loss of the largely unmyelinated intra-epidermal nerve fibers. Though the increase in IENFD in erlotinib-treated diabetic mice may contribute to the enhanced sensitivity to noxious thermal stimuli, improved thermal sensitivity can occur even in the absence of an increase in epidermal fibers [135]. Together, these data support that altered activity of Erb B2 signaling may be a contributing factor underlying diabetes-induced peripheral nerve damage in both myelinated and unmyelinated fibers in Swiss Webster mice.


A
Veh
STZ
12 Weeks
Veh+Veh
STZ+Veh
Veh+Erl
STZ+Erl
21 Weeks
50 µm

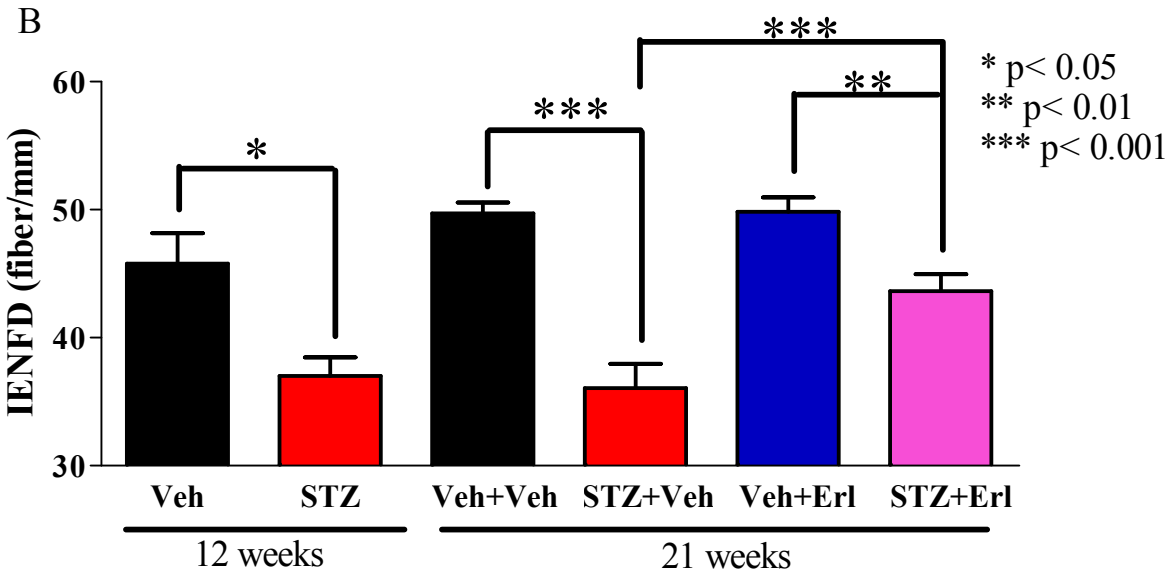


Figure 3.3.3: Inhibition of Erb B2 receptors improved IENFD in diabetic Swiss Webster mice. Footpad samples were collected from the plantar surface of the hind paws after 12 (n = 4 per group) and 21 weeks (n = 6–8 per group) of treatment. **(A):** Representative images of IENFD in non-diabetic (Veh) and diabetic (STZ) mice treated with vehicle (Veh) or erlotinib (Erl). Nerve fibers immunopositive for PGP 9.5 are stained red by the chromagen (arrows) and epidermal cells were stained purple by hematoxylin. **(B):** Quantification demonstrated a significant loss of nerve innervation in diabetic mice prior to drug administration and erlotinib treatment induced a partial recovery in fiber loss.

After 12 and 21 weeks of diabetes, FBG levels and HbA1c were increased, whereas body weight was decreased in diabetic Swiss Webster mice compared to age-matched, vehicle-treated control mice (**Table 3.3.1**). Erlotinib treatment did not affect glucose level or body weight compared to vehicle-treated diabetic mice. These results indicate that the effects of erlotinib on improving the diabetes-induced nerve deficits in both myelinated and unmyelinated nerve fibers were not due to correction of the systemic hyperglycemia, consistent with our previous observation in diabetic C57BL/6 mice [143].

Table 3.3.1: Body weights, FBG and HbA1c values from the Swiss Webster mice

Week	Treatment	FBG (mg/dl)	Weight (g)	(n)	HbA1c (%)	(n)
Week 12	Veh	112 ± 21	37.2 ± 5.4	10	5.0 ± 0.4	6
	STZ	595 ± 12*	30.6 ± 3.7*	11	12.9 ± 0.2*	7
Week 21	Veh+Veh	121 ± 26	42.6 ± 5.4	8	5.1 ± 0.1	3
	Veh+Erl	104 ± 17	43.4 ± 2.7	8	5.0 ± 0.2	3
	STZ+Veh	545 ±106*	34.5 ± 3.5*	8	10.2 ± 1.7*	4
	STZ+Erl	584 ± 40#	37.0 ± 5.5#	6	11.4 ± 1.1#	4

*, $p < 0.05$ vs. Veh or Veh+Veh; #, $p < 0.05$ vs. Veh+Erlotinib (Erl)

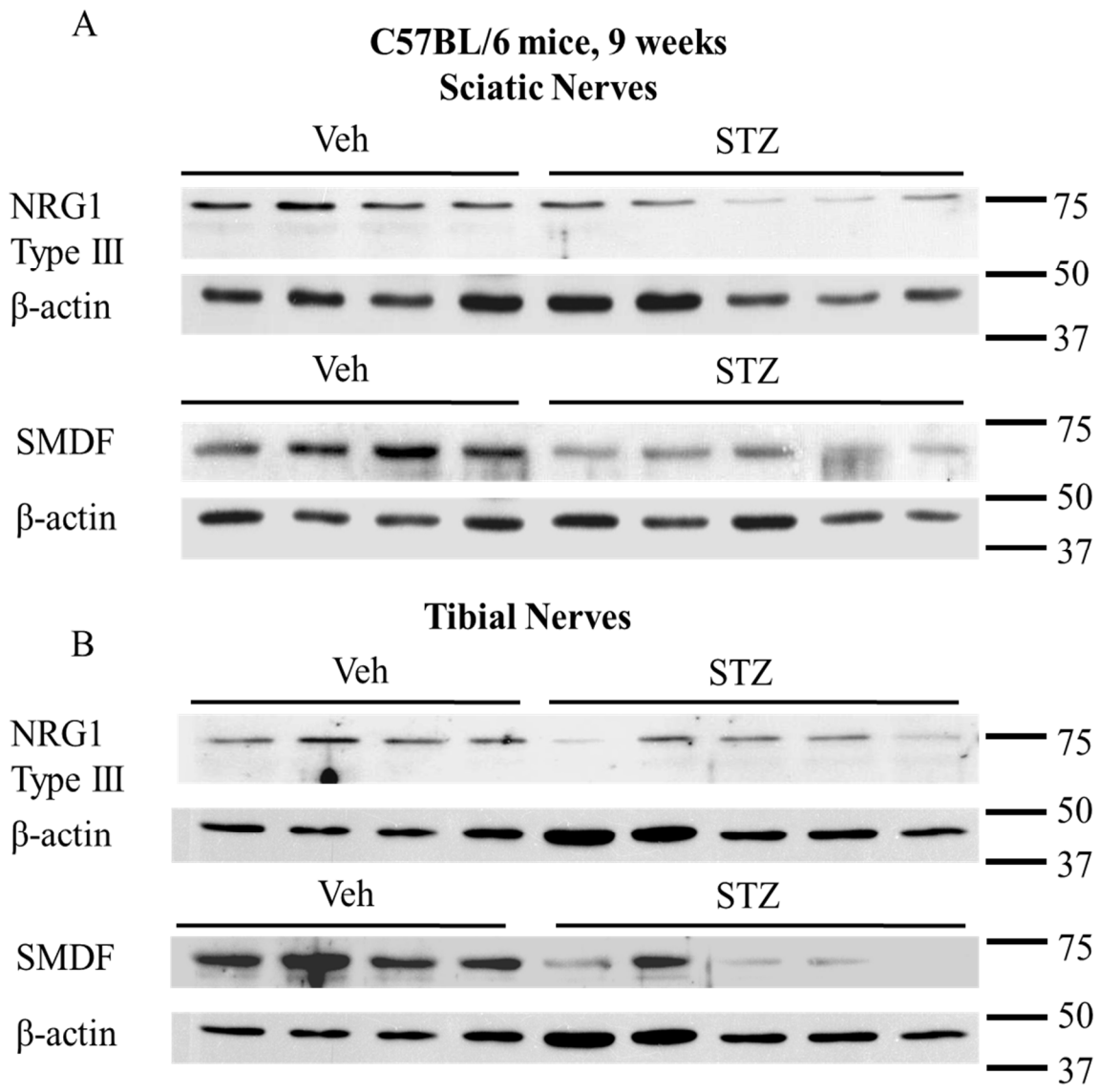
3.4. Diabetes Differentially Alters the Expression of NRG1 Isoforms in Peripheral Nerves

3.4.1. Expression Levels of NRG1 Type III were Decreased in Diabetic Peripheral Nerves

Though aberrant Erb B2 activity can contribute to DPN, it remains unknown whether this may be associated with changes in the expression of NRG1 isoforms which regulate Schwann cell differentiation or dedifferentiation. In order to evaluate the changes of NRG1 Type III expression along with the progression of diabetes, we sacrificed several mice from both diabetic and non-diabetic groups after various durations of diabetes. Peripheral nerves were isolated from control and diabetic mice and protein lysates were separated by SDS-PAGE and probed with two different NRG1 Type III antibodies (discussed in **Materials and Methods**).

In a preliminary study, C57BL/6 mice were rendered diabetic and peripheral nerves were collected after 9 weeks of diabetes. Immunoblot analysis showed that NRG1 Type III levels were more robustly reduced in diabetic tibial versus sciatic nerves, which might be attributed to the more distal location of tibial nerves in the PNS (**Figure 3.4.1.1**). Since DPN progresses in a distal-to-proximal manner, it is possible that 9 weeks of diabetes is sufficient to alter NRG1 Type

III levels in the distal tibial nerves, but has a relatively minor effect on proximal sciatic nerves. However, during the time course in this study, we did not detect any changes of NRG1 Type III expression in sural nerves. Combined with the previous observation that SNCV did not change in short-term diabetic C57BL/6 mice, these data suggest that hyperglycemia may affect myelinated nerves (such as sciatic and tibial nerves) earlier and greater than unmyelinated nerves (such as sural nerves). Given the function of axonal NRG1 Type III to promote Schwann cell differentiation and myelination, these data suggest that diabetes may compromise myelination by decreasing NRG1 Type III levels, especially in myelinated nerves.


A
C57BL/6 mice, 9 weeks
Sciatic Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37
B
Tibial Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37

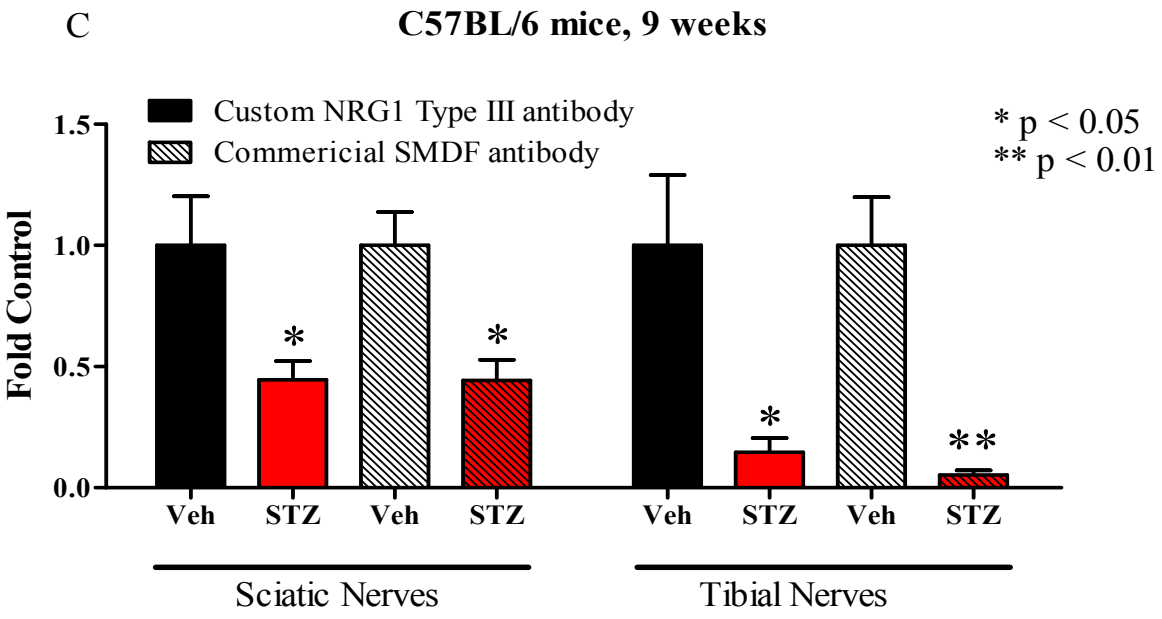
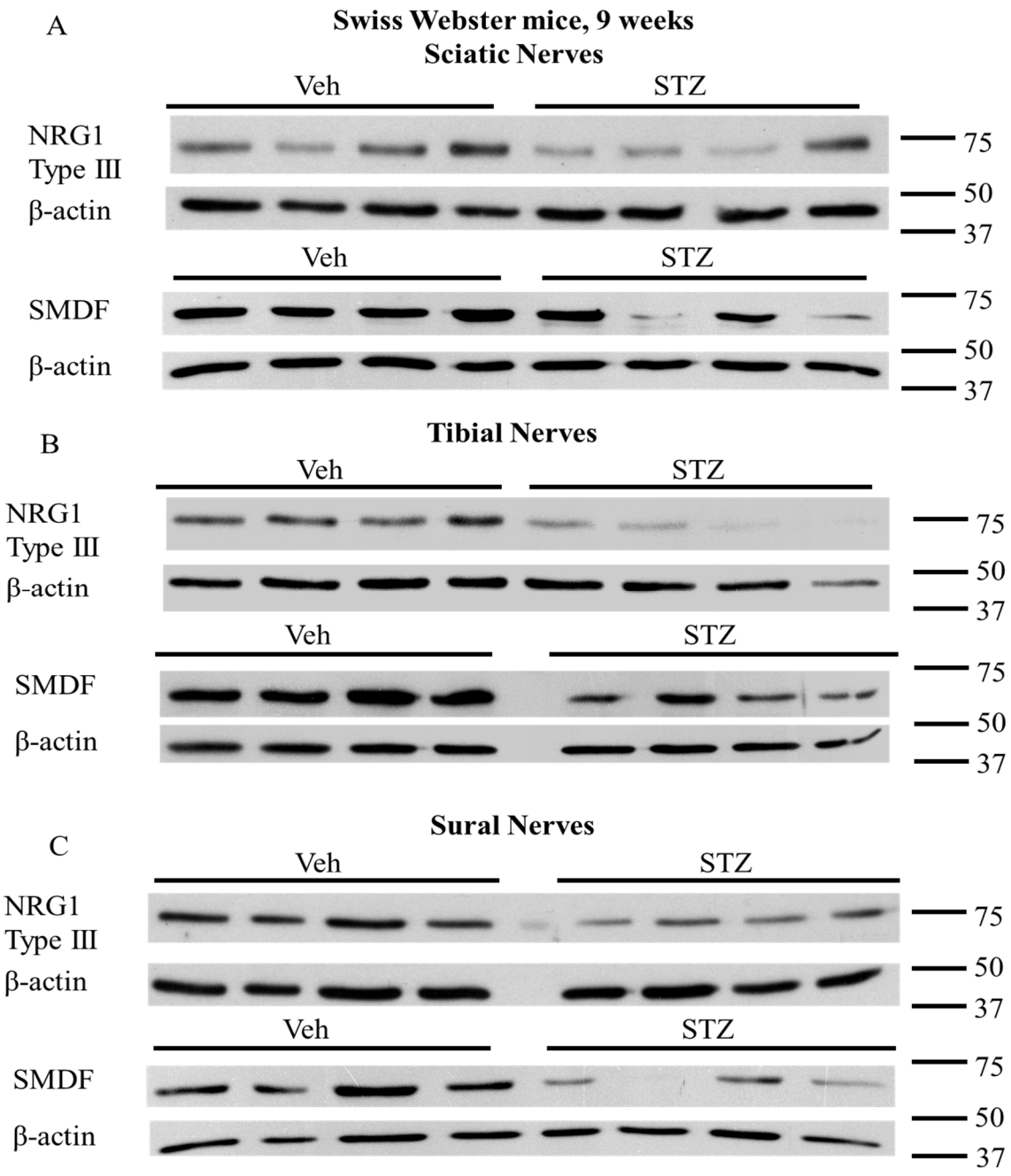


Figure 3.4.1.1: NRG1 Type III levels were decreased in sciatic nerves and tibial nerves after 9 weeks of diabetes in C57BL/6 mice. Wild-type C57BL/6 mice were rendered diabetic for 9 weeks. Sciatic nerves **(A)** and tibial nerves **(B)** were isolated (n = 4-5 per group). Protein lysates were prepared and NRG1 Type III levels were determined by immunoblot analysis using two antibodies. (**C**): Bands were quantified, NRG1 Type III levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$; **, $p < 0.01$ for STZ vs. Veh).

