Mechanism of Fast Axonal Transport Deficits

in Diabetic Neuropathies

BY

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THESIS

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LIST OF MOST COMMON ABBREVIATIONS

CAN	CARDIOVASCULAR AUTONOMIC NEUROPATHY
CVD	CARDIOVASCULAR DISEASE
DM	DIABETES MELLITUS
DN	DIABETIC NEUROPATHIES
DPN	DIABETIC POLYNEUROPATHY
FAT	FAST AXONAL TRANSPORT
FPG	FASTING PLASMA GLUCOSE
GSK3	GLYCOGEN SYNTHASE KINASE 3
IFP	IMPAIRED FASTING GLUCOSE
MAPK	MITOGEN ACTIVATED PROTEIN KINASE
MT	MICROTUBULES
NE	NOREPINEPHRINE
OGTT	ORAL GLUCOSE TOLERANCE TEST
PS	PARASYMPATHETIC
QOL	QUALITY OF LIFE
STZ	STREPTOZOTOCIN
Sy	SYMPATHETIC

SUMMARY

In 2010, the world prevalence of diabetes mellitus (DM) was estimated as 6.4%, affecting 285 million adults. Taken together with the growing incidence of childhood diabetes and undiagnosed cases, by the year 2030 DM is expected to be the biggest pandemic of all time. Due to its high prevalence, currently DM and associated complications represent the main socioeconomic burden worldwide in terms of health care cost, decreased work force, and disability. Diabetic neuropathy (DN) is one of the most common long-term complications of diabetes, which can affect up to 50% of type 2 diabetic (typically insulin independent) patients and nearly 100% of type 1 diabetic (mostly insulin dependent) patients at some point in the course of their disease. DN leads to increased risk of morbidity and mortality, as well as significant reduction in quality of life as a result of end-stage complications such as lower limb amputations, a common consequence of insensate neuropathy, and diabetic heart disease with sudden death, in which cardiovascular autonomic neuropathy is an important contributor. Despite forty years of intensive research that lead to the discovery of many metabolic disturbances and numerous clinical trials, there is still no consensus on global guidelines for DN therapy. Patient care is limited to management of risk factors and symptomatic treatment as there is no available therapy to prevent, halt, or reverse the process of neurodegeneration. The reasons for this shortcoming are likely multifactorial, but high on the list are the lack of relevant animal models to the human condition used for preclinical trials and the lack of consensus on key pathogenic mechanisms that lead to insufficient targeting in clinical trials. Thus, the goal of this thesis was to address some of these issues in three aims.

Aim 1 established and characterized a type 1 DM rat model that better mimics insensate neuropathy, the clinical hallmark of DN. Development of decreased sensitivity to either painful or non-painful stimuli is not only the most prevalent clinical sign of DN but also the behavioral correlate of the anatomical hallmark of neuropathy, namely dying-back axonopathy. Thus, obtaining an animal model with such characteristics is of extreme value for identifying and studying key pathogenic mechanisms responsible for neurodegeneration. In particular, complete loss of sensation is a late phenotype not usually seen in animal models due to their short life span. DM rats may develop some type of insensate neuropathy in combination with pain, and only a few DM mouse models develop complete insensate neuropathy. In this thesis, using a rodent animal model, streptozotocin (STZ) was used to induce type 1 DM. The STZ-induced DM model is the most widely used and best-characterized animal model of type 1 DM. In addition, it has the advantage of not requiring insulin therapy for animal survival and well-being. Fischer 344 was the rat strain of choice, which is less prone to obesity, a factor that can independently cause peripheral neuropathy. Using STZ and the Fisher rodent strain, we are the first to report the development of insensate neuropathy without signs of pain in the rat. This phenotype was developed in a tractable time useful for studies of signaling pathways before and after the onset of neuropathy. This rat model may serve to study key pathogenic mechanisms that lead to dying-back neurodegeneration independently of obesity, pain and insulin therapy.

Using the above animal model, Aim 2 evaluated the development of cardiovascular autonomic neuropathy (CAN) and associated complications, such as

cardiomyopathy co-presenting with sensory neuropathy. In particular, the natural history of human DN indicates that some neuronal fibers, such as autonomic and visceral sensory, may be affected first and others, such as somatic sensory and motor, may be affected later in the course of the disease. Thus, determination of course of appearance of DN for different types of fibers in animal models would not only aid in the description of a more relevant animal model, but would also serve to study specific pathogenic mechanisms that lead to differential neuronal susceptibility to damage. In our animal model, we successfully established the co-presentation of CAN and cardiomyopathy with insensate neuropathy. Thus demonstrating the feasibility of this model as serving as a good experimental framework to study unique pathogenic mechanisms and intrinsic vulnerability of different types of fibers affected in DN.

Aim 3 evaluated the mechanism of fast axonal transport (FAT) deficits in DM nerves by examining the pattern of kinase activation pertinent to axonal transport regulation as well as molecular motor activity. The critical role FAT plays in peripheral neuropathies (PN) has been proven by natural occurring mutations in molecular motors (kinesin and dynein) and associated proteins. These mutations are sometimes sufficient to cause PN of the dying-back type (e.g. hereditary spastic paraplegia), a shared pattern with DN. Thus, FAT deficits may represent a key contributor for the development and/or progression of DN. Although molecular motor mutations have not been associated with DN, FAT deficits in diabetic nerves are prominent. This finding indicates that other factors that disrupt FAT, such as the phosphorylation of the motors, could lead to pathological behavior and to dying-back neuropathy. Phosphorylation of

molecular motors is a well-known regulator of their function, such as binding to microtubules (MT) and vesicles, which translates into changes in FAT. Previous work from Brady lab has identified kinases, such as glycogen synthase kinase 3 beta (GSK3 beta) and mitogen-activated kinases (MAPK) that regulate those functions (e.g. binding to MT/vesicles). These kinases are also involved in pathogenic signaling in diabetes as a result of hyperglycemia and/or lack of insulin. In this work, the pattern of kinase activation found in DM nerves was consistent with lack of insulin signaling (increased GSK3 beta activation, and decreased MAPK activation). Moreover, this pattern correlated with reduced kinesin binding to microtubules mostly for autonomic nerves, at times of sensory and autonomic deficits. These results suggest that differential activation of kinases in specific types of fibers could alter molecular motor functions leading to FAT deficits and dying-back neuropathy.

In conclusion, differential activation of kinases involved in changes in molecular motor's phosphorylation state and function may represent a plausible mechanism to explain FAT deficits in DN. In addition, these altered signaling pathways may serve as targets for therapy, such as inhibitors of GKS3 beta, which are currently being used in preclinical trials for many neurodegenerative diseases. Ultimately, results from this and future studies may lead to the design of novel therapeutic agents that directly target key neurodegenerative processes in the hope of a successful translation into the treatment of human diabetic neuropathy.

I. INTRODUCTION

A. Review of relevant literature

1. Hyperglycemic syndromes and associated complications

a) Glucose homeostasis

Glucose homeostasis is one of the most critical functions in the human body allowing the storage or use of energy sources necessary for basic body and cellular functions. The basis of this tight control is highly dependent of the proper function of several factors that promote glucose absorption from the intestine, production by the liver and uptake, metabolism and storage by peripheral tissues. The main players in this finely orchestrated balance are insulin, produced by beta cells in the pancreas, glucagon, produced by alpha cells in the pancreas, leptin, produced in adipocytes and proliferator-activated receptors (PPARs) [1, 2].

High levels of glucose stimulate insulin secretion from pancreatic beta cells, which promotes glucose uptake (stimulates glucose transporter 4 (GLUT4) translocation to the membrane) in muscle and fat, whereas in the liver it stimulates glycogen synthesis and inhibits glycogenolysis and gluconeogenesis. Insulin also regulates in similar ways the metabolism of lipids and proteins in fat, liver and muscle, promoting their uptake and storage and inhibiting their degradation and release into the circulation. Decreased blood glucose levels lead to glucagon secretion from pancreatic alpha cells of the pancreas, which increases glucose production from the liver [1].

Adipose tissue secrete several adipokines, such as leptin [3], adiponectin [4] and tumor necrosis factor (TNF) alpha, which regulate food intake via direct effects in the central nervous system as well as utilization of glucose in skeletal muscle and insulin sensitivity. Leptin can also regulate glucose homeostasis through the autonomic nervous system [5, 6] or by directly binding to its receptor on the beta cell [7]. Free fatty acids activate PPAR in adipocytes stimulating, via gene transcription, fatty acid oxidation in liver and skeletal muscle, adipogenesis and lipogenesis in the adipose tissue, in addition to increasing insulin sensitivity and promoting glucose uptake in skeletal muscle [8].

The final effect that these factors have on glucose regulation is ultimately derived from a wide array of downstream signaling cascades that are tightly controlled. Due to the high degree of cross talk among the signals and the many players, any small disturbance in this delicate balance can threaten the normal control of glucose homeostasis.

b) Hyperglycemic syndromes

When glucose homeostasis is out of balance a wide spectrum of glucose dysregulation syndromes may appear, namely diabetes, pre-diabetes and metabolic syndrome, all characterized by hyperglycemia [9]. Although these syndromes may represent a continuous spectrum, they are clinically divided into discrete categories based on specific glucose cut-point values (TABLE I). This distinction is derived from the relative risk of developing micro- and macrovascular complications, which are positively correlated with the level of glycemia: the higher the glucose values the higher risk of complications [10].

TABLE I

CLASSIFICATION OF HYPERGLYCEMIC SYNDROMES

	WHO 2006	ADA 2003
Diabetes		
HbA1c	≥ 6.5% (optional) (WHO 2011)	≥ 6.5%
	or	**or**
Fasting glucose	≥7.0 mmol/L (126 mg/dL)	≥7.0 mmol/L (126 mg/dL)
	or	**or**
2-h glucose *	≥11.1 mmol/L (200 mg/dL)	≥11.1 mmol/L (200 mg/dL)
Casual glucose (plus symptoms of hyperglycemia)	Not required	≥11.1 mmol/L (200 mg/dL)
Impaired Glucose Tolerance (IGT)		
HbA1c	Not required	5.7 to 6.4% (ADA 2011)
		or
Fasting glucose	<7.0 mmol/L (126 mg/dL)	Not required
	and	
2-h glucose *	≥7.8 mmol/L and <11.1 mmol/L (140 mg/dL and 200mg/dL)	≥7.8 mmol/L and <11.0 mmol/L (140 mg/dL and 199 mg/dL)
Impaired Fasting Glucose (IFG)		
HbA1c	Not required	5.7 to 6.4% (ADA 2011)
		or
Fasting glucose	6.1 mmol/L to 6.9 mmol/L (110 mg/dL to 125 mg/dL)	5.6 mmol/l to 6.9 mmol/l (100mg/dl to 125 mg/dl)
	and (if measured)	
2-h glucose *	<7.8 mmol/L (140 mg/dL)	Not required
Metabolic syndrome		
Fasting glucose	IGT **or** IFG (WHO 2011)	≥ 5.6 mmol/L (≥100 mg/dL)
(plus other factors)		**or** previously diagnosed type 2 diabetes (IDF 2006)
Normal		
Fasting glucose	< 6.1 mmol/L (110 mg/dL)	<5.6 mmol/L (100 mg/dL)
	and (if measured)	**and**
2-h glucose	<7.8 mmol/L (140 mg/dL)	<7.8 mmol/L (140 mg/dL)

* Venous plasma glucose 2-h after ingestion of 75g oral glucose

WHO: World Health Organization, IDF: International Diabetes Federation, ADA: American Diabetes Association

Diagnosis of the degree of glucose dysregulation is based on different tests including casual plasma glucose, fasting glucose (FG), oral glucose tolerance test (OGTT) and glycated hemoglobin (HbA1c). Casual plasma glucose is the measurement of plasma glucose at any time independent of the nutritional status. FG is the measurement of plasma glucose with overnight fasting (minimum 8 hrs), OGTT is the measurement of plasma glucose after 2h post-glucose load of 75 mg. The degree of plasma glucose concentration can fluctuate over time and the tests need to be repeated several times for an accurate diagnosis. HbA1c reflects average plasma glucose levels over the previous 8 to 12 weeks. It can be done at any time and does not require any special preparation such as fasting [11]. Although HbA1c overcomes the day-to day glucose variability, it is affected by other conditions such as haemoglobinopathies, anemias, and other disorders related to high red cell turnover such as malaria [12]. All the above-mentioned tests present limitations and complimentary tests including both patient- and physician/laboratory-based testing including urine glucose and ketones, and other glycated serum proteins, such as albumin, are also used to diagnose and/or monitor glycemic status [13, 14].

Diabetes, with the highest levels of glycemia, represents the upper end of the spectrum with the highest risk of developing complications. Pre-diabetes, with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), and metabolic syndrome, represent intermediate levels of hyperglycemia. [15]. People with pre-diabetes have a high risk of developing diabetes and associated complications, including peripheral neuropathies that can be prevented by life-style changes [16]. Metabolic syndrome (MetS) (previously known as syndrome X, dysmetabolic syndrome and insulin

resistance) is characterized by hyperglycemia (FPG >100mg/dl), obesity, dyslipidemia, insulin resistance, and hypertension, with at least five different definitions used for diagnosis based on several criteria and cut-point values [17, 18]. Due to complex etiology of MetS, associated complications are a result of other factors in addition to hyperglycemia. The glucose cut-point values used to discriminate among the different syndromes of glucose dysregulation are constantly being updated [19], as well as the cut-point value to define normoglycemia. Thus, at the lower end of the spectrum, the term normoglycemic (FPG <100 mg/dL, most recent American Diabetes Association (ADA) guidelines) represents the lowest risk for developing diabetes and its associated complications.

c) Diabetes mellitus

The name diabetes mellitus (DM) comes from the greek word “diabainein” which means that passes through (dia- means through or entirely and baino-, to go) and the word mellis in latin that means honey, used to describe patients that presented with excessive ”sweet” urination back in the 1700s. This term should be distinguished from diabetes insipidus, meaning polyuria (excessive urination) without taste, a rare condition in which the kidneys produce abnormally large volumes of dilute urine. Diabetes mellitus became so common in the worldwide population that the term diabetes mellitus is used interchangeably with diabetes [20]

The definition of diabetes has evolved over time due to its complex etiology and is used to describe an array of metabolic disorders including carbohydrates, lipids and protein metabolism characterized by hyperglycemia resulting from defects in insulin

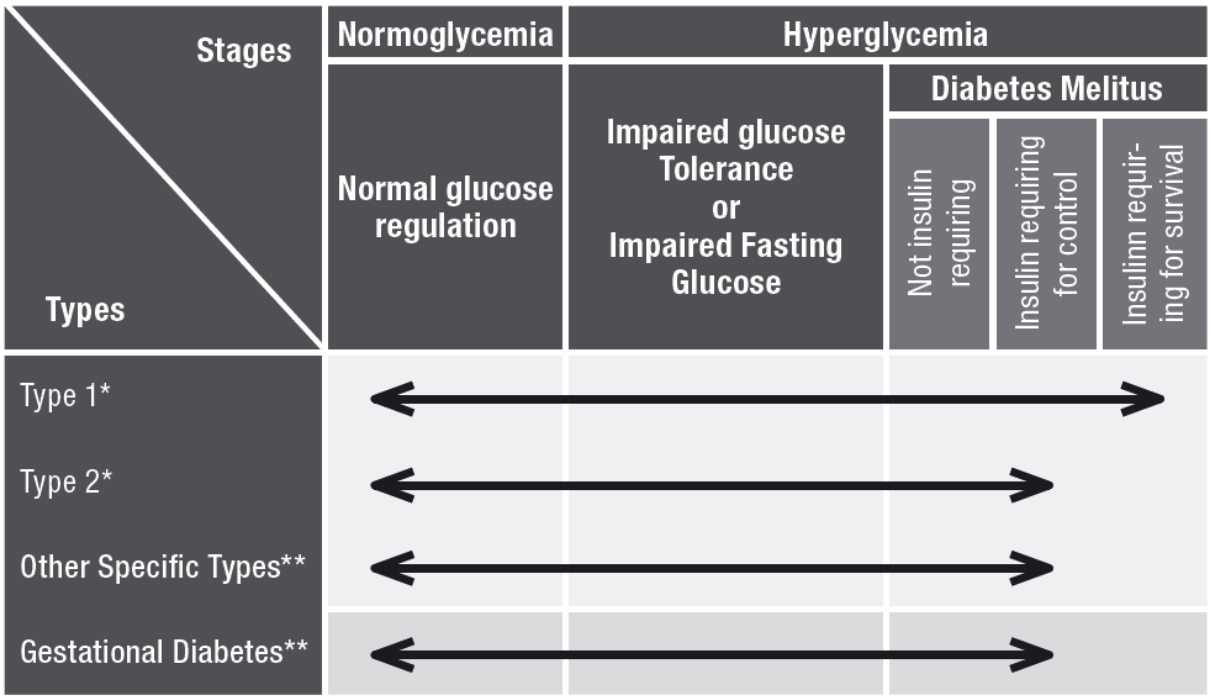
secretion, insulin action or both. There are many types of diabetes but the vast majority fall into two basic categories based on the cause of hyperglycemia and when possible underlying insulin etiologies (TABLE II).

Type 1, accounts for 5 to 10% of all cases, where the cause is an absolute insulin deficiency usually due to autoimmune responses to beta cells (the insulin-producing cells of the pancreas) and requires insulin for survival. Type 2, accounts for 90 to 95% of cases, where the cause is a combination of insulin resistance with different degrees of insulin deficiency due to idiopathic causes and usually presents with obesity. The third most common is gestational diabetes in pregnant women without history of diabetes that could later evolve into type 2 diabetes. Other less common types are based on genetic factors, drug toxicity, endocrinopathies, infections, diseases of the exocrine pancreas, association with specific genetic syndromes and some uncommon forms of immune-mediated diabetes [15].

Manifestation of acute clinical symptoms are common in type 1 diabetes and include: polydipsia, polyuria and unexplained weight loss. Type 2 diabetes can be asymptomatic for several years until diagnosis in advanced stages when symptoms are similar to type 1. Final diagnosis is based on three different tests that account for elevated plasma glucose levels: a) casual plasma glucose concentration $\geq$ 200 mg/dL, b) FPG $\geq$ 126 mg/dL, or c) 2h postload glucose $\geq$ 200 mg/dL during an OGTT. Elevated levels of glycemia, although present in all different types can fluctuate over time and in some cases diabetic patients may become normoglycemic in a transient state called

TABLE II

DISORDERS OF GLYCEMIA: TYPES AND STAGES



from [10]

“honeymoon” remission. However, disturbances in insulin secretion and/or signaling remain to exist and insulin-sensitive tissues continue to be affected [15].

d) Complications of disorders of glycemia

Patients with untreated diabetes can present in the clinic with acute life-threatening complications such as diabetic ketoacidosis (DKA, typical type 1), hyperosmolar non-ketotic coma (HNC, usually type 2), lactic acidosis (LA) and hypoglycemia [21]. DKA and HNC are related to insulin deficiency, hypoglycemia derives from treatment by either oral agents or insulin and LA, rare in diabetes, is a result of acidosis due to excess lactic acid production. Before the isolation of insulin, the hormone responsible for lowering glucose levels, type 1 patients would die soon after diagnosis as a result of the above-mentioned complications [22]. Even today, life expectancy for type 2 diabetic patients is reduced by 5 to 10 years [23]. Over the years, a better prognosis for people with diabetes has been achieved as a result of the constant advancement and innovation in diabetic therapies but even under good glycemic control diabetic patients still suffer from long-term complications [24].

Long-term complications have a complex etiology and affect nearly all organs of the human body via direct effect on the vasculature [25] (micro and macrovascular damage) that serves those organs or directly in the tissues independently of vasculature. These complications include nephropathy (kidney) [26], neuropathy (peripheral nerves) [27], retinopathy (retina) [28], cardiovascular disease (heart) [29], peripheral arterial disease [30], stroke [31] and encelopathies (brain) [32] among others. People with diabetes have a risk of 2 to 4 times higher of suffering heart disease and stroke than the normal population. Near to 75% of adult diabetic patients suffer from

high blood pressure. Diabetes is the first cause of blindness, kidney disease and non-traumatic amputations in the general population. Peripheral neuropathy (PN) is the most common long-term complication in people with diabetes, affecting 50 to 90 % of patients, and the least understood due to its complex heterogeneity.

2. Peripheral neuropathy

Peripheral neuropathy (PN) is a general term used to describe any disorder of the peripheral nervous system (sensory, autonomic and motor). Based on its etiology, PN can be divided into genetic [33-35], acquired [36] or idiopathic (unknown). However, the most common are acquired and can be due to physical injury (trauma) to a nerve, tumors, autoimmune responses, nutritional deficiencies (vitamin B12), certain pharmacological treatments and toxins [37-39] vascular and metabolic disorders. TABLE III shows the most frequent diseases that cause PN.

Diabetes mellitus, represents the most prevalent form of acquired of PN, and it is widely recognized as diabetic peripheral neuropathy, or just diabetic neuropathy. Recently, due the more precise classification of hyperglycemic syndromes, it was found that the majority of patients diagnosed with idiopathic PN had either pre-diabetes or metabolic syndrome [40-42]. Since similar mechanisms may exist in all hyperglycemic-derived PN, some scientists feel the need of including pre-diabetes and metabolic syndrome-derived PN in the same group of “Diabetic Neuropathy” to facilitate their treatment and management.