Similarly, immunoblot analysis showed that 9 weeks of diabetes induced a significant decrease in NRG1 Type III levels in diabetic sciatic, tibial and sural nerves from the Swiss Webster mice. This result was also confirmed by both antibodies (**Figure 3.4.1.2**). Consistent with the results obtained in the C57BL/6 mice, diabetic tibial nerves showed the maximal magnitude of reduction in NRG1 Type III levels in the three types of peripheral nerves. Interestingly, we detected a significant decrease in NRG1 Type III expression levels in diabetic sural nerves after 9 weeks of diabetes, which was not observed in 9-week diabetic C57BL/6 mice. These data indicate that hyperglycemia in diabetic Swiss Webster mice may cause more nerve damage and extend its effects to unmyelinated nerve fibers.


A
Swiss Webster mice, 9 weeks
Sciatic Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37
B
Tibial Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37
C
Sural Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37

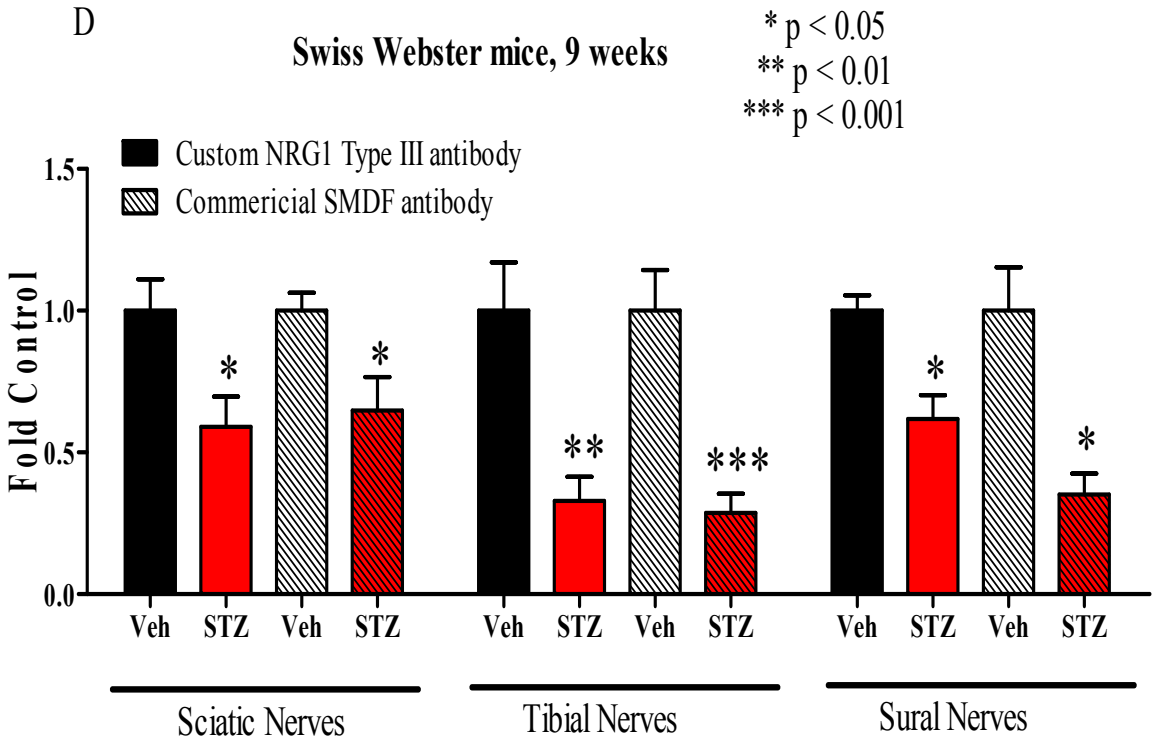
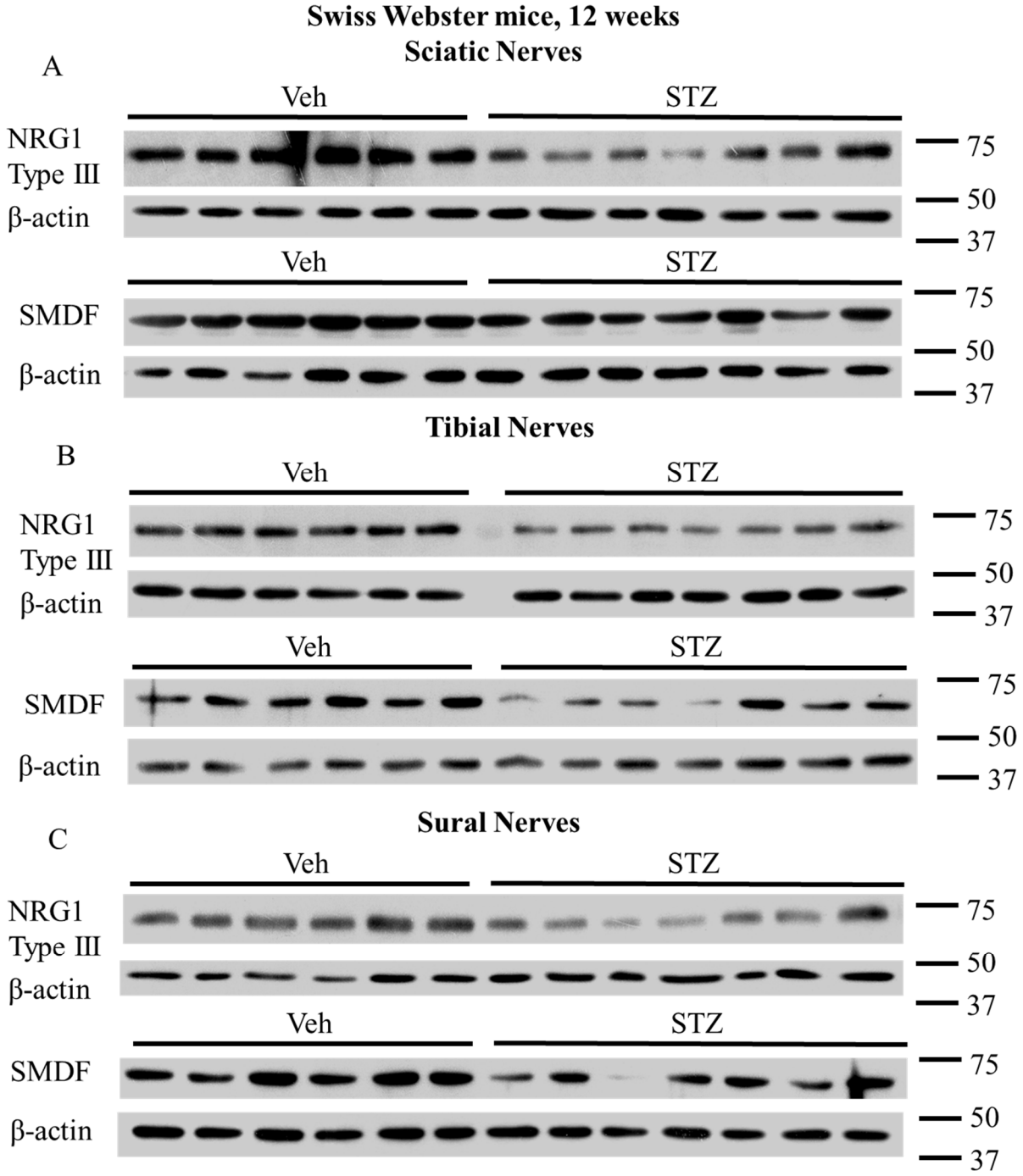


Figure 3.4.1.2: NRG1 Type III levels were decreased in sciatic nerves, tibial nerves and sural nerves after 9 weeks of diabetes in Swiss Webster mice. Swiss Webster mice were rendered diabetic for 9 weeks. Sciatic nerves **(A),** tibial nerves **(B)** and sural nerves **(C)** were isolated (n = 7-8 per group). Protein lysates were prepared and NRG1 Type III levels were determined by immunoblot using the two antibodies. **(D):** Bands were quantified, NRG1 Type III levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for STZ vs. Veh).

With disease progression, 12 weeks (**Figure 3.4.1.3**) and 16 weeks (**Figure 3.4.1.4**) of diabetes induced a considerable decrease in NRG1 Type III expression levels in tibial and sural nerves, confirmed by two antibodies. Results in the 16 sciatic nerve samples were inconsistent between the antibodies but the origin of this discrepancy could not be ascertained. Nonetheless, these data suggest that chronic hyperglycemia could mitigate the expression of the pro-myelinating isoform (Type III) of NRG1 family in both myelinated and unmyelinated nerve

fibers. This decrease might be associated with sensory hypoalgesia and the comprised nerve function (decreased MNCV and SNCV).



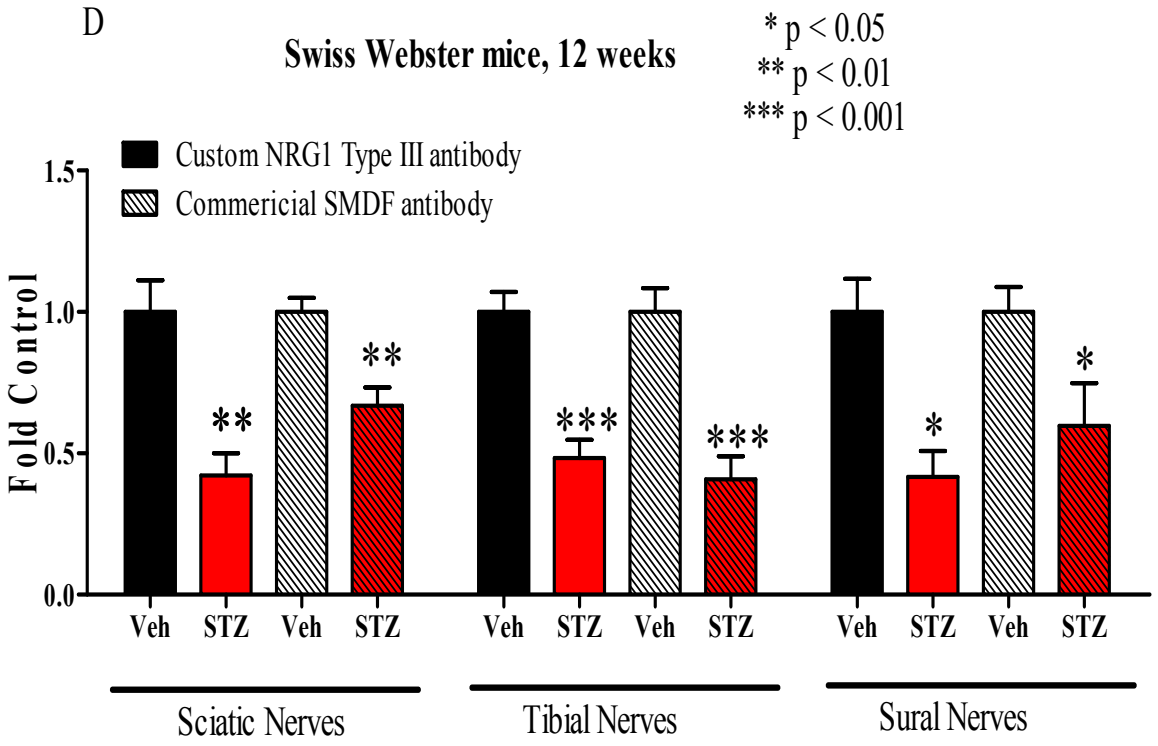
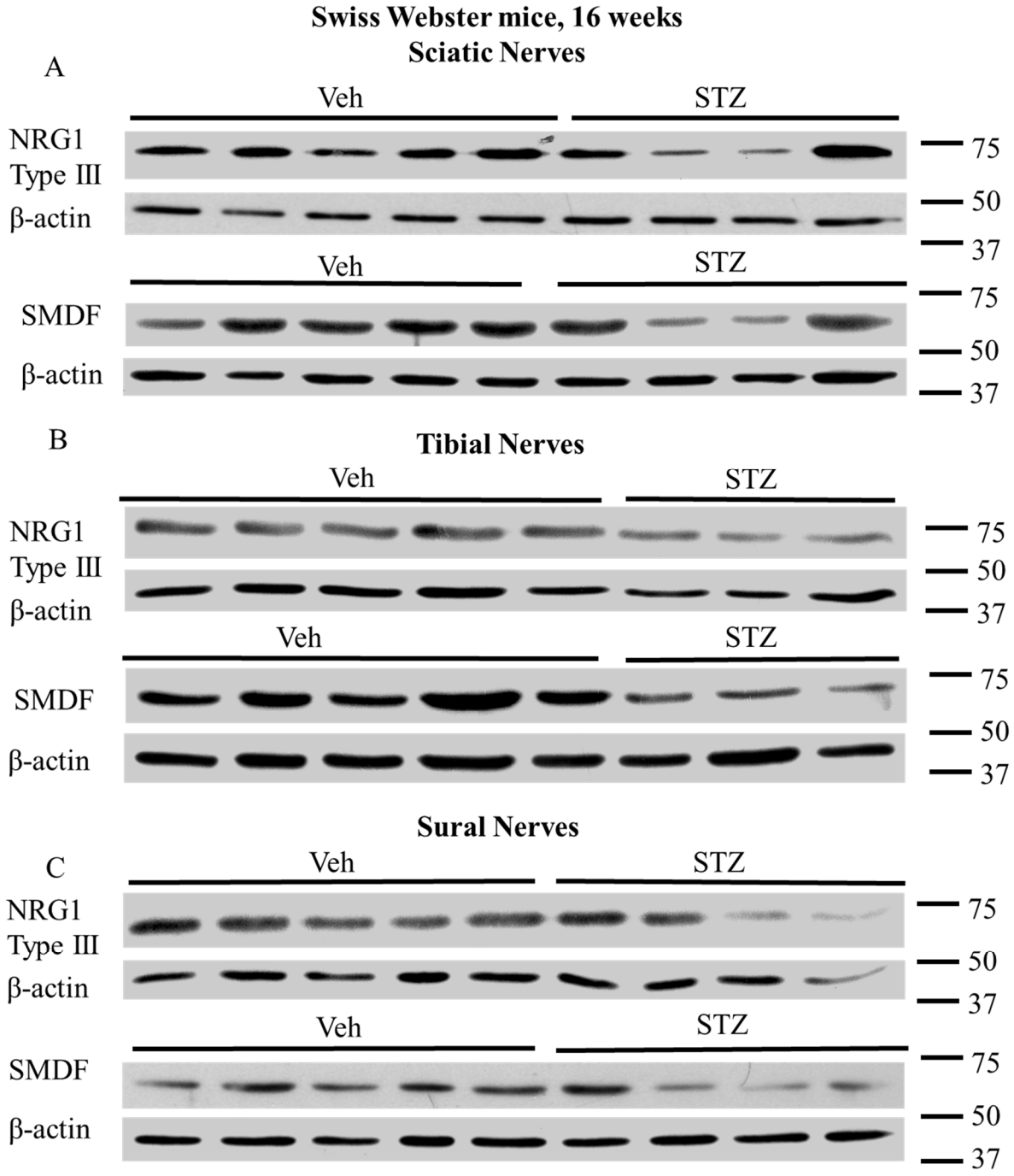


Figure 3.4.1.3: NRG1 Type III levels were decreased in sciatic nerves, tibial nerves and sural nerves after 12 weeks of diabetes in Swiss Webster mice. Swiss Webster mice were rendered diabetic for 12 weeks. Sciatic nerves **(A)**, tibial nerves **(B)** and sural nerves **(C)** were isolated (n = 5-7 per group). Protein lysates were prepared and NRG1 Type III levels were determined by immunoblot analysis using the two antibodies. **(D):** Bands were quantified, NRG1 Type III levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for STZ vs. Veh).