TABLE III

DISEASE DERIVED-PERIPHERAL NEUROPATHY

Pure genetic	**Systemic diseases**	**Infectious diseases**
Charcot Marie Tooth (CMT)	Diabetes	Human immunodeficiency virus (HIV)
Hereditary sensory neuropathy (HSN)	Renal insufficiency	Chagas
Hereditary sensory and autonomic neuropathy (HSAN)	Nutritional deficiency	Botulism
Hereditary neuropathy with liability to pressure palsies (HNPP)	Chronic liver disease/Alcoholism	Leprosy
Hereditary motor neuropathy (HNP)	Amyloidosis	Diphteria
Hereditary neuralgic amyotrophic (HNA)	Malabsorption	
Spinal muscular atrophy (SMA)	Hypothyroidism	**Immune-mediated**
Channelopathy associated insensitivity to pain	Acromegaly	Coelic disease
Fabry's disease	Chronic obstructive pulmonary disease	Guillan-Barre syndrome
Allgrove's disease	Sarcoidosis	Connective tissue disease
Tangier disease	Carcinoma	Sjogren's disease
Navajo Indian neuropathy	Lymphoma	Systemic lupus erythematosus
Leucodystrohy	Myeloma	Rheumatoid Arthritis
Refsum disease	Monoclonal gammopathy of unknown significance	Cryoglobulinaemia
		Chronic inflammatory demyelinating neuropathy (CIDP)
Associated with other neurological diseases		
Parkinson's		

Diabetic neuropathies have become a worldwide economic burden due to the high prevalence of diabetes and the lack of available therapies [43]. Although diabetic neuropathies have been a subject of intense basic research for almost 40 years, there is still no consensus in the key pathogenic mechanisms that lead to neurodegeneration [44].

3. Diabetic neuropathy

a) Definition of diabetic neuropathy

Diabetic neuropathy is defined as “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after exclusion of other causes." [45]. Diabetic patients have a higher risk of developing other types of PN, independent of the diabetic state, many of which have available treatments. Those cases should be referred as “PN in diabetic patients”, and the term “diabetic neuropathy” should be used only when derived directly from the diabetic state [46]. For example, the drug metformin used as a glucose lowering agent, can cause vitamin B12 deficiency, which in turn causes neuropathy [47]. Thus, proper classification of PN observed in diabetic patients is essential for proper treatment. The earlier the intervention, the more likely the prevention of end-stage complications.

b) Classification of diabetic neuropathy

DN represents a heterogeneous group with no clear consensus for classification. TABLE IV shows the three most popular classifications of DN based on clinical symptoms [48, 49], a combination of clinical and anatomical features and another one that reflects the heterogeneous presentation of PN in diabetes [50, 51].

DN can be classified into mononeuropathy, mononeuropathy multiplex or polyneuropathy. Mononeuropathy implies a focal lesion of a single peripheral nerve usually caused by trauma, focal compression, or entrapment. The most common ones are carpal tunnel syndrome and ulnar neuropathy. Mononeuropathy multiplex refers to multiple separate noncontiguous peripheral nerves affected simultaneously. Polyneuropathy refers to the involvement of different types of nerves together presenting with a wide range of symptoms [52].

Moreover, DN can be further classified based on several clinical and anatomical observations. These include: a) demyelinating or axonal (electrophysiological measurements); b) chronic, acute or relapsing (rate of progression and frequency); c) (mostly, mixed or pure) motor, sensory, or autonomic (degree of fiber involvement); d) large and small fiber (based on signs and symptoms); e) distal, proximal (distance), f) symmetrical or diffuse (pattern of degeneration), and g) focal or multifocal (target involvement).

TABLE IV

THREE CLASSIFICATION SYSTEMS FOR DIABETIC NEUROPATHY

A: Pure Clinical Classification

Polyneuropathy

Sensory

- Acute sensory
- Chronic sensorimotor

Autonomic

- Cardiovascular
- Gastrointestinal
- Genitourinary
- Other

Proximal motor (amyotrophy)

Truncal

Mononeuropathy

Isolated peripheral

Mononeuritis multiplex

Truncal

B: Clinical and Anatomical Classification

Length dependent diabetic polyneuropathy

- Distal symmetrical sensory polyneuropathy
- Large fiber neuropathy
- Painful symmetrical polyneuropathy
- Autonomic neuropathies

Focal and multifocal neuropathies

- Cranial neuropathies
- Limb neuropathies
- Proximal DN of the lower limbs
- Truncal neuropathies

Non diabetic neuropathies more common in diabetes

- Pressure palsies
- Acquired inflammatory demyelinating polyneuropathy

C: Other

Rapidly reversible

- Hyperglycemic neuropathy

Generalized symmetrical polyneuropathies

- Chronic sensorimotor neuropathy (diabetic polyneuropathy)
  - Small fiber neuropathy
  - Large fiber neuropathy
- Acute sensory neuropathy
- Autonomic neuropathy

Focal and multifocal

- Cranial neuropathy
- Thoracolumbar radiculoneuropathy
- Focal limb neuropathy -mononeuropathy and entrapment syndromes
- Proximal motor neuropathy(amyotrophy)
- Superimposed chronic inflammatory demyelinating neuropathy

Adapted from [53]

In terms of prevalence, distal symmetrical polyneuropathy (DSPN) is the most widespread type, affecting nearly 30% of type 2 diabetic patients and nearly 100% of type 1 diabetics [54]. For type 1 patients, DSPN represents the most prevalent neuropathy of true diabetic origin, thus many times is commonly referred as diabetic peripheral neuropathy or diabetic polyneuropathy, both abbreviated as DPN in the literature, or just type 1 diabetic neuropathy. In this thesis, the term DPN, was used to refer to distal symmetric polyneuropathy. DPN progresses with the disease and can be sometimes managed by controlling typical diabetic risk factors [45]. The fully expressed syndrome of DPN is a symmetrical distal lower limb sensorimotor polyneuropathy of a dying-back type [55], predominantly sensory and autonomic early in the progression of the disease and with minor motor involvement in late stages.

c) Diabetic polyneuropathy: risk factors and management

The most common risk factors for development of DPN are hyperglycemia, glycosylated hemoglobin, increased height, cigarette smoking and duration of diabetes [56]. However, emerging data from epidemiological studies showed that even under tight glycemic control, DPN still develops, and that other potentially modifying independent factors such as obesity, hypertriglycerides and hypertension correlated with DPN development [57-59]. In particular, elevated triglycerides were found to be not only associated with diabetic sensory neuropathy [60] but with diabetic cardiovascular autonomic neuropathy as well [61]. Since then, other factors such as lack of insulin [62] and lack of C-peptide (by-product of insulin) [63] have been suggested to play an important role in the development of DPN. Finally, a recent report by Elliot *et al*.,

demonstrated that common cardiovascular risk factors can predict large fiber neuropathy [64].

In terms of prevention and management, presence of certain types of complications may also predict others and provide a framework to monitor and prevent irreversible outcomes. For instance, sensory neuropathy robustly associates with both micro- and macroangiopathy [65]. Moreover, presence of certain types of neuropathy can also serve as an indication that nerve damaging is already occurring and may help monitor and diagnose other types of neuropathies at the subclinical stage. For example, retinopathy strongly correlates with small fiber corneal neuropathy, which can be assessed by corneal confocal microscopy before signs of keratopathy (impaired corneal sensation, reduced tear secretion) appear [66]. Indices of corneal neuropathy correlate with sensory neuropathy [67] and may even predict them, potentially becoming a non-invasive surrogate marker for early detection of human DN [68]. Recent evidence also indicates that cardiovascular autonomic neuropathy (CAN) strongly correlates with sensory neuropathy [69-71]. Moreover, emerging data from the Diabetes Control and Complication Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) studies show that both sensory neuropathy and CAN still develop under tight glycemic control, suggesting a common pathogenic mechanism independent of hyperglycemia [72]. In addition, increasing evidence exists showing that CAN may be a key contributor to cardiomyopathy, in particular left ventricular dysfunction, in the absence of atherosclerosis and hypertension, especially in type 1 patients [73-76] but also in type 2 patients [77, 78]. Precise assessment of CAN is obtained with high-end techniques such as heart rate variability and expensive imaging studies [76], which may

not be widely available. Thus, assessment of left ventricular dysfunction by more accessible echocardiographic methods may represent an alternative method to predict or monitor co-presenting subclinical neuropathies, when a high-tech sensitive method to test for CAN is not available.

d) Clinical pathology of diabetic polyneuropathy

DPN is manifested by painful and/or insensate neuropathy with varying degree of autonomic dysfunction, although the latter is not routinely included in DPN clinical assessment. The different types of sensory symptoms are used as the first line of DPN evaluation in the outpatient clinic to better judge the risk of complications and necessity for specialist referral (TABLE V) [45]. Stage 0/1 implies either no clinical neuropathy or asymptomatic subclinical neuropathy that can only be detected in special neurophysiological laboratories, but are not routinely performed. Stage 2 describes presence of clinical neuropathy with varying symptoms. Based on the type of symptoms, DN can be further subdivided into three subcategories: a) chronic painful, with pain usually at night, but absent sensation to several modalities and reduced or absent reflexes; b) acute painful, less common and with a diffuse pattern of pain; and c) painless with complete/partial sensory loss, characterized with no symptoms or numbness of feet, reduced thermal sensitivity, painless injury and signs of reduced or absent reflexes. Stage 3 describes patients with late complications of clinical neuropathy, such as foot lesions, neuropathic deformity, and non-traumatic amputation. Other more discrete stages and subdivisions have also been described [50, 79].

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TABLE V

STAGING OF DIABETIC POLYNEUROPATHY

Stage	Characteristics
Stage 0: No clinical neuropathy	No symptoms or signs
Stage 1: Subclinical neuropathy	No symptoms or signs
Stage 2: Clinical neuropathy	
▪ Chronic painful	▪ Positive symptomatology increasing at night: burning, shooting, stabbing pain ▪ Pins and needles± ▪ Absent sensation to several modalities ▪ Reduced or absent reflexes
▪ Painless with complete/partial sensory loss	▪ No symptoms or numbness/deadness of feet; reduced thermal sensitivity; painless injury ▪ Signs of reduced or absent sensation ▪ Absent reflexes
Stage 3: Late complications of clinical neuropathy	▪ Foot lesions e.g. ulcers ▪ Non-traumatic amputation

from [80]

Staging of DN has been developed to provide a useful working framework for easy diagnosis in the general clinic and better patient management and education. Lack of awareness and inappropriate management of diabetic neuropathy (DN) leads to unnecessary morbidity and substantial healthcare costs [43]. Complications associated with DPN represent a significant reduction in quality of life (QOL) [81-83], increased risk of lower limb amputation [84], and increased risk of mortality due to sensory [85, 86] and autonomic neuropathies, in particular cardiovascular autonomic neuropathy (CAN) [87]. Diabetic patients with CAN have an elevated relative risk of mortality of 3.45 over patients without CAN [88].

e) Natural history of diabetic polyneuropathy

The natural history of fiber-specific clinical manifestations, may represent differential resistance to damage of the different nerve fibers determined by the degree of myelination and/or intrinsic protein repertoire [51]. Figure 1 shows the variable degree of clinical manifestations in relationship to dysfunction of different types of neuronal fibers.