Swiss Webster mice, 16 weeks
Sciatic Nerves
A
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37
B
Tibial Nerves
Veh
STZ
NRG1
Type III
β-actin
75
50
37
Veh
STZ
SMDF
β-actin
75
50
37
Sural Nerves
C
Veh
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75
50
37
Veh
STZ
SMDF
β-actin
75
50
37

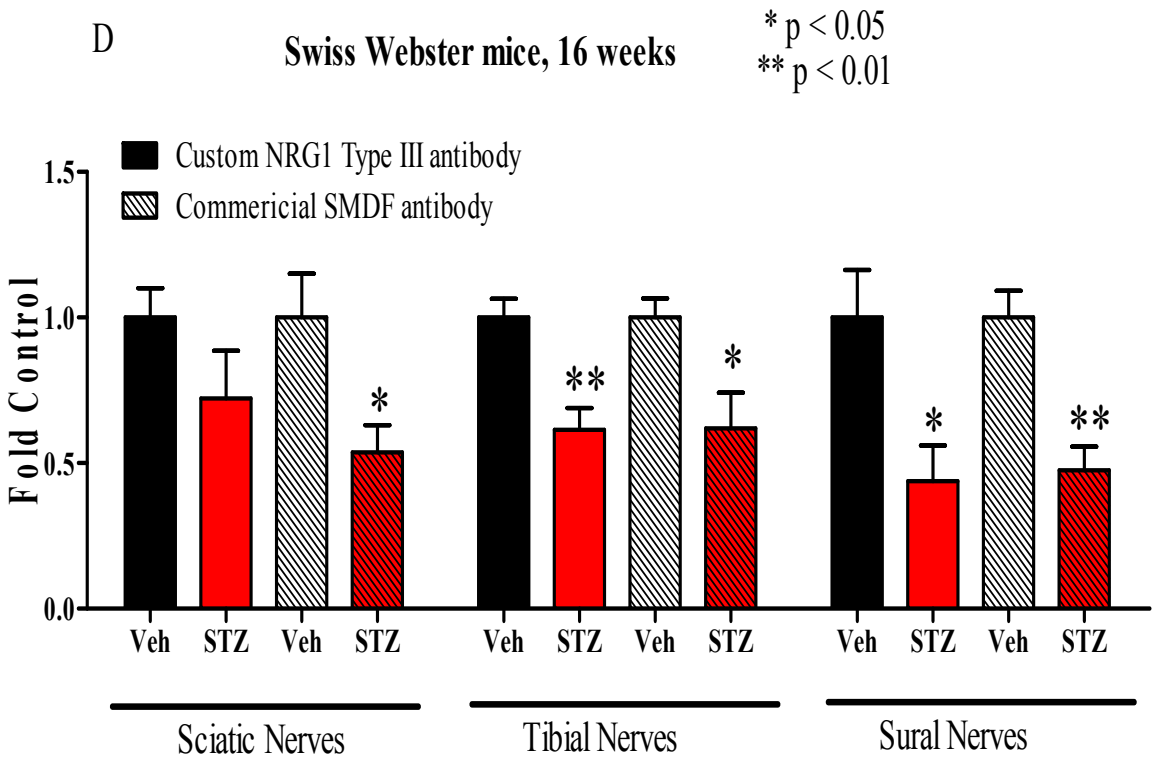


Figure 3.4.1.4: NRG1 Type III levels were decreased in sciatic nerves, tibial nerves and sural nerves after 16 weeks of diabetes in Swiss Webster mice. Swiss Webster mice were rendered diabetic for 16 weeks. Sciatic nerves (**A**), tibial nerves (**B**) and sural nerves (**C**) were isolated (n = 7-9 per group). Protein lysates were prepared and NRG1 Type III levels were determined by immunoblot analysis using the two antibodies. (**D**): Bands were quantified, NRG1 Type III levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$; **, $p < 0.01$ for STZ vs. Veh).

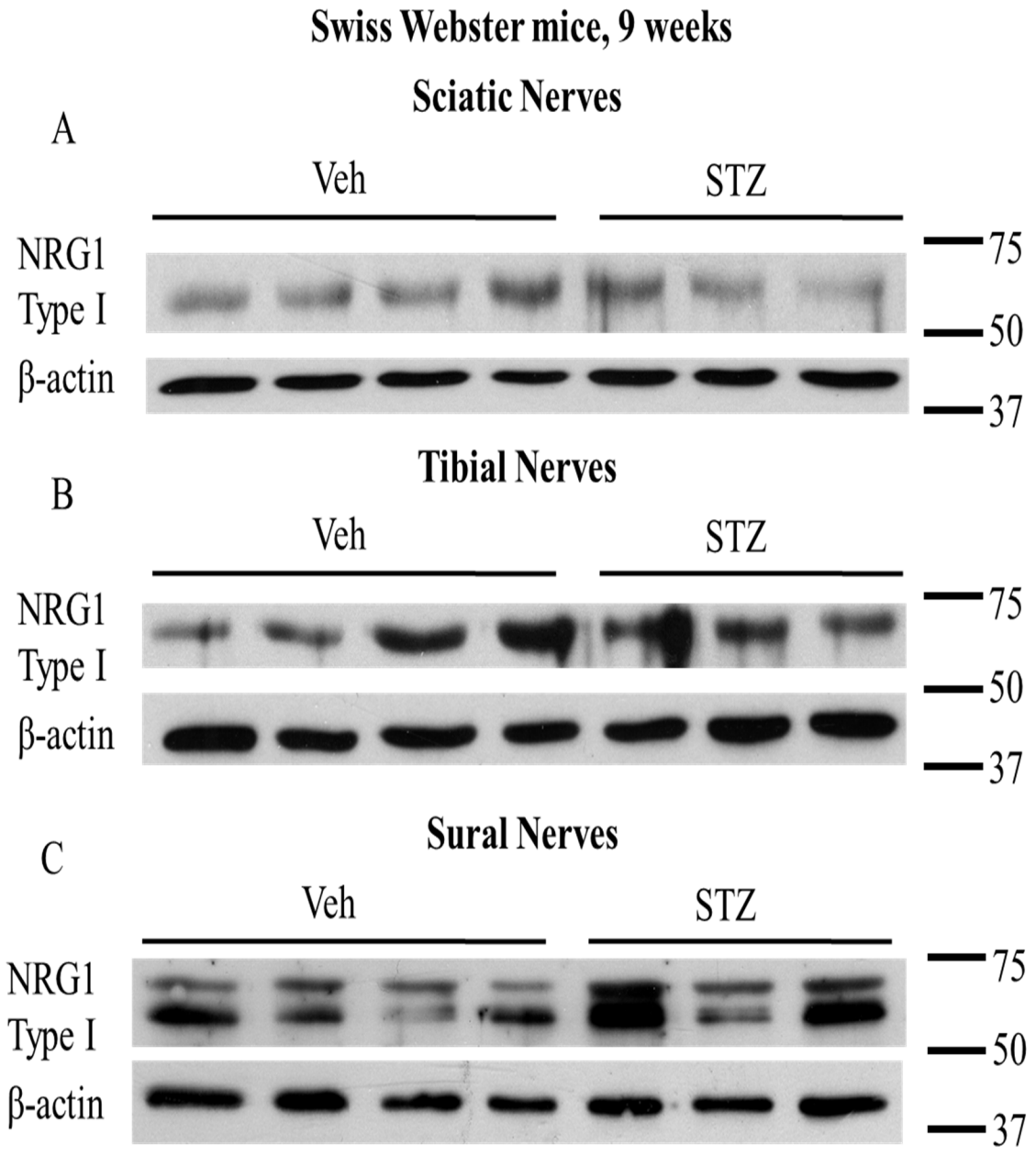
3.4.2. Expression Levels of NRG1 Type I were Increased in Diabetic Peripheral Nerves

Though a significant decrease in NRG1 Type III levels in diabetic peripheral nerves were observed, a lower ligand expression (NRG1 Type III in this case) could not be the mechanism underlying enhanced activation of Erb B2. Therefore, we evaluated the changes of another isoform in the NRG1 family, NRG1 Type I. The expression levels were detected by an Abcam

NRG1 Type I antibody against the peptide sequence near N-terminus of NRG1 Type I, which differs from N-terminal fragments of NRG1 Type III.

Nine weeks of diabetes induced a moderate but significant increase (1.5-fold) in NRG1 Type I expression levels in sural nerves (**Figure 3.4.2.1**), but not in sciatic or tibial nerves. With disease progression, the expression of NRG1 Type I further increased (2.5-fold) in sural nerves after 12 weeks of diabetes (**Figure 3.4.2.2**). In addition, a 2.3-fold increase of NRG1 Type I was detected in diabetic tibial nerves after 12 weeks of diabetes, but no significant change was observed in diabetic sciatic nerves.

Though we did not detect significant changes of NRG1 Type I expression after 16 weeks of diabetes, the transient increase in NRG1 Type I expression levels after 9 weeks and 12 weeks of diabetes might be one of the underlying mechanisms to over-activate Erb B2 receptors in diabetic nerves. In addition, both diabetic sural nerves and tibial nerves have a significant induction in NRG1 Type I expression, but not in diabetic sciatic nerves. It is possible that thinly-myelinated and unmyelinated nerve fibers (sural and tibial nerves) are more vulnerable to diabetes-induced changes of NRG1 Type I expression levels. Given the demyelinating functions of NRG1 Type I *in vitro*, enhanced NRG1 Type I expression might contribute to diabetes-induced glial dedifferentiation and eventually demyelination *in vivo*, though normal myelin thickness of sciatic nerves was observed in NRG1 Type I conditional overexpressing mice (under the control of Thy 1.2 promoter, which is active in postnatal motoneurons and DRG neurons).


Swiss Webster mice, 9 weeks
Sciatic Nerves
A
Veh
STZ
NRG1
Type I
β-actin
75
50
37
Tibial Nerves
B
Veh
STZ
NRG1
Type I
β-actin
75
50
37
Sural Nerves
C
Veh
STZ
NRG1
Type I
β-actin
75
50
37

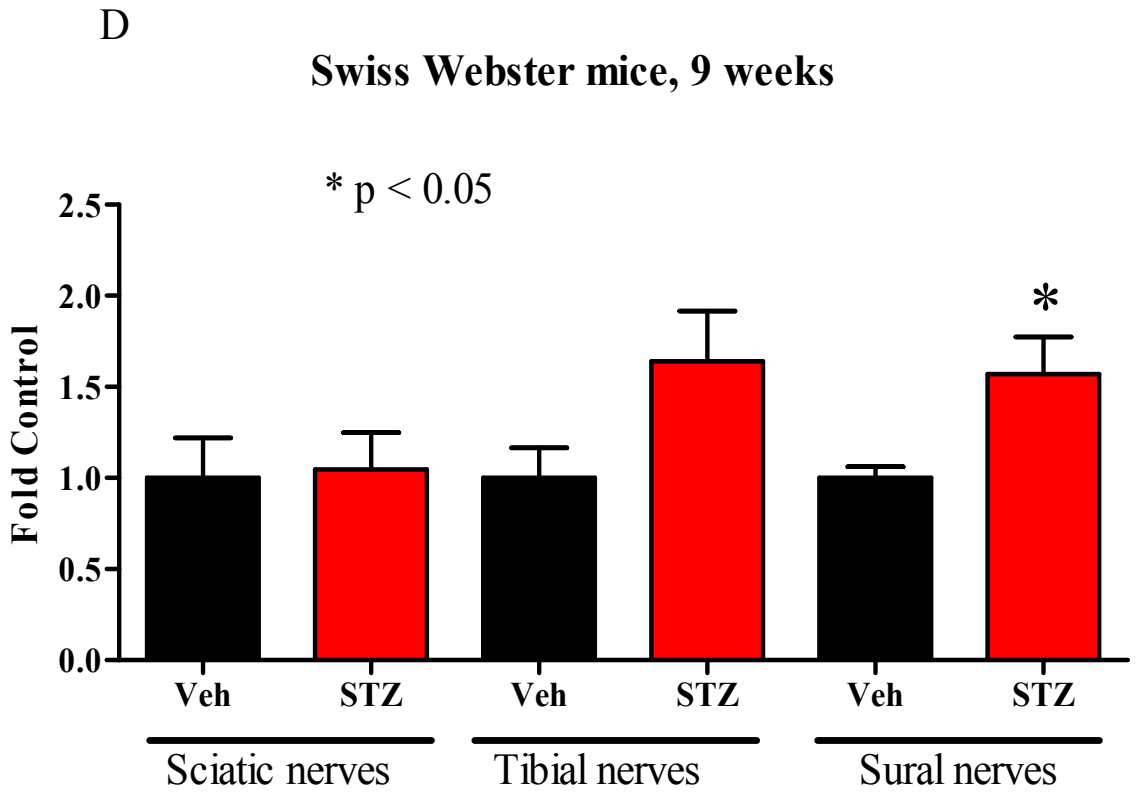
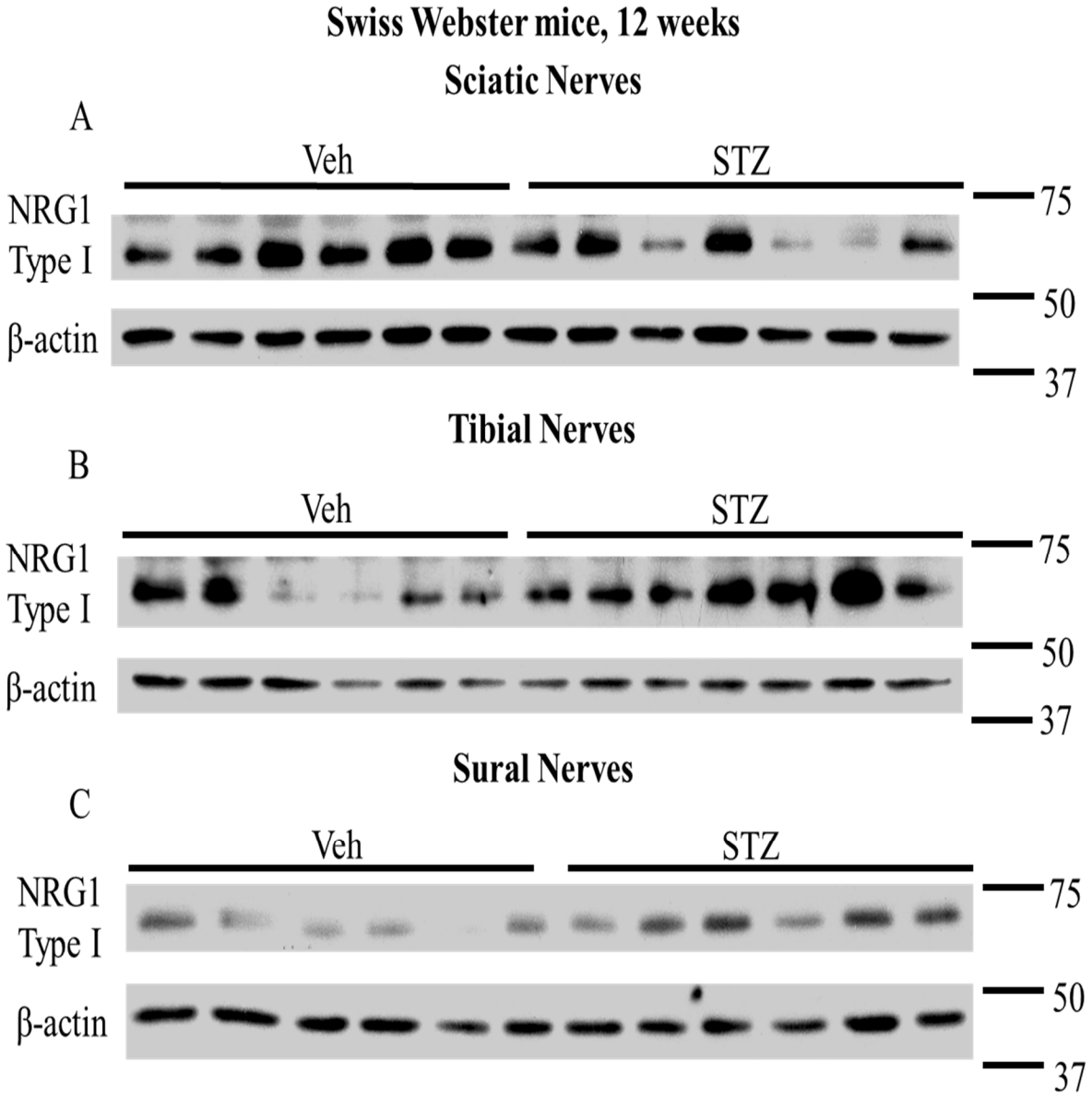


Figure 3.4.2.1: NRG1 Type I levels were increased in sural nerves after 9 weeks of diabetes in Swiss Webster mice. Swiss Webster mice were rendered diabetic for 9 weeks. Sciatic nerves (**A**), tibial nerves **(B)** and sural nerves **(C)** were isolated (n = 6-7 per group). Protein lysates were prepared and NRG1 Type I levels were determined by immunoblot analysis. (**D**): Bands were quantified, NRG1 Type I levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$ for STZ vs. Veh).


Swiss Webster mice, 12 weeks
Sciatic Nerves
A
Veh
STZ
NRG1
Type I
β-actin
75
50
37
Tibial Nerves
B
Veh
STZ
NRG1
Type I
β-actin
75
50
37
Sural Nerves
C
Veh
STZ
NRG1
Type I
β-actin
75
50
37

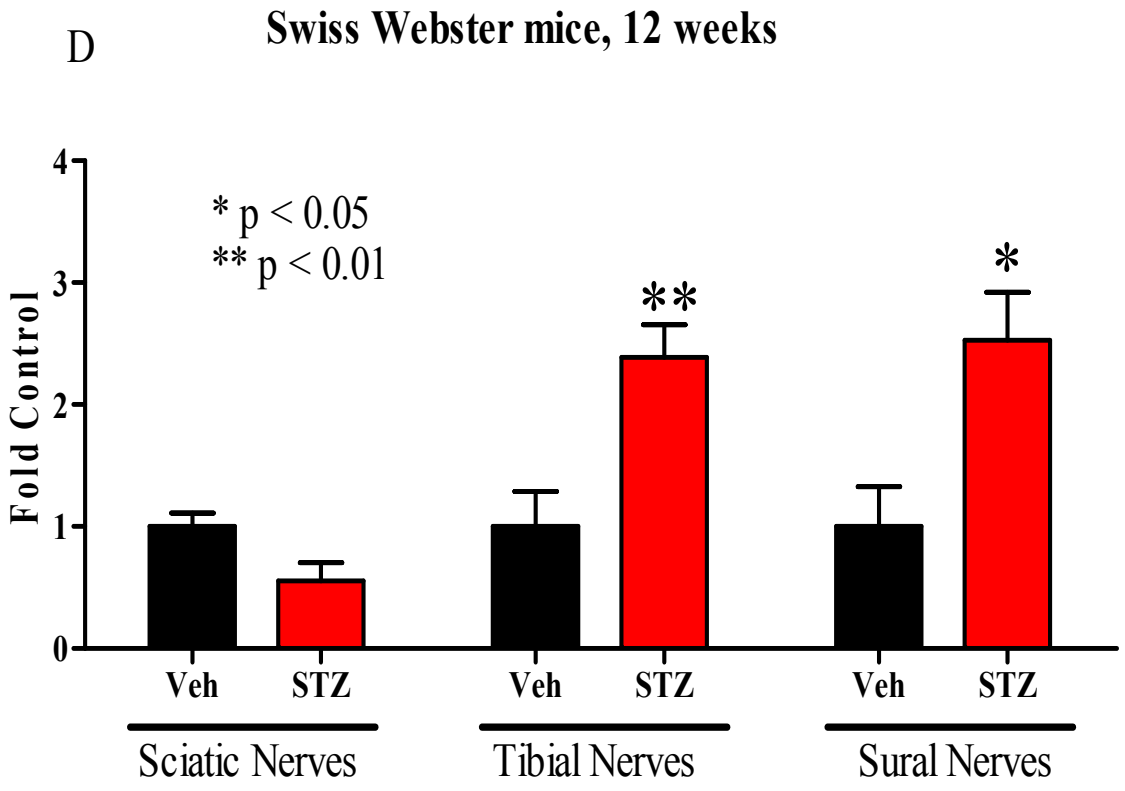


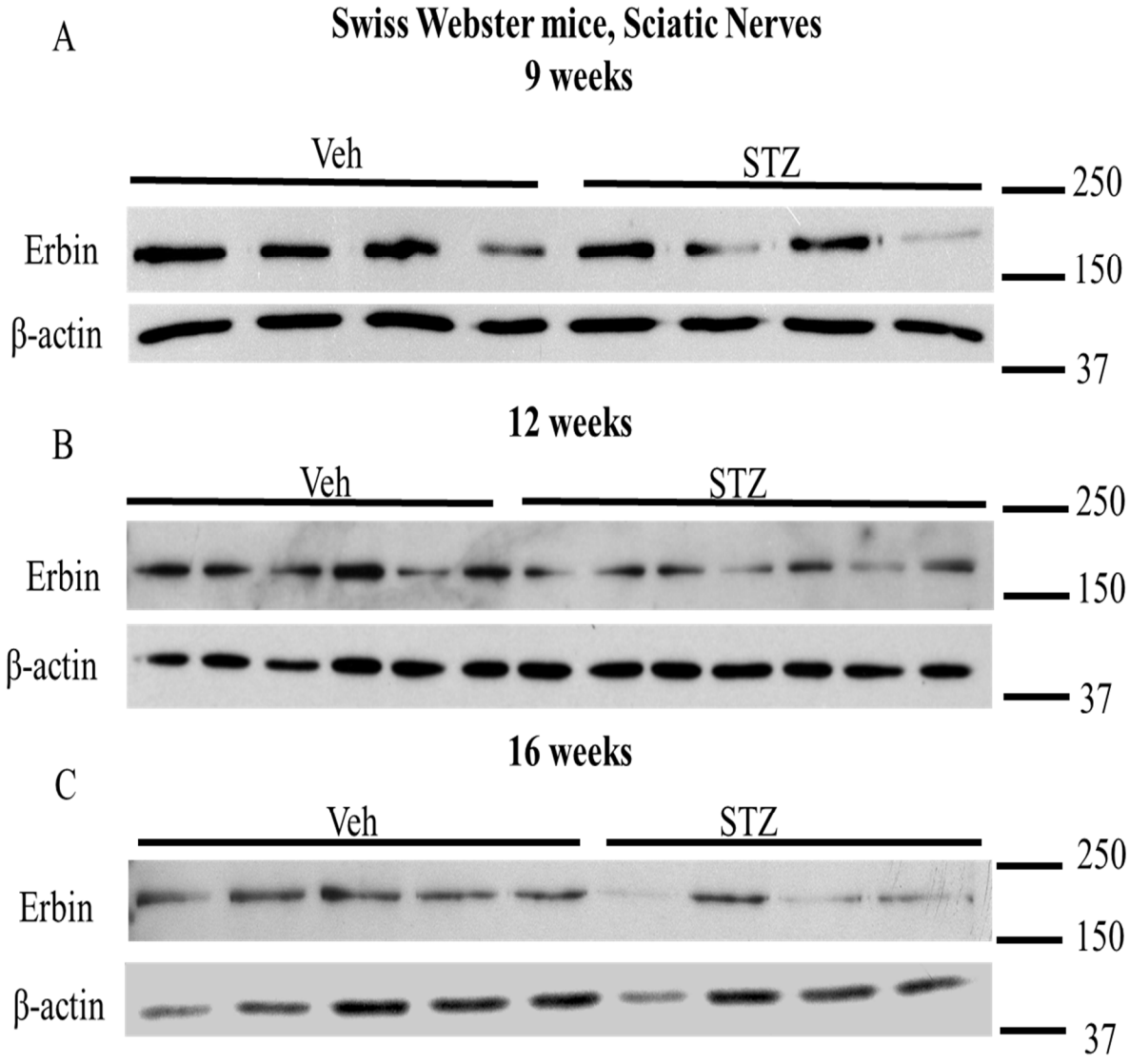
Figure 3.4.2.2: NRG1 Type I levels were increased in sural nerves and tibial nerves after 12 weeks of diabetes in Swiss Webster mice. Swiss Webster mice were rendered diabetic for 12 weeks. Sciatic nerves (**A**), tibial nerves **(B)** and sural nerves (**C**) were isolated (n = 6-7 per group). Protein lysates were prepared and NRG1 Type I levels were determined by immunoblot analysis. (**D**): Bands were quantified, NRG1 Type I levels were normalized to β-actin and expressed as a fold of the levels in control nerves (*, $p < 0.05$; **, $p < 0.01$ for STZ vs. Veh).

Together with the observation of decreased NRG1 Type III in diabetic peripheral nerves, these data suggested that diabetes could differentially alter the expression of NRG1 isoforms by decreasing the pro-myelination isoform (NRG1 Type III) and increasing a demyelinating isoform (NRG1 Type I). This disrupted balance between NRG1 Type III and NRG1 Type I in both myelinated and unmyelinated nerve fibers could contribute to the neuropathic symptoms (sensory deficits, nerve dysfunction and loss of fiber innervation) observed in diabetic Swiss Webster mice. However, after 21 weeks of diabetes with 8 weeks of erlotinib treatments, we did

not detect any changes in expression levels of NRG1 isoforms either by chronic hyperglycemia or erlotinib. Whether this lack of change is attributed to potential metabolic mechanisms or compensation from the growth factor interaction remains unclear.

3.5. Diabetes Decreases Erbin Expression and Increases p42/p44 MAPK Activity

Given the negative regulation of erbin on myelination and Erb B2 receptor activation, we hypothesized that a decrease in erbin expression may contribute to the potential overactivation of Erb B2 signaling and demyelination in diabetic mice. Erbin is an Erb B2-interacting protein that can function as a negative regulator of receptor signaling, in part by inhibiting p42/p44 MAPK activity [288]. Thus, a decrease in erbin expression may also contribute to potential dysregulation of Erb B2 signaling in diabetic nerve. Diabetic sciatic nerve showed a decreased expression of erbin after 12 and 16 weeks of diabetes (**Figure 3.5.1**).


A
Swiss Webster mice, Sciatic Nerves
9 weeks
Veh
STZ
Erbin
β-actin
250
150
37
B
12 weeks
Veh
STZ
Erbin
β-actin
250
150
37
C
16 weeks
Veh
STZ
Erbin
β-actin
250
150
37