Autonomic deficits seem to occur early in DM and include symptoms such as diarrhea, constipation, excessive sweat, etc [89]. Other autonomic deficits, such as irregular heart rate control, may remain undiagnosed until more severe manifestations are evident.

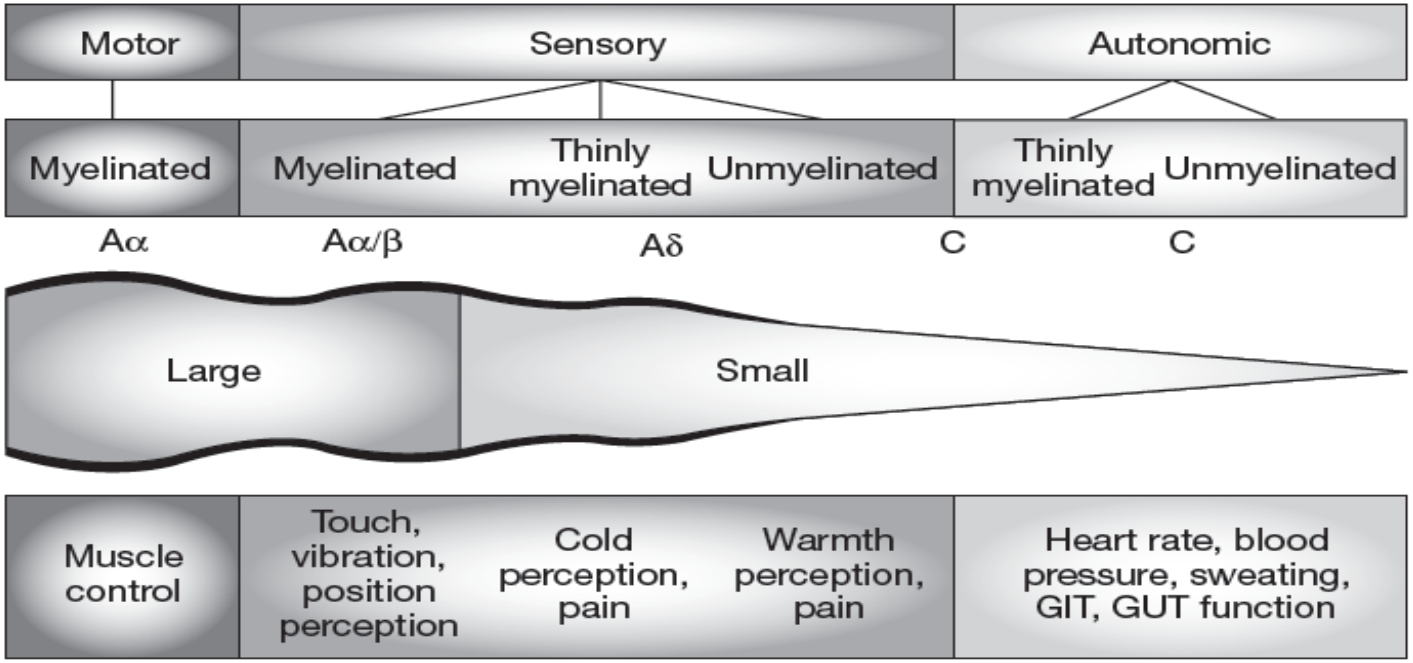

Figure 1. Natural history of clinical manifestations based on the type of fibers affected

Sensory manifestations include negative symptoms (loss of feeling) and positive symptoms (pain), the first one being more characteristic of large fibers and the latter one of smaller ones. It is uncertain whether there is a regular sequence of fiber involvement, with small fibers affected initially and larger fibers later, or whether the small- and large-fibers types represent either side of a continuous spectrum of fiber damage [50]. Approximately 20-30% of all diabetic neuropathies are painful and these patients seek medical treatment early. The distressing nature of symptoms and difficulties in their treatment have given rise to the false impression that diabetic neuropathy is usually a painful condition. Hypoalgesia is often not recognized medically until secondary problems have developed such as foot ulcers, infections and gangrene.

Although DPN is classically defined as a sensorimotor neuropathy, motor manifestation is rare, occurring mainly at late stages and in older age with mild symptoms. Significant muscle weakness in diabetic patients may indicate the presence of other types of neuropathies such as lumbosacral radiculoplexopathy, a common consequence of weight loss and other neuromuscular neuropathies independent of diabetes [90]. However, functional deficits such as motor nerve conduction velocities may occur early in the disease without any significant symptoms and may predict the advent of sensory and autonomic neuropathy [91].

Intermediate cases of glycemia, also manifest PN and may help understand the natural history of nerve degeneration. Patients with intermediate levels of glucose may present early symptoms of DN, while patients with long-standing diabetes may have more advanced DN in combination with other neuropathies as a result of other complications. In this sense, several reports indicate that PN in patients with pre-

diabetes and metabolic syndrome is manifested by sudomotor impairment, representing early autonomic dysfunction [92], reduced skin innervation, many of them experiencing pain and only a small percentage with motor impairment [40]. This indicates that small fibers may be affected first in the course of DN. There is also evidence that cardiovascular autonomic neuropathy is already present in cases of intermediate glucose levels [93].

f) Anatomical pathology of diabetic polyneuropathy

Independent of the type of symptoms, there is a common pattern in the process of neurodegeneration occurring in human diabetic nerves [94]. Pathologically, all three components, sensory [95], autonomic [96] and motor [97], seem to share the same slowly progressive degeneration characterized anatomically by a typical dying-back phenotype [55]. Early events include damage to the most distal terminals first, followed by target denervation (skin and other organs), with subsequent patterns of degeneration towards more proximal sites eventually leading to cell loss [98, 99]. Late degeneration events include segmental and paranodal demyelination, axonal degeneration and regeneration [100], with the presence of vacuoles and Wallerian degeneration [101, 102]. Motor neurons seem to be rather refractory to axonal damage but clinically they show reduction in nerve conduction velocities [53], as well as motor unit denervation in animal models [97]. Anatomical pathology directly correlates with the different stages of neuropathy (Figure 2)

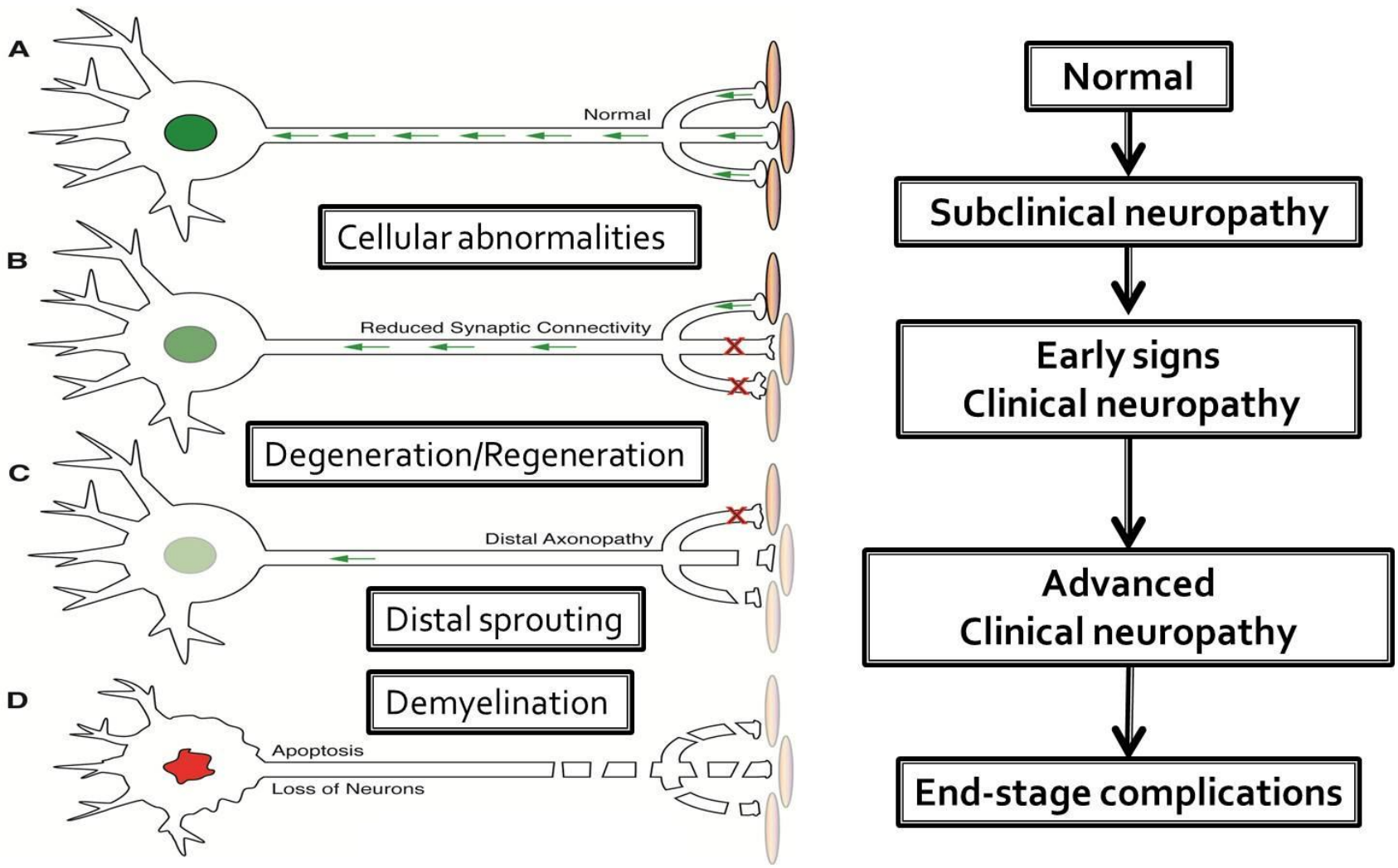

Figure 2. Schematic representation of anatomical and clinical correlates in diabetic polyneuropathy

Early stages, mostly reversible, account for biochemical and physiological deficits, but without any overt clinical signs (subclinical neuropathy). Intermediate stages account for early anatomical deficits, such as skin denervation with some level of demyelination, where clinical signs start to emerge. This stage might be reversible or not. Late stages, mostly irreversible account for more pronounced anatomical deficits, usually manifested with complete loss of neuronal function. This stage is irreversible and patient care is focused on preventing end-stage complications such as gangrene and lower limb amputations [103]. The degree of reversibility is also dependent on the distal to proximal degeneration. The more distal the degeneration, the greater the chance of finding a therapy that could reverse the degeneration process. Once degeneration is observed more proximally with signs of neuronal death, there is probably no chance of treatment.

g) Molecular pathology of diabetic polyneuropathy

Despite a good understanding of the anatomical and clinical pathology of nerve degeneration in DN and a long list of molecular pathologies, there is no consensus in what constitutes the most significant pathogenic mechanism responsible for neurodegeneration. Several signaling pathways seem to interplay in the complex heterogeneity of pathogenic mechanisms described in DN, with a high level of crosstalk. These pathogenic mechanisms derive directly from the diabetic state, namely hyperglycemia, lack of growth factor support [104] and only recently described hyperlipidemia [105]. Other factors may also play a role such as hypercholesterolemia and hyperleptinemia [44], although their signaling pathways are less clear.