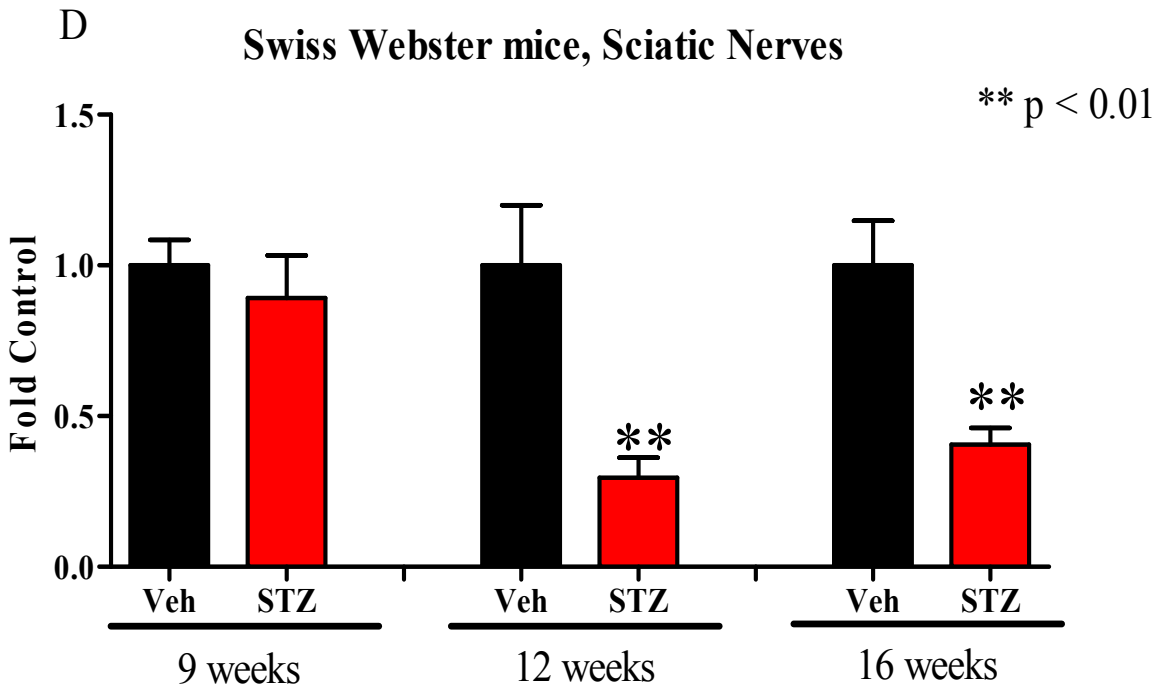
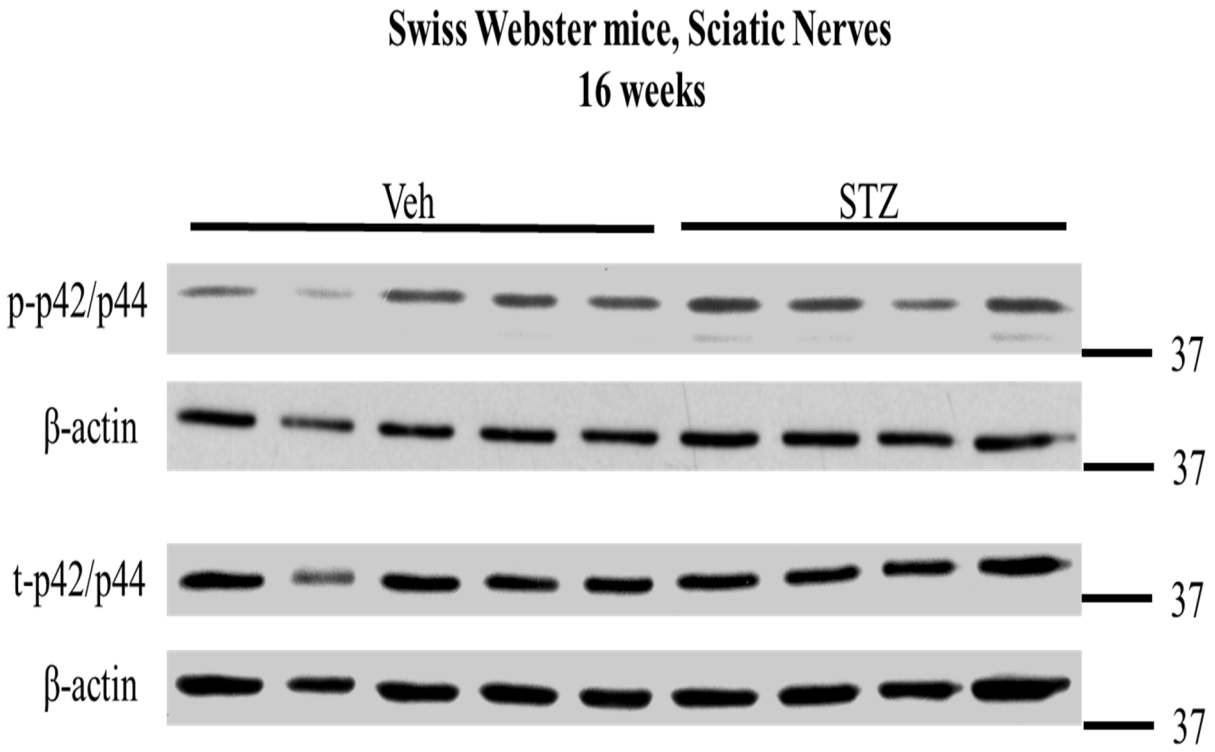


Figure 3.5.1: Erbin levels were decreased in sciatic nerves in diabetic Swiss Webster mice. Swiss Webster mice were rendered diabetic. Sciatic nerves were isolated after 9 weeks (**A**), 12 weeks (**B**) and 16 weeks (**C**) of diabetes (n = 6-9 per group). Protein lysates were prepared and Erbin levels were determined by immunoblot analysis. (**D**): Bands were quantified, Erbin levels were normalized to β-actin and expressed as a fold of the levels in control nerves (**, $p < 0.01$ for STZ vs. Veh).

Considering the inhibitory effects of erbin on p42/p44 MAPK (ERK) pathway, we measured p42/p44 MAPK activity by immunoblot analysis. Phospho-p42/p44 MAPK and total-p42/p44 MAPK levels were normalized to corresponding β-actin levels. Then the p42/p44 MAPK activities were determined by the ratio of phospho-p42/p44 MAPK divided by total-p42/p44 MAPK expression. Enhanced p42/p44 MAPK activities were only detected in sciatic nerves after 16 weeks of diabetes (**Figure 3.5.2**).



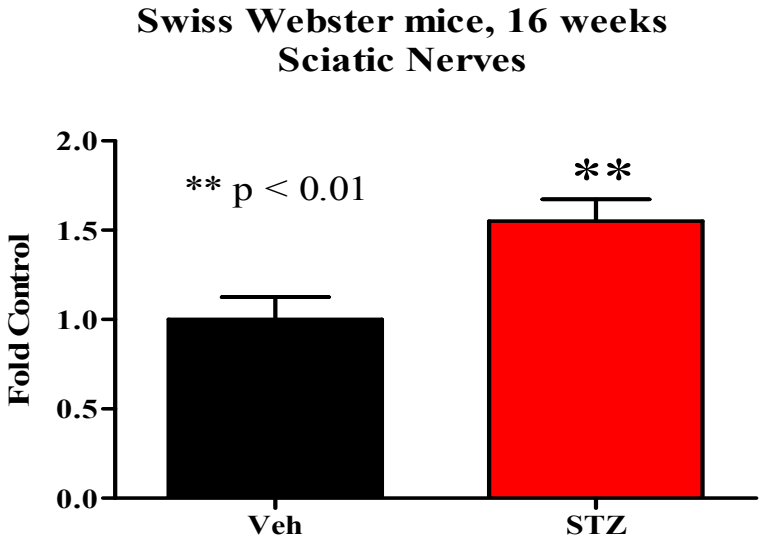
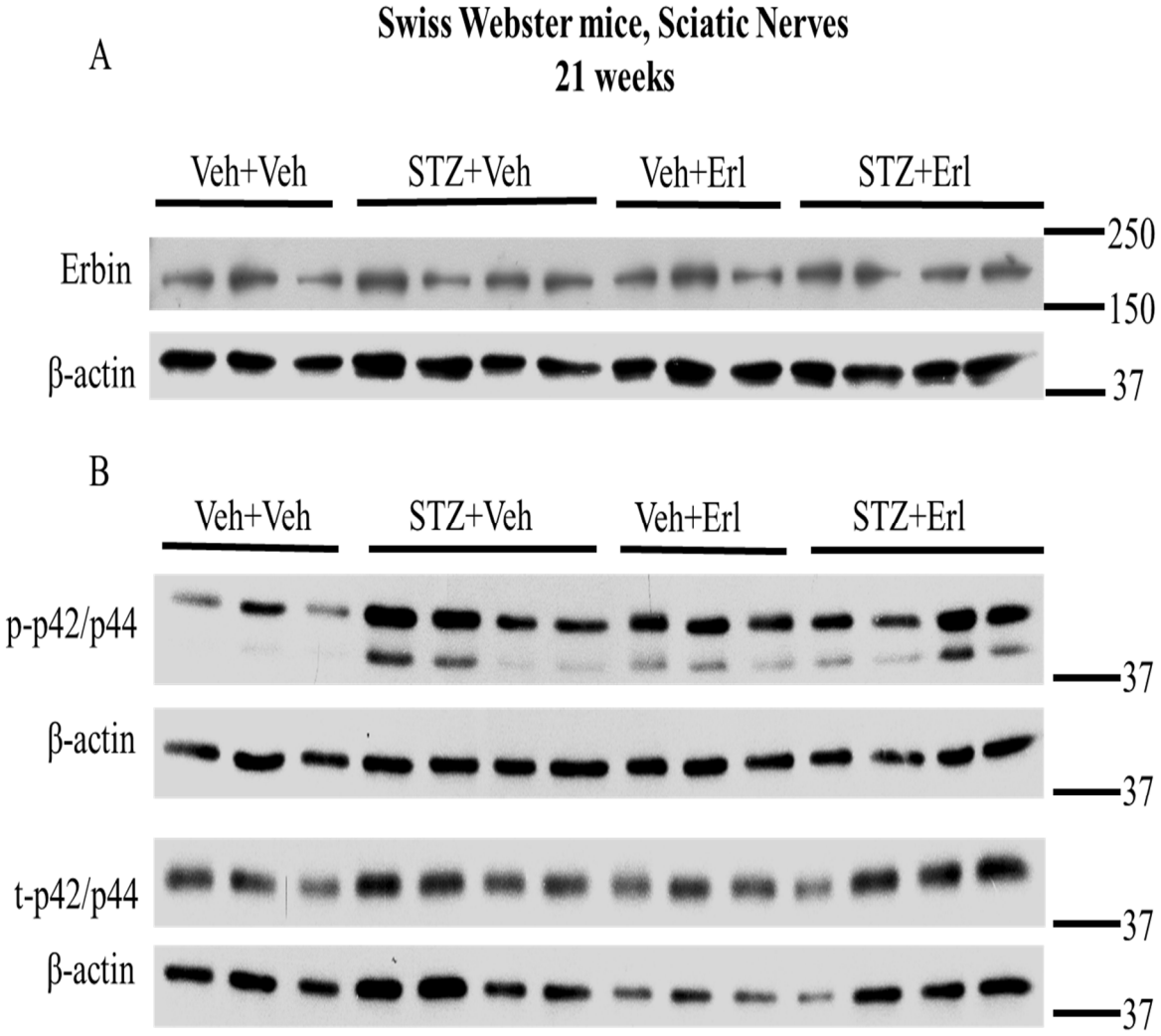


Figure 3.5.2: p42/p44 MAPK activities were enhanced in sciatic nerves after 16 weeks of diabetes. Sciatic nerves were isolated control and diabetic mice (n = 8-9 per group). Protein lysates were prepared and p42/p44 MAPK activities were determined by immunoblot. Quantification demonstrated a significant increase in p42/p44 MAPK activation at 16 weeks (**, $p < 0.01$ for STZ vs. Veh).

With disease progression, we also observed a significant increase in p42/p44 MAPK activities in both sciatic (**Figure 3.5.3 B**) and sural nerves (**Figure 3.5.4 B**), whereas erbin levels were decreased only in sural nerves (**Figure 3.5.4 A**) after 21 weeks of diabetes. In addition, 8 weeks of erlotinib treatment completely suppressed the extent of p42/p44 MAPK phosphorylation in diabetic sural nerve (**Figure 3.5.4 B**), but this did not reach significance in diabetic sciatic nerve (**Figure 3.5.3 B**). Longer periods and a higher or more frequent dose of erlotinib treatment might extend the inhibitory effects p42/p44 MAPK activity in sciatic nerve and induce greater recovery. However, in spite of the complete suppression on p42/p44 MAPK activation by erlotinib treatment, this was associated with only a partial recovery in the physiologic measures of DPN. Thus, monotherapy targeting the MAPK pathway may not be sufficient to fully reverse the pathophysiological sensory deficits in DPN patients.


A
Swiss Webster mice, Sciatic Nerves
21 weeks
Veh+Veh
STZ+Veh
Veh+Erl
STZ+Erl
Erbin
β-actin
250
150
37
B
Veh+Veh
STZ+Veh
Veh+Erl
STZ+Erl
p-p42/p44
β-actin
t-p42/p44
β-actin
37
37
37
37

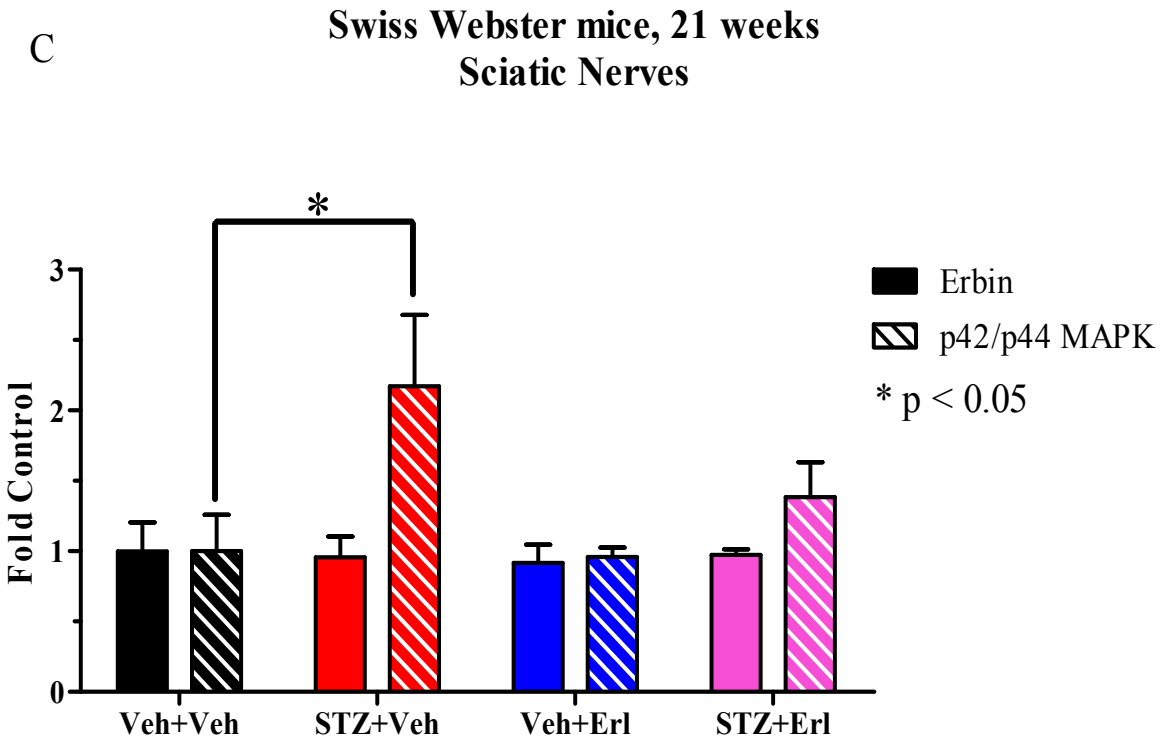
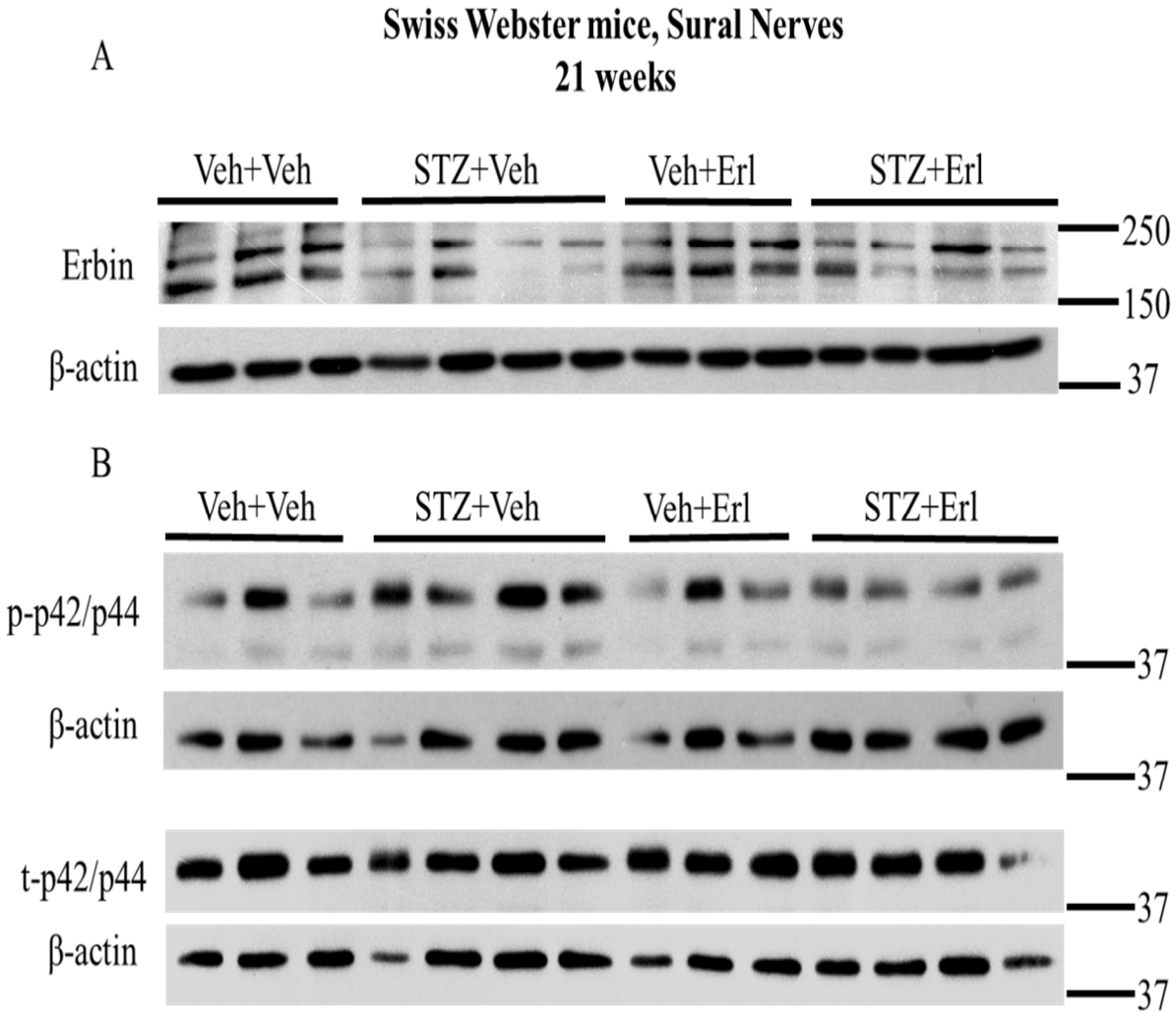


Figure 3.5.3: Diabetes induced p42/p44 MAPK pathway activation in sciatic nerves. Swiss Webster mice were rendered diabetic. Sciatic nerves were isolated from vehicle or erlotinib-treated control and diabetic mice (n = 3-4 per group) after 21 weeks of diabetes with 8 weeks of erlotinib treatments. Protein lysates were prepared and erbin levels **(A)** and p42/p44 MAPK levels **(B)** were determined by immunoblot. **(C):** Quantification demonstrated an increase in p42/p44 MAPK activation in vehicle-treated diabetic mice (*, $p < 0.05$).


Swiss Webster mice, Sural Nerves
21 weeks
A
Veh+Veh
STZ+Veh
Veh+Erl
STZ+Erl
Erbin
β-actin
250
150
37
B
Veh+Veh
STZ+Veh
Veh+Erl
STZ+Erl
p-p42/p44
β-actin
t-p42/p44
β-actin
37
37
37
37

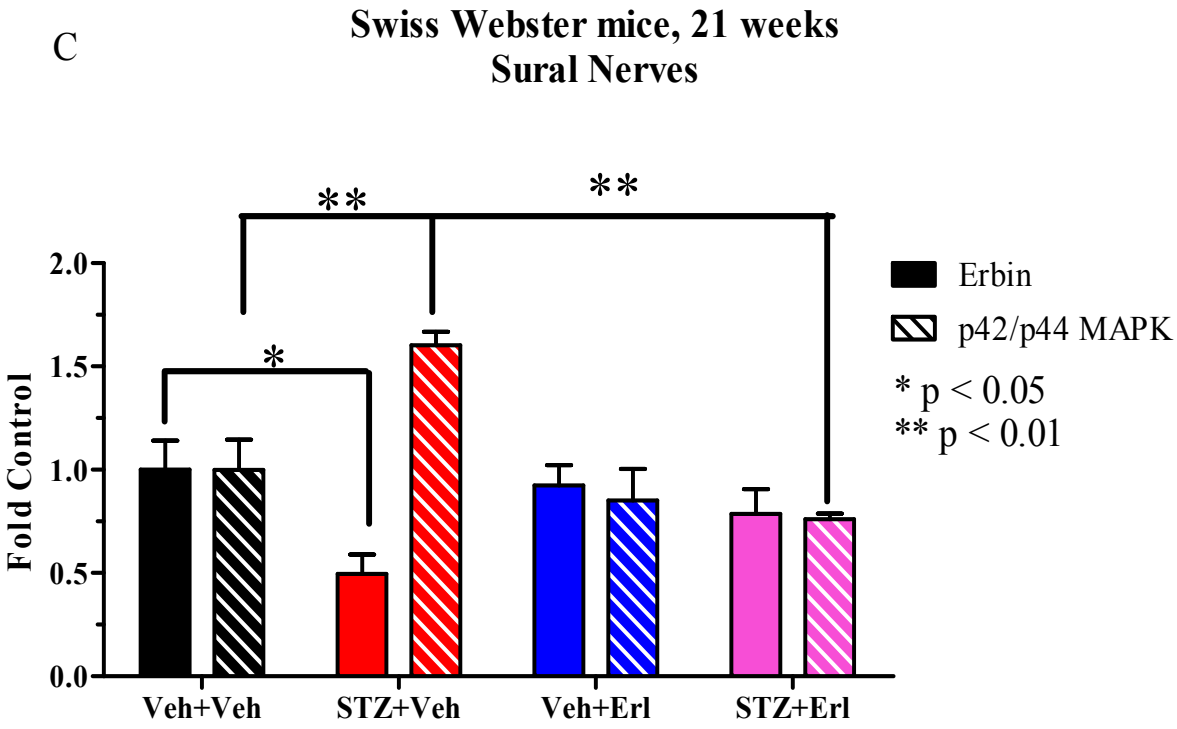


Figure 3.5.4: Inhibition of Erb B2 with Erlotinib suppressed diabetes-induced p42/p44 MAPK pathway activation in sural nerves. Swiss Webster mice were rendered diabetic. Sural nerves were isolated from vehicle or erlotinib-treated control and diabetic mice (n = 3–4 per group) after 21 weeks of diabetes with 8 weeks of erlotinib treatments. Protein lysates were prepared and erbin levels **(A)** and p42/p44 MAPK levels **(B)** were determined by immunoblot. **(C):** Quantification demonstrated a significant decrease of Erbin and an increase in p42/p44 MAPK activation in vehicle-treated diabetic mice. Erlotinib treatment suppressed p42/p44 MAPK activation in Erlotinib-treated diabetic mice (*, $p < 0.05$; **, $p < 0.01$).

Chapter 4: Discussion

4.1. Activation of NRG1-Erb B2 Signaling in Diabetic Peripheral Neuropathy

Though it has been established that NRG1-Erb B signaling plays an essential role in the development of the PNS and the regeneration after nerve injury, little is known about whether diabetes may alter NRG1-Erb B signaling in peripheral nerve. We were the first to report that a transient aberrant activation of Erb B2 receptors is one of the contributing factors to the development of DPN, since 3-week treatments of Erb B2 receptor inhibitor erlotinib fully reversed the mechanical hypoalgesia and MNCV deficits after 12 weeks of diabetes in C57BL/6 mice [143]. The current study used outbred Swiss Webster mice that develop more robust diabetic neuropathy, especially in myelinated nerve fibers. Though we only observed a partial recovery in mechanical hypoalgesia and MNCV deficits after 8 weeks of erlotinib treatments, it is important to note that we detected a significant improvement in thermal sensitivity and SNCV, which has not been reported in the previous study [143]. In addition, erlotinib treatments induced a greater recovery in thermal sensitivity and SNCV (70%) compared to that in mechanical sensitivity (56%) and MNCV (39%). Whether this observation is attributable to differences between the mouse strains is unclear. Nonetheless, these data demonstrate that NRG1-Erb B2 signaling activation may contribute to Schwann cell dysfunction in DPN.

More importantly, the current study is the first to demonstrate that diabetes can alter the expression of NRG1 isoforms in peripheral nerves. While we observed a reduction in NRG1 Type III levels in diabetic sciatic, tibial and sural nerves, the level of NRG1 Type I was increased in tibial and sural nerves after 9-12 weeks of diabetes. Since inhibition of Erb B2 signaling with erlotinib partially reversed the sensory deficits associated with prolonged diabetes,

the elevated expression of NRG1 Type I in diabetic nerves might be sufficient to induce Erb B2 receptor activation and contribute to the progression of DPN. Moreover, the decreased NRG1 Type III and increased NRG1 Type I expression observed in diabetic tibial and sural nerves (but not in diabetic sciatic nerves) implies that distal and more thinly myelinated fibers may be more susceptible to early disruption of NRG1 signaling. This would be consistent with recent results showing a greater severity of oxidative stress in distal sural nerve compared to sciatic nerve [302].

NRG1 Type III is a membrane-anchored precursor protein which needs to be proteolytically processed to become an active signaling molecule. The β-secretase BACE1 and the α-secretase TACE are two enzymes that process NRG1. NRG1 Type III processed by BACE1 produces a membrane-associated, N-terminal fragment that promotes myelination [269], whereas TACE cleaves NRG1 Type III within the EGF-like domain and inactivates it, resulting in hypomyelination [274]. BACE cleavage also produces the C-terminal fragment which can be degraded by γ-secretase. Though the C-terminal fragment is not necessary for myelination, it may be translocated to the nucleus of neurons and can repress apoptosis and promote survival [275]. Diabetes-induced changes in the expression of NRG1 Type III produced by BACE cleavage were verified using two antibodies which targeted either the CRD located within the ~75 kDa N-terminal fragment or an epitope within the C-terminal fragment (discussed in **Materials and Methods**). Interestingly, we did not consistently observe the presence of unprocessed NRG1 Type III in nerves from either control or diabetic mice. This suggests that the decrease in the expression of the N- and C-terminal fragments of NRG1 Type III were not due to a decrease in BACE levels or dysfunction of proteolytic processing. Since transcriptional and/or translational modifications play a critical role of regulating the protein expression of

neurotrophins, it may also occur to NRGs and changes in the rate of transcription and/or translation could contribute to the decrease in NRG1 Type III. However, we also observed an increase in NRG1 Type I in diabetic nerves. Since both isoforms are the product of alternative splicing of a single transcript, it is possible that diabetes-induced changes in mRNA processing may alter the isoform expression pattern. In this regard, diabetes has been shown to alter expression of transcriptional variants of the *Slo* gene that may contribute to erectile dysfunction [303]. However, diabetes may have tissue specific effects on the expression of NRG1 isoforms. For example, the levels of NRG1 Type I were decreased in diabetic rats with cardiomyopathy [304] and impaired NRG1-Erb B signaling may contribute to the pathogenesis of diabetic cardiomyopathy, increasing susceptibility to heart failure [305].

The degeneration of sensory neurons in DPN is clearly associated with an alteration in neurotrophic support and disrupted NRG1-Erb B2 signaling, presumably in Schwann cells, may be interconnected with altered neurotrophism. BDNF is released from Schwann cells and its expression is decreased in diabetic rats [227]. Treatment with BDNF prevented nerve conduction slowing and damage to large motor fibers [228]. A clear relationship exists between BDNF and NRG1 signaling since BDNF can stimulate the secretion of soluble forms of NRG1 [239], whereas transgenic blockade of endogenous Erb B2 via expression of a DN-Erb B4 in non-myelinating Schwann cells was sufficient to decrease the expression of BDNF [250]. In addition, NT-3 and ciliary neurotrophic factor (CNTF) stimulate proliferation of DRG progenitor cells through inducing NRG1 secretion in a dose-dependent manner [306]. These data indicate that a tight relationship exists between neurotrophins and NRGs. Though it remains unclear whether changes in neurotrophin levels may have contributed to the altered expression of NRG1 isoforms observed in diabetic nerves in the current study, elucidating the effect of diabetes on the

activity of neurotrophins and NRGs in dedifferentiating and regenerating Schwann cells may provide fundamental insight into the potential for pharmacologically regulating Erb B2 signaling at specific disease stages to improve nerve function. Lastly, recent data also suggests that axonal expression of NRG1 Type III can negatively regulate the expression of Schwann cell-derived NRG1 Type I [307]. Although these results were obtained in the context of a decrease in NRG1 Type III due to axonal loss following nerve crush, axonal loss is not a hallmark of the rather early stage of DPN modeled in our study. Thus, a diabetes-induced alteration in the expression of NRG1 Type III without frank axonal loss may be sufficient to promote the expression of NRG1 Type I. However, additional work is required to determine if the negative regulation of NRG1 Type I expression by axonal NRG1 Type III may be recapitulated following a peripheral nerve injury that is solely metabolic.

4.2. Role of Erbin in Diabetic Peripheral Neuropathy

Erbin plays an important role in myelination functions since erbin null mice have a decrease in Erb B2 levels and NRG1-induced myelination [290]. Erbin also serves as a negative regulator of p42/p44 MAPK signaling by disrupting the interaction between Ras and Raf [288, 308]. Consistent with this relationship, diabetes decreased erbin levels in sciatic and sural nerves and this correlated with an increase in the activity of p42/p44 MAPK. Although erlotinib treatment inhibited the activation of p42/p44 MAPK in sural nerves without increasing erbin expression, it is not possible to directly link this change in MAPK activity to the improved sensory endpoints following erlotinib treatment since the drug would be expected to blunt all signaling through Erb B2. Though activation of the MAPK pathway has been associated with demyelination [258, 309], myelin loss is not a hallmark of DPN in rodent models. Therefore, the activation of this pathway is either of insufficient magnitude and duration to promote demyelination in rodent

nerve or contributes to other aspects of DPN. However, the contribution of p42/p44 MAPK activity to DPN in both rodent and human DPN is unclear and these enzymes showed variable activation in sural nerve biopsies obtained from diabetic patients undergoing amputations [310].

In contrast, DRG/Schwann cell co-cultures provide a well-characterized *in vitro* model to investigate the function of erbin and downstream p42/p44 MAPK pathway during the developmental and myelinating stages. Myelinated DRG/Schwann cell co-cultures were prepared and infected with an erbin shRNA -expressing lenti-virus for 16 hrs. Cell lysates were collected 48 hrs after infection and protein lysates were separated by SDS-PAGE.

Though studies have shown that NRG1-Erb B signaling is mostly dispensable for maintenance of myelinated peripheral nerves [276], immunoblot analysis (**Figure 4.2.1**) showed that depletion of erbin in DRG/Schwann cell co-culture induced a remarkable reduction in P0 levels in a dose-dependent manner. Though erbin has been recognized as binding partner to various proteins and is involved in protein-protein interactions, whether erbin could directly bind to and regulate myelin proteins such as myelin basic protein (MBP) or P0 needs further confirmation. In addition, we previously demonstrated that hyperglycemia sensitizes the myelinated DRG/Schwann cell co-cultures to NRG1 Type I-induced demyelination [299]. However, it remains unknown whether this exacerbated demyelination is associated with hyperglycemia-induced downregulation of erbin and corresponding p42/p44 MAPK activation.