Hyperglycemia can lead to a variety of pathways that interconnect with each other, including activation of polyol pathway [106], increased reactive oxygen species [107], oxidative/nitrosative stress pathways [108, 109], increased advanced glycation end-products [110]. Activation of these pathways can translate into several cellular and metabolic [44] abnormalities that include altered function of critical enzymes and proteins essential to maintain normal nerve function [111], such as sodium/potassium pump, in addition to endothelial dysfunction [25, 112, 113], mitochondrial dysfunction [114], increased inflammation [115], altered transcription [116], reduced regeneration [117], and deficits in axonal transport. Among the common signaling transducers of those events are kinases, such as mitogen activated protein kinases (MAPK) [118] and protein kinase C (PKC) [119] (Figure 3). Additionally, long-term glucose variability may be also an important factor in the development and progression of DN, not only due to periods of hyperglycemia but hypoglycemia as well [120]. Thus, glucose toxicity can critically affect overall neuronal function and predispose the neuron to selective axonopathy [121] by other factors. Such factors may include insulinopenia and growth factors deficiency. This observation is in accordance with results obtained from the Diabetes Control and Complications Trial (DCCT) that shows that even under tight glycemic control, neuropathy still develops [57, 58].

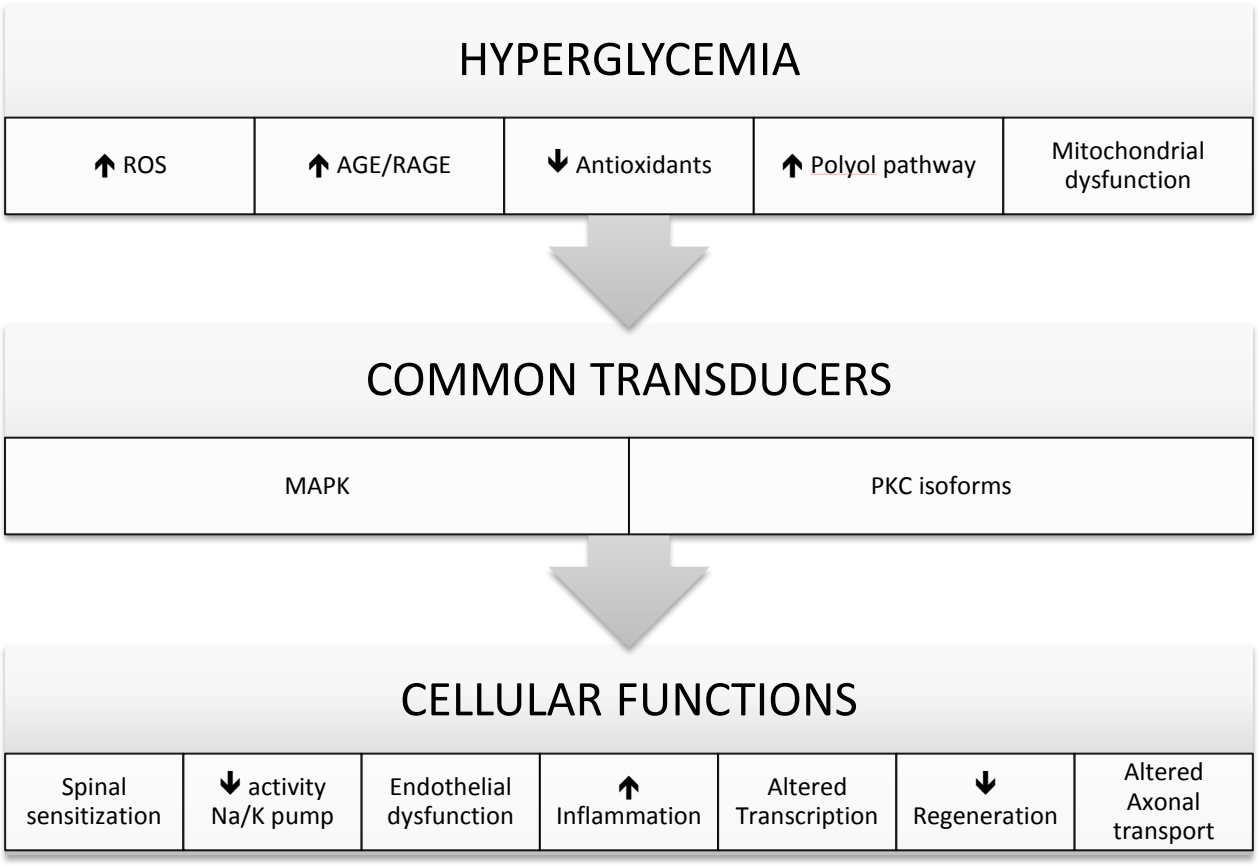

Figure 3. Common pathogenic mechanisms derived from hyperglycemia

Insulin, besides playing a critical role in glucose, lipids and protein homeostasis [1], also serves as an important growth factor to neurons supporting their development and function [122]. Lack of growth factor signaling in diabetes includes deficiency in insulin [62], C-peptide [123] and neurotrophic factors [104, 124], all of which have been implicated in the pathogenesis of DN [125] (Figure 4). Lack of trophic support and signaling may in part explain the dying-back phenotype [44] as a result of deficits in trophic factors transcription, altered expression and distribution of receptors and reduced axonal transport [126]. Deficits in axonal transport may have more deleterious effects at distal portions of the axon and may represent a critical factor in the character of neurodegeneration. Interestingly, recent evidence suggest that lack of insulin signaling may prevail over glucose toxicity in DN [127] and that growth factor deficiency may be a key step in the development and progression of DN [128]. Downstream signaling cascades derived from the insulin and other growth factor receptors and include the phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase 3 beta (GSK3) beta pathway and Ras/Mek/MAPK pathway, among many others [129]. These pathways have a high degree of specificity and numerous sites for crosstalk due to the promiscuous character of growth factor receptors, different types of insulin receptor substrates (IRS) [130] and their differential phosphorylation from positive and negative feedback loops [131] together with tissue-specific actions [132].

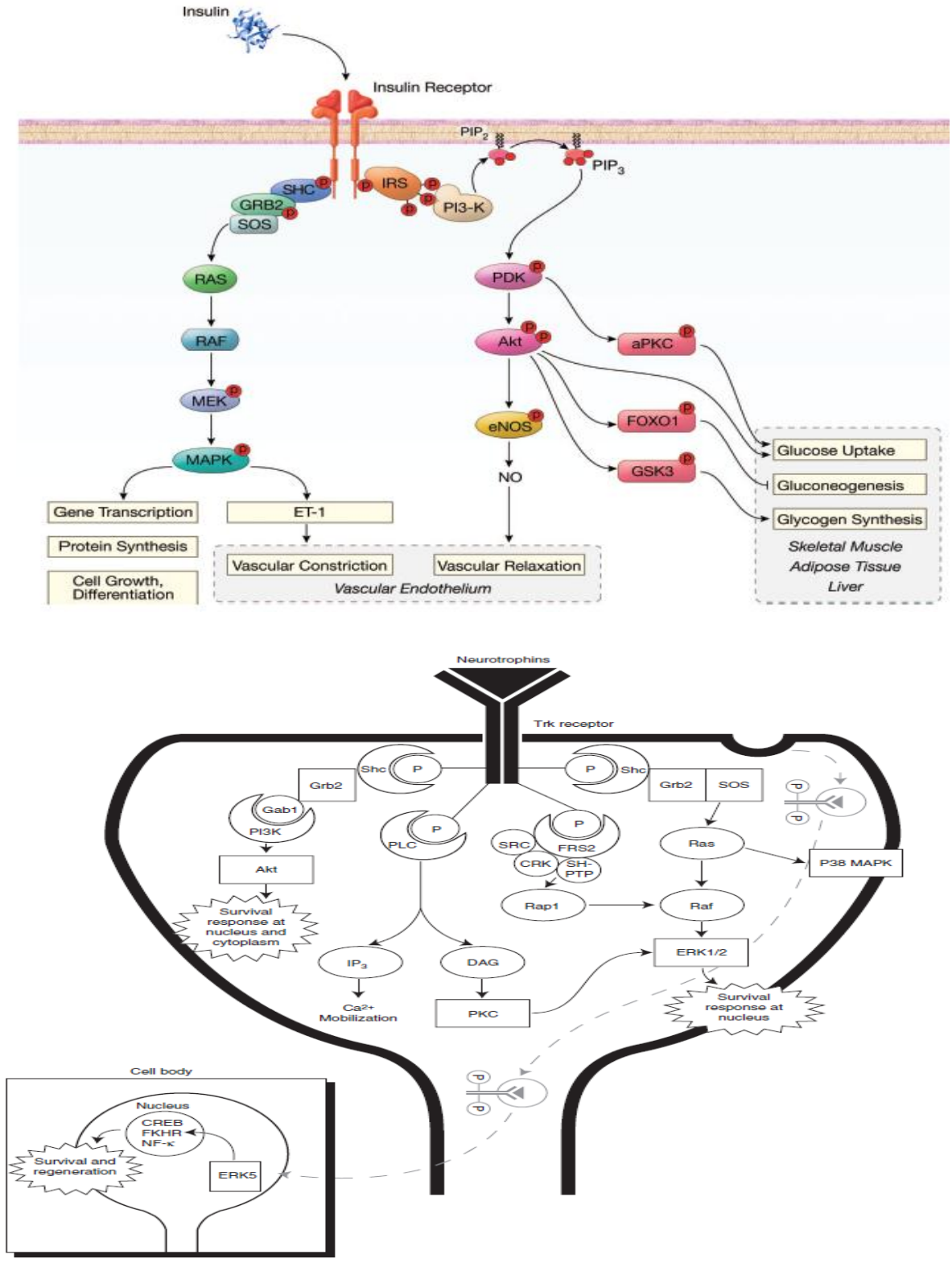

Figure 4. Common insulin and neurotrophin signaling pathways from [104, 133]