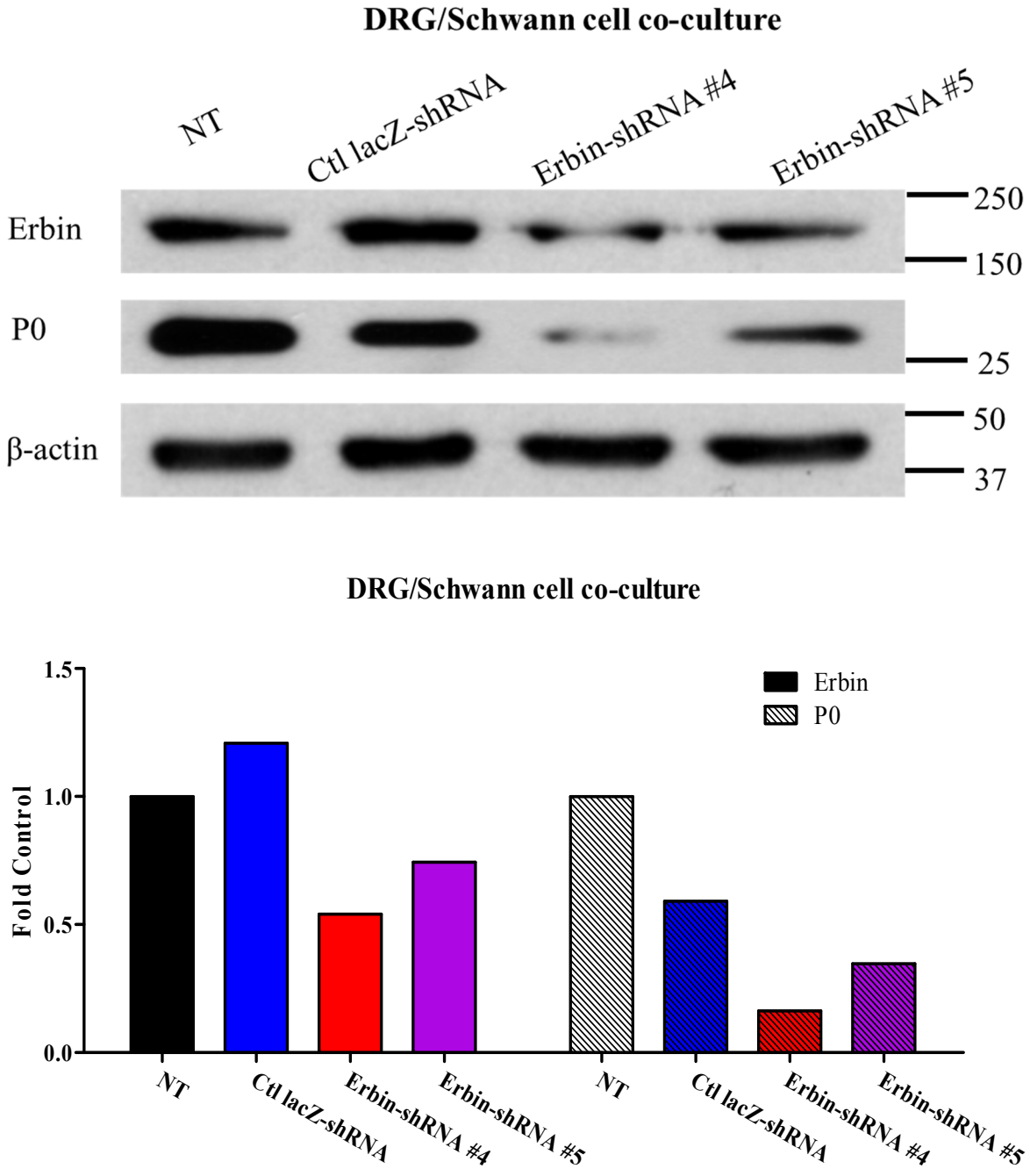


Figure 4.2.1: Erbin is necessary for maintenance of myelination in fully myelinated DRG/Schwann cell co-cultures. Mouse DRG/Schwann cell co-cultures were prepared and infected with vehicle (NT), control lacZ-shRNA and two colons of erbin-shRNA for 16 hrs. Protein lysates were collected 48 hrs after infection and were separated by SDS-PAGE. Immunoblot analysis were quantified, erbin and P0 levels were normalized to β-actin and expressed as a fold of the levels in non-treated cells

In summary, we propose that impaired NRG1-Erb B2 signaling contributed to the onset and progression of DPN. Hyperglycemia differentially altered the expression of NRG1 isoforms. While a decreased pro-myelinating NRG1 Type III expression in diabetic nerves may compromise nerve function and contribute to sensory deficits, an increased expression of demyelinating NRG1 Type I in diabetic tibial and sural nerves may be sufficient to alter the activation of Erb B2 receptor. Furthermore, erbin levels were downregulated in diabetic sciatic nerves and this corresponded with an increase in p42/p44 MAPK pathway activity, which could promote the Schwann cell dedifferentiation and demyelination. Inhibition of Erb B2 signaling with erlotinib partially reversed several pathophysiologic aspects of DPN including a pronounced sensory hypoalgesia, nerve conduction velocity deficits and the loss of epidermal nerve fiber innervation (**Figure 4.2.2**).

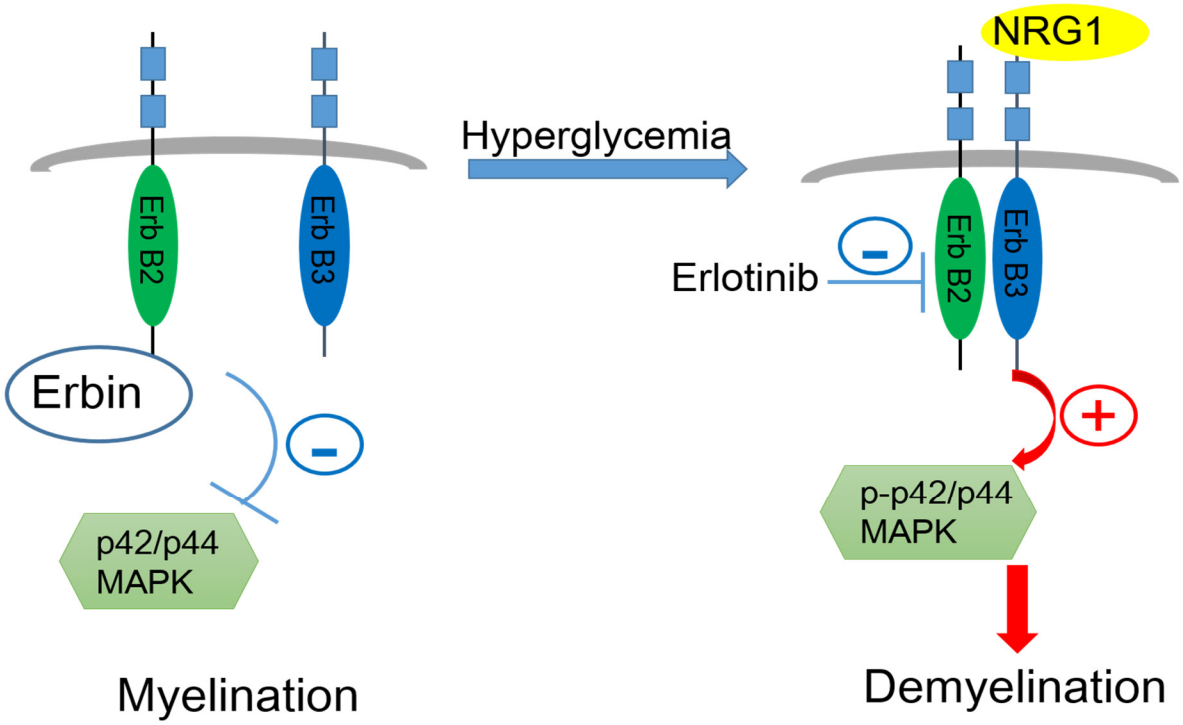


Figure 4.2.2: Proposed mechanism that is associated with the enhanced Erb B2 activation and erlotinib-mediated recovery in diabetic peripheral neuropathy. Hyperglycemia

differentially alters the expression of NRG1 isoforms and downregulates erbin levels, resulting in an increase in p42/p44 MAPK pathway activation, which could promote the Schwann cell dedifferentiation and contribute to the nerve dysfunction and demyelination. Erb B2 receptor inhibitor erlotinib reveres pathophysiologic deficits and suppressed p42/p44 MAPK activation in diabetic peripheral neuropathy.

4.3. Mitochondrial Dysfunction and NRG1-Erb B2 Signaling

Mitochondria is the key organelle regulating cellular energy production to adapt to fluctuating APT demands. In diabetes, excessive glucose is metabolized through glycolysis, the TCA cycle and oxidative phosphorylation, producing an over-abundance of ROS. In particular, glucose uptake in neurons is mediated primarily by GLUT-3, is insulin-independent and a high extracellular glucose concentration will distribute glucose equally across the plasma membrane driven via equilibrative transport. In addition, hyperglycemia-driven over activation of cellular signaling pathways such as AGE formation, enhanced polyol pathway and increased PKC activation could further contribute to ROS generation. Accumulated ROS can impair mitochondrial function by damaging mitochondrial DNA, proteins and membrane potential. Many studies have shown that the mitochondrial inner membrane potential of adult sensory neurons from STZ-induced diabetic animals was depolarized [150, 311], suggesting deficits in mitochondrial function. Consistent with these data, we also observed a decrease in mitochondrial bioenergetics in sensory neurons obtained from diabetic Swiss Webster mice.

With the introduction of the Seahorse Bioscience Analyzer, it is feasible to investigate neuronal bioenergetics in cultured neurons. Diabetic Swiss Webster mice showed an impaired mitochondrial respiration only after 16 weeks of diabetes (**Figure 4.3.1**) [312]. This impairment was not due to changes of mitochondrial number or mass. However, whether the late onset of impaired mitochondrial bioenergetics is associated with the enhanced MAPK activation observed

in sciatic nerves after 16 weeks of diabetes remains unclear. Nonetheless, these data suggest that reduced respiratory capacity, especially in neurons, limits their adaptive ability to meet energetic demand and renders the cells more susceptible to secondary stress such as the overproduction of ROS. The exhausted ATP supply in the distal nerve compartment could contribute to defective axon regeneration and nerve fiber re-innervation. In addition, unmyelinated axons have been found to be more energetically demanding than myelinated axons [313].

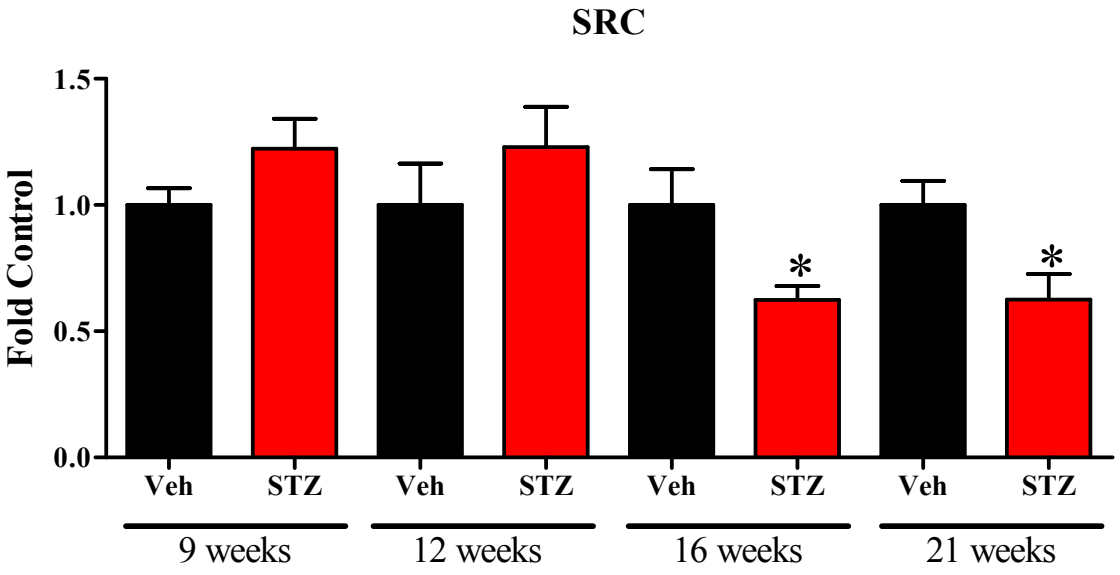


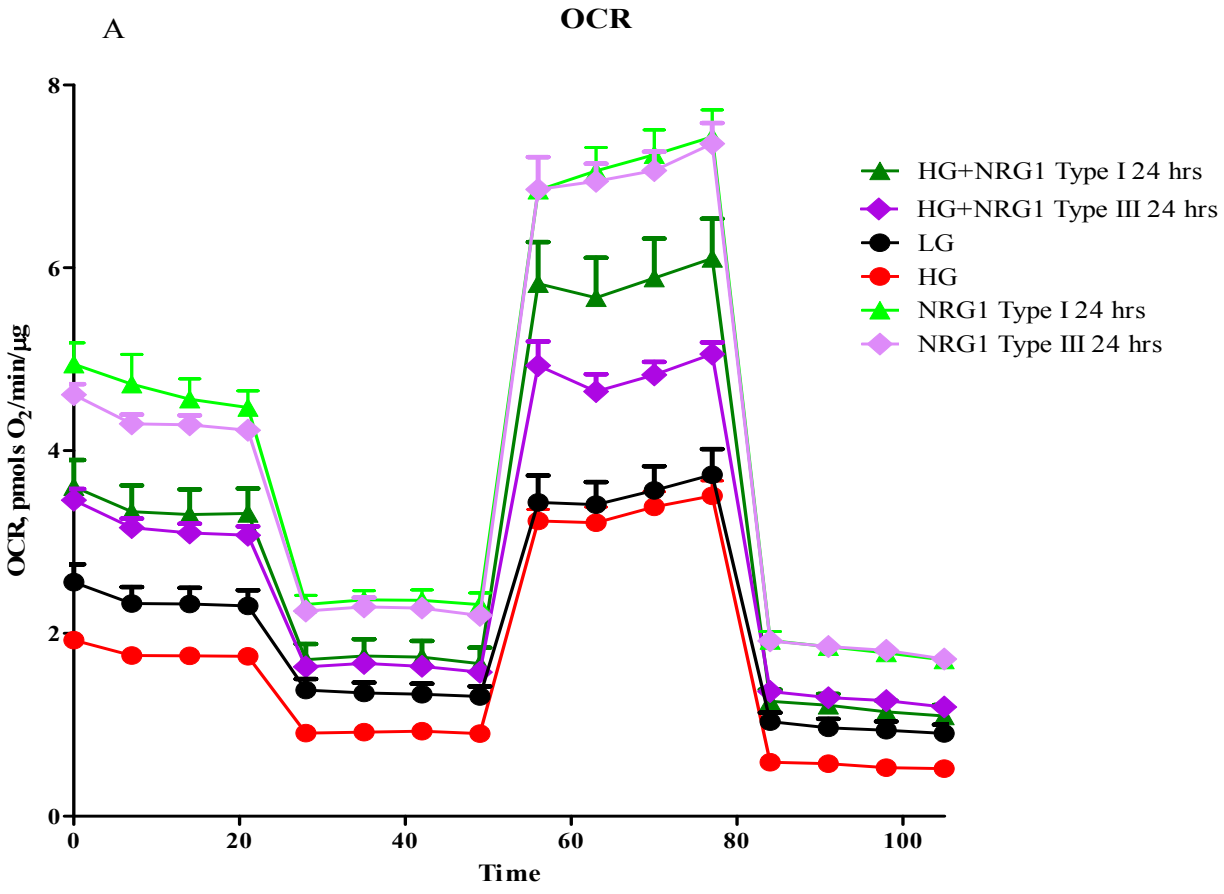
Figure 4.3.1: Diabetes induced the onset of mitochondrial bioenergetic deficits in DRG neurons. Swiss Webster mice were rendered diabetic. Adult lumber DRG neurons (L4-L6) were collected after 9, 12, 16 and 21 weeks of diabetes. Spare respiratory capacity (SRC) was measured by an XF96 Extracellular Flux Analyzer. After 16 weeks of diabetes, SRC was significantly decreased in diabetic DRG neurons compared to time-matched non-diabetic controls (*, $p < 0.05$ for STZ vs. Veh).