One popular kinase in the insulin pathway is GSK3 beta, known to play key roles in not only diabetes but also many neurodegenerative diseases with high potential for drug therapy [134-136]. Moreover, recent reports show that insulin/growth factor treatment in animal models of DN can revert some neurodegenerative parameters [137] via GSK3 inhibition [138]. Furthermore, crosstalk between insulin and glucose signaling pathways may exist and may act synergistically in the neurodegenerative process of DN [62, 139].

h) Therapies for diabetic polyneuropathy

Several clinical trials based mostly on glucose toxicity pathways and some growth factors deficiency have been performed [140]. Many of them have been ineffective, others had serious side effects, only a couple with minor benefits (alpha lipoic acid (antioxidant) [141] and one aldose reductase inhibitor (polyol pathway) [142]) are being used in countries other than U.S.A., and the recently promising C-peptide (growth factor) [143] is still in different clinical trial stages. Additionally, as new signaling pathways are being discovered new emerging drugs are also considered for new clinical trials [144]. Thus, apart from the two drugs with only minimal effects on some indices of nerve dysfunction, there is no available therapy to treat neuronal degeneration.

This failure can be explained by several factors, but some critical ones are evident. First, due to the multifactorial pathogenesis of DN, monotherapies that target one mechanism may not prevent other mechanisms from occurring. Thus, dual peptides that target cross-talk molecules affected by several mechanisms may be more effective [145]. Second, combination therapies may be needed to target non-overlapping pathways. However, for combination therapies to successfully translate into clinical trials with minimum side effects, a consensus in what constitutes the most critical pathogenic

mechanisms need to be reached. Moreover, that lack of a common agreement in the pathogenic mechanism and ineffective clinical trials may also reside on the animal models used to test existing hypothesis [146, 147]. The majority of animal models used for preclinical trials failed to reproduce the hallmark of DN, namely loss of sensation [148]. Along the same line, mechanism derived from these animals may only explain symptoms of pain, which are more or less manageable but cannot explain the key pathogenic mechanisms that lead to sensory loss. Therefore, current pharmaceutical approaches are solely aimed at ameliorating disabling symptoms such as pain [149].

Furthermore, the pathogenic mechanisms proposed so far cannot fully explain the process of neurodegeneration in diabetic nerves, namely ischemia, endothelial dysfunction, and oxidative stress, among others. One mechanism that could better explain the dying-back phenotype of the nerves affected in diabetes might be trophic factor deficiency and defects in axonal transport [150]. On one hand, trophic factor deficiency was thought to be primarily important for the reduced/improper character of regeneration of diabetic nerves [117]. On the other hand, neurotrophic factors and related molecules are also known to be important in the development and maintenance of neurons, thus their deficiency may also play equally important roles in initiating the degeneration process, and that processes of degeneration and regeneration may coexist. In this sense, fast axonal transport is highly linked to trophic actions and defects in both of them may be important in the neurodegeneration process. In addition, deficits in fast axonal transport may result from signaling pathways derived from both glucose toxicity [151] and growth factor deficiency [152].

4. Axonal transport

a) Axonal transport description

Among the many abnormalities described in diabetic nerves, axonal transport deficits may not only contribute but also be sufficient to explain the dying-back phenotype characteristic of diabetic peripheral neuropathies. Due to their extensive length and trophic-dependency, peripheral nerves are highly reliant on intracellular transport to carry essential molecules from the cell bodies to different parts of the axon and distal terminals, as well as back to the cell body for activation of different cellular processes. This type of intracellular transport is widely recognized as fast axonal transport (FAT) and it is mostly based on microtubule-dependent movement of the molecular motors kinesin [153], responsible for anterograde direction (away from the cell body), and cytoplasmic dynein [154], for retrograde direction (towards the cell body). Specificity and timely local delivery of cargoes depends on the ability of molecular motors to bind to microtubules, cargoes, associated proteins and ATPase function [155]. FAT ensures proper function of neurons, as wells as proper maintenance of their connectivity tree for communication with their microenvironment facilitating the processes of development , survival, plasticity and regeneration [156].

b) Axonal transport regulation

Post-translational modifications represent a crucial point of regulation of the function of proteins, phosphorylation being the most common, and most subject to regulation by differential activation of kinases and phosphatases. The large portion of the genome that accounts for kinases and phosphatases suggests the versatility

needed to accommodate the high degree of phosphorylation regulation in cellular functions, axonal transport being one of them.

Molecular motor proteins exist as phosphoproteins in vivo and differential phosphorylation in their functional domains represent crucial points of regulation of their function [157]. The state of phosphorylation of specific functional domains can change their affinity for binding to MT, vesicles and associated proteins or alter their intrinsic ATPase, ultimately leading to changes in FAT that may or may not be specific to certain cargoes. Several kinases have been identified and shown to directly or indirectly alter the phosphorylation state of molecular motors causing changes in their function, such as GSK3 beta and cyclin-dependent kinase 5 (Cdk5) [158, 159]. Tight local balance of the activity of kinases and phosphatases, normally allows transport and delivery of a variety of materials at the proper time and space [160] and when this regulation is out of balance, FAT defects appear and may lead to neurodegeneration.

c) Role of axonal transport in neurodegeneration

Deficits in fast axonal transport deficits have been shown to play crucial roles in many neurodegenerative diseases [156, 161, 162]. Natural occurring human mutations and genetic manipulation in animal models of specific domains of molecular motors are sufficient to produce dying-back degeneration in sensory and motor neurons [163]. In particular, a particular type of hereditary spastic paraplegia (HSP10), a hereditary peripheral neuropathy with motor and sensory deficits, is caused by mutations in a specific kinesin isoform that causes inhibition motor binding to microtubules [164]. This study suggests that other mechanisms that can cause inhibition of microtubule binding

and other molecular motors' function, may also lead to neuropathy in non-genetic cases.

Indeed, in other neurodegenerative diseases of non-genetic origin, altered kinase activities derived from disease-modifying pathways can shift the normal function of molecular motors to a disease one. Several specific kinases have been linked to neurodegeneration by either directly or indirectly phosphorylating molecular motors and altering their normal activity. These include: c-jun activated kinases (JNK) in polyglutamine expansion diseases [165, 166], casein kinase 2 (CK2) in Alzheimer's disease [167] and protein kinase C (PKC) isoforms in Parkinson's disease [168, 169]

5. Axonal transport deficits in diabetic neuropathies and possible mechanisms

Axonal transport deficits have been extensively described for both sensory and autonomic nerves in animal models of DN. These deficits occur at both late and early stages of diabetic neuropathies, probably affecting different processes. Late events are associated with deficits in neuronal regeneration, as indicated by reduced transport of cytoskeleton and structural proteins [170]. Early events are associated with reduced transport of neurotransmitters [171] and trophic factors [126, 172-175] linked to early neuronal dysfunction before signs of overt degeneration are evident. Although fast axonal transport deficits are very well established in diabetic nerves, no mechanism has ever been shown to explain this deficit.

Recently, many pathogenic mechanisms of FAT deficits have been described in other neurodegenerative diseases that include the regulation of molecular motors function by kinases and phosphatases. Activity of many of these kinases, such

as GSK3 beta, p38 and JNK are known to be altered in diabetic nerves as a result of hyperglycemia and/or lack of insulin/trophic factor signaling. These kinases and others may serve as a pathogenic link between deficits of FAT and neurodegeneration in diabetic nerves.

B. General hypothesis and specific aims

I proposed that FAT deficits due to DM-induced changes in kinase signaling pathways and altered molecular motor activity may serve as a key pathogenic mechanism that can better explain the dying back phenotype of DN in both sensory and autonomic nerves. This novel mechanism may represent a crucial target for designing innovative therapeutic drugs aimed directly at main processes of neurodegeneration in diabetic nerves.

Aim 1: ESTABLISH A TYPE 1 DIABETIC ANIMAL MODEL THAT MIMICS THE PHENOTYPE OF INSENSATE NEUROPATHY IN A TRACTABLE TIME

Diabetic neuropathy (DN) manifests clinically by insensate signs with or without pain. Loss of sensation represents the hallmark of the disease and the clinical correlate of anatomical deficits which progresses with the length of the disease. Although insensate neuropathy is the most prevalent, especially in type 1 patients, it is the least reproduced in animal models. Loss of sensation seems to be a late event in animal models and does not always develop in animals commonly used for research due to the short life span. Only recently, some mice models mimicked signs of hypoalgesia but in combination with hyperalgesia as well. Promising drug therapies in animals that later failed in the clinic were based on painfulness models with no signs of loss of sensation and obesity-prone strains, not typical of type 1 diabetes. In order to find successful

therapies aimed at the degeneration mechanisms, a model of diabetic neuropathy in a lean rat strain that mimics loss of sensation in a tractable time to study pathogenic mechanisms is highly needed. Fischer 344 represents a promising strain as a DN rat model. First, it is mostly inbred, thus having a less variable genetic background. Second, it is less prone to obesity, a factor that can independently cause peripheral neuropathy. Streptozotocin (STZ) is widely used as a chemical agent to induce diabetes in animals models and one of the best characterized for DN anatomical and functional changes. I proposed that Fischer 344 rats treated with STZ would develop typical signs of diabetes and would show insensate signs of neurodegeneration typical of human diabetic neuropathy in a tractable time to study pathogenic mechanisms.

Aim 2: CHARACTERIZE THE DEVELOPMENT OF CARDIOVASCULAR AUTONOMIC NEUROPATHY AND ASSOCIATED COMPLICATIONS

Cardiovascular autonomic neuropathy (CAN) is a common but less recognizable aspect of DN. In diabetic patients, autonomic deficits may be present even before signs of sensory deficits. However, this phenotype is less clear in animal models and is not always measured. Autonomic and sensory neuropathy share similar anatomical and physiological deficits, suggesting a common pathogenic mechanism. Therefore, co-evaluation of autonomic and sensory deficits in animal models is crucial to understand pathogenic mechanisms responsible for degeneration and contributing factors for differential neuronal susceptibility to damage. Cardiomyopathy is a common complication associated with CAN, although this relationship is not commonly study in animal models. I proposed that STZ Fischer 344 rats show signs of cardiovascular autonomic neuropathy and associated complications, such as cardiomyopathy.

Aim 3: ELUCIDATE THE PATTERN OF KINASE ACTIVATION PERTINENT TO AXONAL TRANSPORT REGULATION AND EVALUATE THE FUNCTION OF MOLECULAR MOTORS AS A PATHOGENIC MECHANISM OF FAT DEFICITS IN DIABETIC POLYNEUROPATHY

Pathogenic mechanisms in diabetic polyneuropathy include changes in signaling pathways derived from hyperglycemia as well as reduced insulin signaling and neurotrophism. Fast axonal transport is tightly linked to trophic signaling pathways essential for neuronal function and preservation. Moreover, FAT deficits have been widely described in diabetic nerves not only regarding neurotrophins but also signaling molecules and neurotransmitters, further compromising the basic function of the neuron. Furthermore, mutations in molecular motors (kinesin and dynein) are sufficient to cause dying-back degeneration in other neurological diseases. This suggest that FAT deficits may play a crucial role in the development of DN. Proper function of FAT is achieved by activation and deactivation of specific kinases capable of phosphorylating molecular motors and affecting their function, such as binding to vesicles and microtubules. Several kinases have been identified, such as GSK3 beta, JNK and p38, in the regulation of FAT in health and disease. These kinases are also altered in diabetes having the potential of altering normal FAT regulation by changing molecular motor's function. I proposed that a novel pathogenic mechanism of FAT would be present in diabetic nerves that include alteration of one or more kinase signaling pathways important for FAT regulation as well as altered molecular motors' activity at times of evident peripheral neuropathy.

C. Significance

The prevalence of diabetes is increasing at high-speed rates and if this trend continues it has the potential to become the biggest pandemic of all time [176, 177]. Diabetic neuropathy (DN) is of the most common long-term complication of diabetes, which can affect nearly all of diabetic patients at some point in the length of their disease leading to increased morbidity and mortality as well as significant reduction in quality of life. Despite many attempts, there is no current available treatment for DN.

Pathogenic mechanisms proposed so far cannot totally explain the dying-back phenotype characteristic of DN, thus alternative mechanisms need to be explored. As indicated by mutations studies in other types of neurodegenerative diseases and in particular other peripheral neuropathies, axonal transport deficits may be sufficient to explain the dying-back phenotype that other described mechanisms have failed to reach. Impaired axonal transport has been described extensively in diabetic nerves but further research was abandoned due to the lack of a target mechanism.

This work represents the first attempt to describe a putative mechanism of FAT deficits in DN as a result of the common signaling pathways altered in diabetes in which GSK3 beta may play a crucial role. This work also provides a working framework for future investigation into more specific details of the mechanism of FAT in DN. Results from this and future studies can lead to the design of novel therapeutic targets aimed directly at key neurodegenerative processes in diabetic neuropathy that could translate into successful therapies for human diabetic neuropathy.

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II. ESTABLISH A TYPE 1 DIABETIC ANIMAL MODEL THAT MIMICS THE PHENOTYPE OF INSENSATE NEUROPATHY IN A TRACTABLE TIME

A. Summary

Lack of available therapies in diabetic neuropathy (DN) may be a consequence of an incomplete reproduction of DN in animal models. Manifestation of human DN include both pain and loss of perception, the latter being the hallmark of the disease. Many of the available animal models reproduce signs of pain but fail to develop signs of sensory loss. Although both type 1 and type 2 diabetic patients develop DN, DN is more prevalent in type 1 patients, and study of type 1 animal models may shed more light into the principal causes of the disease. The present study showed successful establishment a streptozotocin (STZ) rat model of type 1 diabetes mellitus (DM) that mimics insensate neuropathy for thermal and tactile stimuli in the absence of pain.

B. Introduction

1. Animal models of diabetes mellitus

In diabetes research, there are many animal models of induced, genetic and spontaneous origin that reproduce different aspects of the disease and reflect the principal metabolic disturbances, including carbohydrate, lipid and protein metabolism. Many of these models are shown in TABLE VI [178-181].

Chemically induced models are the most widely used because of their availability and chemicals selective toxicity to beta cells of the pancreas (insulin producing cells).

Because of residual insulin secretion some of them do not require insulin for survival, they are also relatively inexpensive and easy to maintain. Hyperglycemia, sometimes variable, develops as a result of insulin deficiency rather than insulin resistance and other cytotoxic effects may be present in other organs. Despite these useful elements, these models of insulin insufficiency have been variable, partly as a result of strain or species differences, and do not always replicate key features of the human condition. Other models are similarly problematic. Diet- and environmentally-induced DM models develop diabetes as a result of obesity and usually present a wide a spectrum of dysglycemia. Surgically-induced DM avoids the cytotoxicity of a drug but animals may develop gastrointestinal problems and severe cases of hypoglycemia due to lack of enzymes and counter-regulatory hormones present in the excised pancreatic tissue. There are also animal models of spontaneous DM, which are more similar to human DM of unknown etiology. Although they are mostly inbred, many of them require special care and have a high risk of mortality. Finally, transgenic and knockout DM models, although expensive, have been useful to investigate the *in vivo* effect of a single gene/mutation and dissect the complex genetics of type 2 diabetes and autoimmune contributions for type 1 diabetes [178].

In addition to reproducing the common diabetic features, many of the above-mentioned DM models are used to study DM long-term complications. Selection of the animal type largely depends on the types of studies being performed. Aimed at the study of peripheral neuropathies both mice and rats are mostly used. For type 1 DM, the most well characterized models are BB/W rats and STZ-induced diabetic rats. For type 2 DM, db/db mice and BB/Z rat are most often used.

TABLE VI

COMMON DIABETIC ANIMAL MODELS

Type 1	Type 2
Chemically induced	
STZ	Low dose STZ
STZ+ Complete Freud adjuvant	Neonatal STZ
Alloxan	Low dose Alloxan
	GTG treated obese mice
Surgically derived	
Pancreatectomy	Partial pancreatomy
	VHM lesioned dietary obese diabetic rat
Spontaneous/Genetically derived	
NOD mouse	BB/Z
	Obese: ob=Lepdb, ob2J
BB/W rat	Diabetes: db=Lepdb, db2J, db3J,dbad,dbpas,db5j
LETL rat	Agouti: A, Ay, Avy, Aiy, Asy, Aiapy
New Zealand white rabbit	Tubby: tub
Keeshond dog	Fat: Cpefat
Chinese hamster	Zucker Fatty: Fa
Celebes black ape	Zucker Diabetic Fatty
(Macacca nigra)	Koletsky JCR:LA- Corpulaent Rat: Fa (Cp)
	New Zeland Obese mouse
	Japanese KK Mouse
	Nagoya-Shibata-Yasuda Mouse
	PBB/Ld Mouse
	Otsuka-Long-Evans-Tokushima Fatty Rat
	Goto-Kakisaki rat
	djungarian (Siberian) hamster
	South african hamster
	Polygenic by hibrid crosses: BSB Model; AKR/Jx SWR/J Model; GK crosses
Transgenic/knockout	
TCR MHC haplotypes	Insulin Resistance: IR; IRS 1,2; GLUT; Hexokinase II; TNF alpha; Fatty Acid binding protein 2, RAD
	Insulin secretion: GLUT-2;Glucokinase;Hepatic Nuclear Factor; Islet amyloid polypeptide
	Increased body fat: UCP1; beta 3 adrenergic receptors

GTG: Gold Thioglucose; NOD: non-obese diabetic; BB/Z or W: Bio Breeding Zucker or Wistar; LETL: Long Evans Tokushima Lean; VHM: ventromedial hypothalamus; IR: insulin receptor; GLUT: glucose transporters; RAD: Ras associated ; TNF: tumor necrosis factor; STZ: streptozotocin; UCP: uncoupling protein 1; TCR: T cell receptor; MHC: major histocompatibility complex

The BB/W (BioBreeding/Worchester) rat spontaneously develops diabetes in an outbred Wistar rat line (BioBreeding (BB) Laboratories, 1976) [182]. The responsible gene is linked to the major histocompatibility complex [183], as is human type 1 diabetes, and is autosomal recessive with 50% penetrance. The hyperglycemia develops secondary to autoimmune destruction of beta cells within the pancreatic islets of Langerhans. The BB/W diabetic rats are non-obese and develop the disease around the age of sexual maturity. The autoimmune syndrome is more severe than in human type 1, and great care must be taken to avoid death from infection, most commonly involving the pulmonary tract.

The Streptozotocin (STZ)-induced diabetes model, originally characterized in 1963, is described as an atypical type 1 model [184]. STZ is a toxin that selectively enters beta cells in the pancreas and produces deoxyribonucleic acid (DNA) damage leading to apoptosis [185], thereby creating a hypoinsulinemic, hyperglycemic animal within two to four days of injection [186]. STZ is hydrophilic and cannot cross the lipid bilayer of the plasma membrane or the blood brain barrier. It is taken by the cell via GLUT2 glucose transporter, which is expressed mostly in beta cells of the pancreas, but it is also found in small intestine, liver and kidney [187]. One advantage of using STZ is that humans are rather resistant to STZ toxicity due to the low levels of GLUT2 expression and human GLUT2 low transport capacity [188, 189]. The degree of beta cell destruction may vary with the dose used and many STZ animals may secrete residual insulin sufficient for survival [190]. STZ model is generally regarded as in-between type 1 and type 2, since presents destruction of beta cells (type 1) but does not require insulin for survival (type 2). However, this model involves hypoinsulinemia,

rather than insulin insensitivity and therefore has more in common with type 1. In addition, many standard rat strains used for STZ models are prone to obesity, which may complicate the pathophysiology further. The most common rat strains used are Sprague-Dawley (SD), Wistar and Fischer 344 [191]. One marked difference among them is their weight gain after sexual maturity. As shown in Figure 5, Wistar rats gain the most weight. SD rats represent an intermediate case but after 16 weeks (not shown in the graph), their weight is similar to that of naturally obese rats. Fischer 344 rats maintain roughly the same weight during their mature life and may represent a better control for DM studies. The route of STZ administration represents another important variable in the generation of diabetes. Different studies in the literature report the use of intraperitoneal, femoral, and tail vein injections (TABLE VII). STZ has a very short half-life, it is highly unstable and only active for 20 min in plasma, therefore a system that would deliver the drug immediately into the bloodstream would facilitate the diabetogenic effect of the drug [192]. Intraperitoneal injections although faster to perform and needing no anesthesia may affect the STZ final concentration and can elicit an inflammatory response. Femoral vein injection requires a minor surgery with deep anesthesia and assisted post-surgery recovery. Tail vein injection offers significant advantages such as easy vein access without surgery, and a more accurate and fast systemic blood level of the drug [193].