Given the tight interrelationship between Schwann cells and neurons in the PNS, Schwann cell abnormalities may also contribute to mitochondrial dysfunction in DPN. However, little is known about the Schwann cell mitochondrial deficits in DPN. Enlarged mitochondria in Schwann cells of both myelinated and unmyelinated nerve fibers were observed in sural nerve

biopsy samples from diabetic patients with progressive neuropathy [314]. In addition, the transgenic mice (Tfam-SCKO) with Schwann cell-specific deletion of mitochondrial transcription factor A (Tfam) developed a progressive peripheral neuropathy including nerve conduction velocity deficits and extensive muscle denervation [315]. These transgenic mice also have axonal degeneration with early preferential loss of small unmyelinated fibers, which was followed by prominent demyelination of large nerve fibers. The mitochondria isolated from sciatic nerves in Tfam-SCKO mice showed a 60% decrease in the activity of cytochrome oxidase that is encoded by mitochondrial DNA, but normal activity of succinate dehydrogenase that is encoded by nuclear DNA. Surprisingly, this severe mitochondrial DNA depletion and respiratory chain abnormalities in Tfam-SCKO mice was not associated with Schwann cell proliferation or survival. Furthermore, our lab previously found that hyperglycemia increased the expression of a range of proteins that contributed to mitochondrial dysfunction and compromised the efficiency of mitochondrial respiration in cultured primary Schwann cells [316]. These data suggest mitochondrial function in both Schwann cell and neurons is necessary to maintain the axon-glia integral relationship.

Though exogenous Schwann cell-derived neurotrophins (NGF, IGF-I and NT-3) prevent depolarization of the mitochondrial inner membrane potential in diabetic sensory neurons, whether NRGs could support mitochondrial integrity in Schwann cells remains unclear. Therefore, we treated cultured primary Schwann cell with low glucose (5.5 mmol/L) or high glucose (25 mmol/L) for 6 days and sub-groups were treated with 10 ng/ml NRG1 Type I or 10 ng/ml NRG1 Type III during the last 24 hours. Mitochondrial bioenergetics were measured using the Seahorse Bioscience XF96 Analyzer. Interestingly, both NRG1 Type I and NRG1 Type III increased SRC to a similar extent, compared to control in low glucose medium (**Figure**

4.3.2). However, hyperglycemia significantly impaired NRG1 Type III-induced but not NRG1 Type I-induced improvement in mitochondrial bioenergetics. Similarly, one group found a synergistic effect of increasing in mitochondrial density by NRG1 Type I (20 ng/ml) and IGF (100 ng/ml) in Schwann cell cultures [317]. Whether exogenous NRG1 Type I treatment stimulated endogenous IGF secretion in our study needs further confirmation. In addition, hyperglycemia specifically impaired NRG1 Type III-increased OCR and SRC. More studies are needed to investigate whether NRG1 Type I and NRG1 Type III might enhance mitochondrial bioenergetics through different mechanisms and whether the pathway stimulated by NRG1 Type III is more susceptible to hyperglycemia.



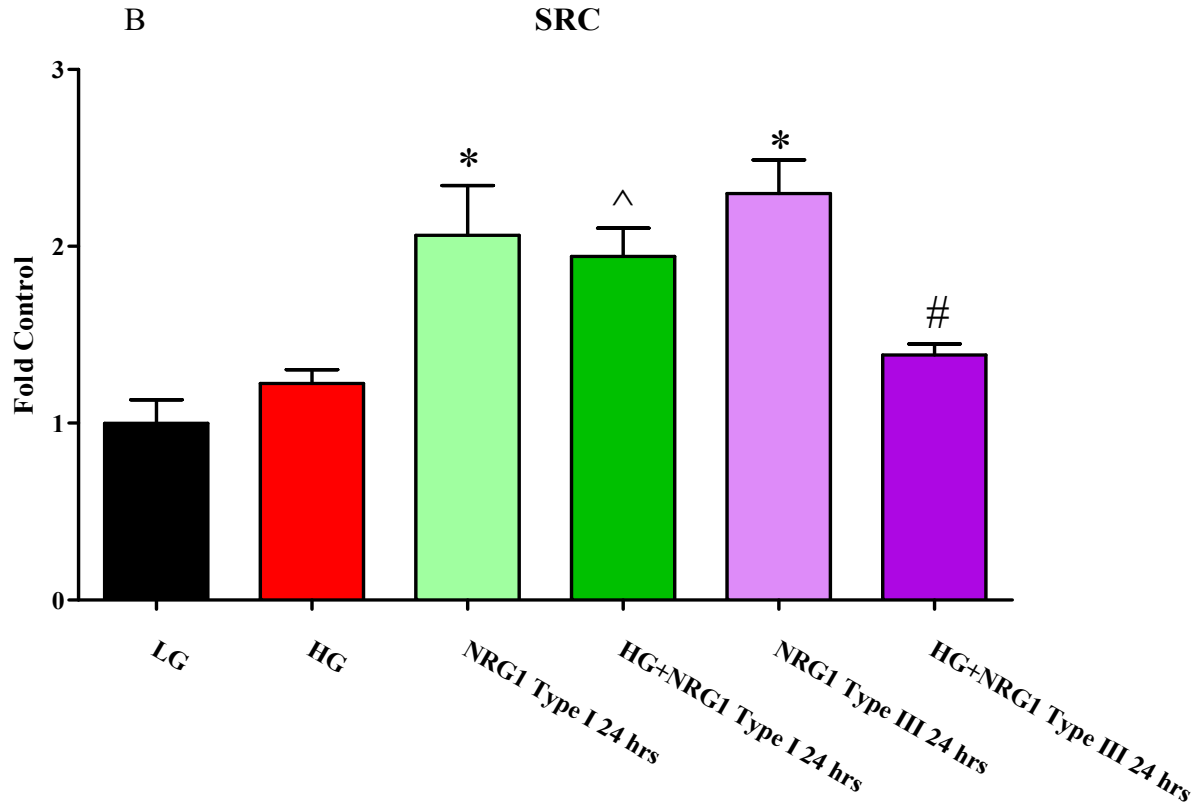


Figure 4.3.2: Hyperglycemia impaired NRG1 Type III-induced but not NRG1 Type I-induced increase in mitochondrial bioenergetics in cultured primary Schwann cells. Schwann cell cultures were treated with low glucose (LG) or high glucose (HG) medium for 6 days and sub-groups were treated with 10 ng/ml NRG1 Type I or 10 ng/ml NRG1 Type III for 24 hours. Oxygen consumption rate (OCR) and Spare respiratory capacity (SRC) were measured by an XF96 Extracellular Flux Analyzer. Bothe OCR and SRC were increased by the treatment of both NRG1 isoforms. However, hyperglycemia significantly impaired NRG1 Type III-induced but not NRG1 Type I-induced increase in mitochondrial bioenergetics (*, $p < 0.05$ vs. LG; ^, $p < 0.05$ vs. HG; #, $p < 0.05$ vs. NRG1 Type III).

Upon NRG1 stimulation, Erb B2 receptors have been shown to translocate to mitochondrial through associating with the mitochondrial heat shock protein 70 (mtHSP70) in Erb B2-postive cancer cells. This translocation negatively regulated mitochondrial respiratory function and rendered the cancer cells more resistant to ErbB2-targeting antibody trastuzumab [318]. To determine if a similar translocation event may occur in Schwann cells, Erb B2 receptor levels were measured in a mitochondrial fraction isolated from Schwann cell treated with 10 ng/ml

NRG1 Type I. In contrast to the results in cancer cells, immunoblot analysis (**Figure 4.3.3**) showed a decrease in Erb B2 receptor levels upon NRG1 Type I treatment in both mitochondrial and cytosolic fractions. A lower level of Erb B2 receptors in mitochondria might contribute to a higher mitochondrial respiratory function. However, we detected a time-dependent decline in NRG1 Type I-activated p42/p44 MPAK pathway (**Figure 4.3.4**), suggesting that the signaling cascades activated by NRG1 Type I differ between acute and prolonged treatment. Therefore, it is not possible to directly link the decrease in mitochondrial Erb B2 receptor induced by 1 hour NRG1 Type I treatment to the increased mitochondrial bioenergetics induced by 24 hours NRG1 Type I treatment. Surprisingly, we detected a predominant expression of Erb B2 receptors in mitochondrial fraction compared to the cytosolic fraction in untreated cells. This suggests that the presence of ligand recruits the receptor to the plasma membrane and initiates the signaling cascades. Furthermore, Schwann cells have been hypothesized to be mainly glycolytic and rely on non-oxidative catabolism of glucose to meet their energy needs [319]. Whether this enrichment of Erb B2 receptors in mitochondria at basal level contributes to minimize mitochondrial activity is unclear.

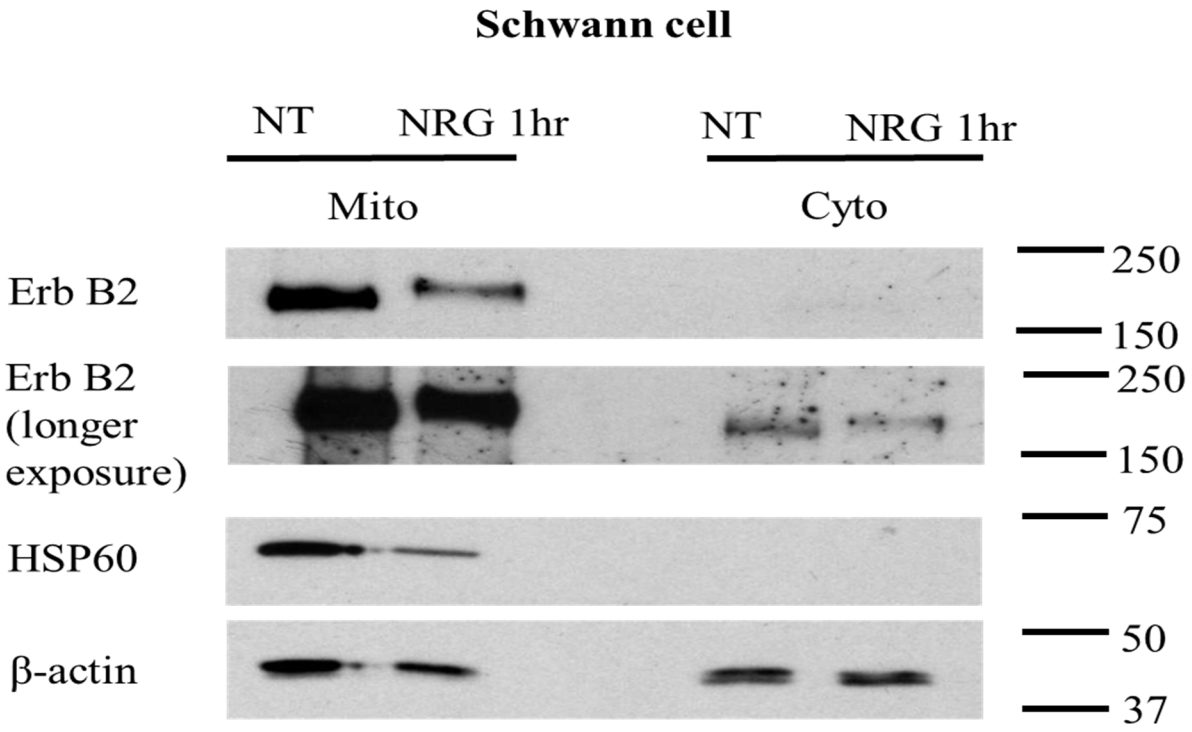


Figure 4.3.3: Erb B2 receptor levels were decreased in both mitochondrial and cytosol fractions upon NRG1 Type I treatments. Schwann cell cultures were treated with vehicle or 10 ng/ml NRG1 Type I for 1 hour and protein lysates were collected and separated by SDS-PAGE. HSP60 was a marker for mitochondrial fraction and β-actin served as a loading control.

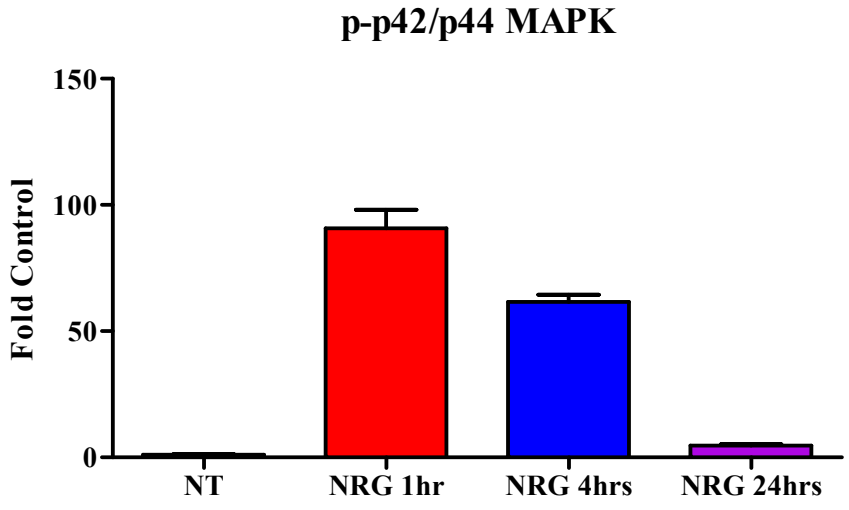


Figure 4.3.4: NRG1 Type I-induced activation of p42/p44 MAPK pathway decreased in a time-dependent manner in cultured primary Schwann cells. Schwann cell cultures were

treated with vehicle or 10 ng/ml NRG1 Type I for 1, 4, and 24 hours and protein lysates were collected and separated by SDS-PAGE. Immunoblot analysis were quantified, activities of p42/p44 MAPK were expressed as a fold of the levels in non-treated cells.

4.4. Conclusions

In summary, our data support that diabetes may alter Erb B2 signaling in peripheral nerves by altering the balance in NRG1 isoform expression and downregulating the expression of erbin, an adapter protein that can function as a negative regulator of p42/p44 MAPK signaling via Erb B2 receptors. Though a limitation of our study is that we cannot ascertain if changes in NRG1 isoforms are necessary or sufficient to contribute to DPN, altered signaling through the NRG1-Erb B2 ligand-receptor pair may contribute to dysfunction of both myelinated and unmyelinated fibers since diabetic mice treated with Erb B2 receptor inhibitor erlotinib exhibited an improvement in both mechanical and thermal sensitivity. Given the complex role of neuregulins in controlling both myelination and demyelination, these data suggest that an altered neuregulinism may contribute to myelin pathologies that develop in human DPN.

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