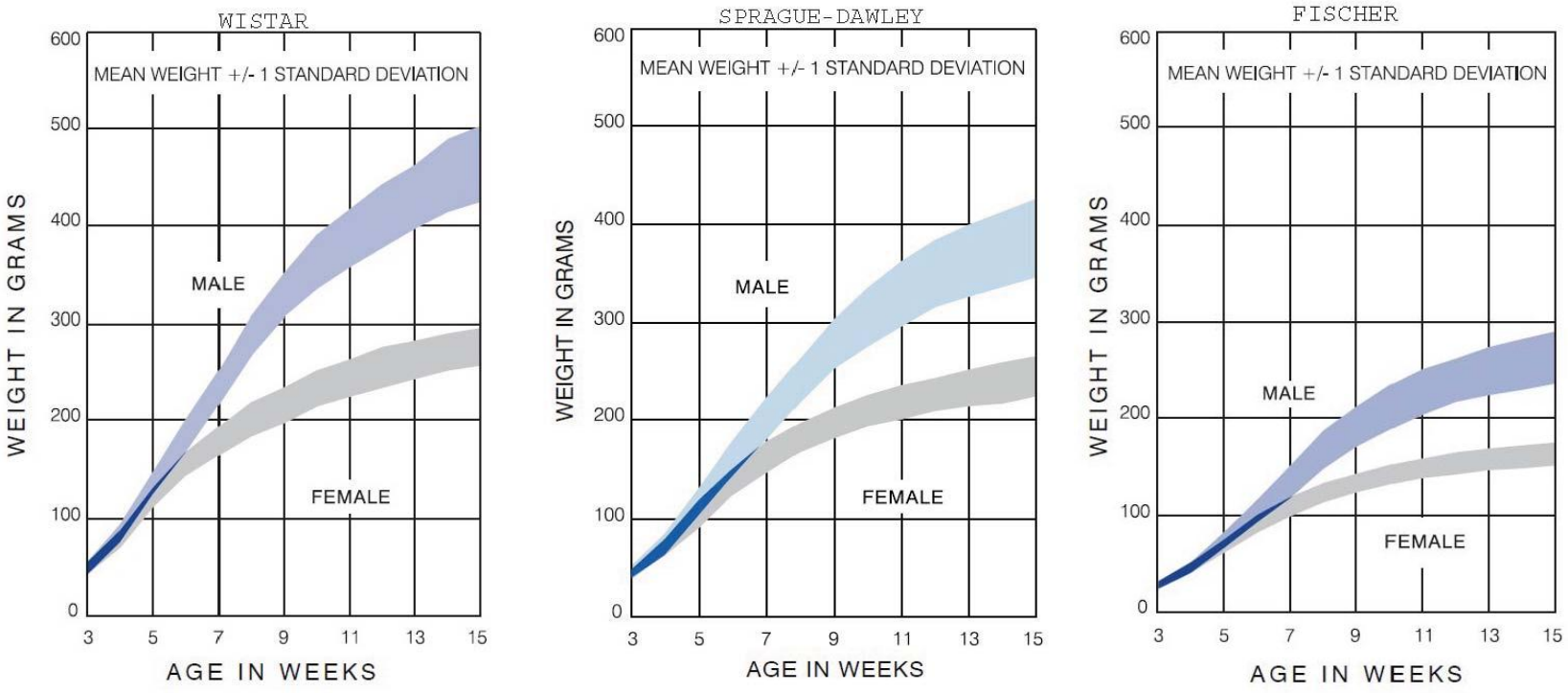


Figure 5. Typical weight of the most typical rat strains used in diabetic studies from [191]

TABLE VII

SELECTION CRITERIA FOR STZ ADMINISTRATION

Criteria	**Tail vein**	**Femoral Vein**	**Intraperitoneal**
Ease of access	external	requires surgery	external
Injection volume (ml)	0.5	0.3	5-10
Level of anesthesia	light	deep	none
Level post-procedural care	medium	high	none
Efficacy drug delivery	high	high	medium
Accuracy dose administration	high	high	medium
Risk of infection/damage	low	medium	high

The most thoroughly studied type 2 animal model is the db/db mouse, derived from an inbred mouse strain in 1966 [194]. This autosomal recessive genetic disorder is 100% penetrant with an onset at approximately 3 weeks of age. The animals are obese and die typically within 6 months of developing diabetes. The genetic cause of this form of diabetes was shown to be a defect in the leptin receptor, creating an unabated appetite and feeding behavior. Soon, the animal's insulin secreting capabilities are overextended, and hyperglycemia and insulin insensitivity develops, generally mimicking the insulin insensitivity seen in type 2 diabetics.

Another emerging type 2 model for the study of DN is the BB/Z rat [195]. This strain was created by classical breeding methods used to introduce the defective leptin receptor gene from insulin-resistant Zucker fatty rats into the inbred BB/W strain background. The BB/Z rat spontaneously develops type 2 insulin-resistant diabetes thatis preceded by obesity and accompanied by hyperinsulinemia, hyperlipidemia, and hypercholesterolemia.

2. Animal models of diabetic neuropathy

To explore fully the different aspects of diabetic neuropathies experimentally, an appropriate animal model must be chosen based on the extent of neuronal damage or dysfunction, in addition to reproduction of the common features of diabetes. TABLE VIII shows the most common animal models used in the field of DN [196], each of which replicates some aspects of the degeneration process.

The BB/W rat develops several neuropathic changes such as slow nerve conduction velocity and most distinguishable from other type 1 models, severe structural changes in both somatic sensory and motor nerves as well as autonomic

TABLE VIII

ANIMAL MODELS OF DIABETIC NEUROPATHY

Abnormalities	Type 1 diabetes		Type 2 diabetes	
	STZ rat	BB/W rat	db/db mouse	BB/Z rat
Functional				
Nerve conduction velocity	+++	+++	+	+
Structural				
Axonal dystrophy	++	+++	+	+
Axoglial disjucntion	++	+++	-	-
Paranodal demyelination	++	+++	+	+
Segmental demyelination	+	+	++	++
Wallerian degeneration	+	+	++	++
Fiber loss	-	++	-	-

STZ: Streptozotocin,

-= absence, +: mild; ++=moderate; +++=severe

from [196]

nerves, similarly to human type 1 diabetes. The STZ rat model of diabetes is relatively resistant to axonal damage, even long-term, [196], but exhibits early abnormalities within sensory [197] and autonomic [198] neurons. Thus, STZ animals represent a useful model to study early pathogenic mechanisms for sensory and autonomic nerves independent of other biochemical changes that may be co-presenting at the time of severe axonal damage.

In contrast to other diabetic models, the db/db mouse develops impairments in nerve conduction velocities that are more pronounced for motor than sensory nerves, although there are differences depending on the genetic background [199] This model develops mild structural damages and progressive axonal atrophy without fiber loss [200]. As with other type 2 models, the BB/Z rats develop a mild progressive axonal atrophy without fiber loss and mild deficits in nerve conduction velocities [127].

Successful drug treatments in animal models have so far not translated into successful clinical trials. Many scientists think that this failure results from the fact that the animal models do not resemble closely enough the human disease, thus other models are constantly being developed, such as NOD mice, ob/ob mice, ZDF rat and GK rat [201]. However, the solution may not lie on finding the perfect animal model, which may never happen, but rather on a better description of existing animal models based on more precise and uniform criteria. Such criteria should be based on discrete and measurable parameters directly comparable across all animal models. These parameters should accurately describe the development and severity of DN based on multiple anatomical, biochemical and functional measurements. Assessment of the success of a particular animal drug treatment should follow similarly well-defined and

uniform guidelines. In fact, the diversity of models may very well resemble the diversity found in the human population, thus a positive drug treatment in several animal models based on the same criteria may have a higher chance of a successful clinical trial. Along these lines, a recent paper published in 2008 proposes unifying criteria to properly define the development of diabetic neuropathy as well as its severity in animal models [202].

3. Common biomarkers of neuropathy used in DN animal models

The diagnosis of diabetic neuropathies in humans is complex and involves a comprehensive clinical examination by a neurologist, biopsies and quantitative sensory, motor and autonomic nerve tests. Likewise, animal models of DN present a syndrome difficult to quantify, although several useful neurological endpoints have been reported [201]. The most well-established endpoints include: nerve morphometry, electrophysiology, nerve conduction velocities, quantitative sensory tests and only recently, intraepidermal nerve fiber analysis.

Nerve morphometric studies use transverse sections or teased nerve fiber preparations to examine the fiber quantity, density, diameter, axon diameter to myelin sheath ratio, axonal atrophy or regeneration, and myelin irregularities such as segmental or paranodal demyelination [96, 203]. Other morphometric studies in ganglia and nerves [198] are also useful to analyze the extent of neurodegeneration in the autonomic system [204]. This type of methods typically is only useful for postmortem analyses and is not well suited for analysis of disease progression.

Nerve conduction velocities (NCV) are used to analyze the overall function of motor [205] and sensory nerves [206]. Classical NCV measurements are obtained by

dividing the distance between stimulating and recording electrodes by the time (latency) for the compound action potential (CAP) to travel this distance. The NCV calculated by using latency carries information mostly for the nerve fibers having the largest axon diameters and the fastest conduction velocities, whereas the smaller axons are under-represented. The velocity is also sensitive to fiber size, myelination status, nodal and internodal length, and axonal resistance, thus a high variability in the measurements is usually observed [207]. Recent methods based on conduction velocity distribution (CVD) obtained by a computer-based mathematical model attempts to increase the sensitivity and specificity of the test [208].

Quantitative sensory testing includes assessment of the degree of sensitivity to touch, cold and hot temperature, vibration and pressure [148]. Several responses can be obtained such as allodynia (perception of pain due to a stimulus that does not normally elicit pain), hyperalgesia (extreme reaction to a normally painful stimulus), hypoalgesia (decreased sensitivity to a painful stimulus) and decreased non-painful sensation. For mechanical responses the most common tests used include Von Frey filaments, pinprick and Randal-Stelitto. Flexible Von Frey filaments are used to study responses to non-noxious mechanical stimuli, whereas rigid Von Frey and pinprick test (needle probing) are used to study responses to noxious ones. The Randall-Sellito test is used to assess responses under different pressures on the paw (rat) or tail (mouse). For thermal responses, a variety of tests can be used including tail flick, hot plate and Hargreaves method. Tail flick test, which does not have an anatomical equivalent in humans, measures the latency for tail movement away from a water bath at different temperatures. Hot-plate test provides rapid stimulation of all paws, tail, and in some

cases -as when used in obese animals- parts of abdominal skin. The Hargreaves method, with radiant heat stimulation applied to the plantar surface of a single paw, measures the latency of paw withdrawal. The tail-flick and hotplate tests are less sensitive in detecting thermal hyper or hypoalgesia, thus the Hargreaves test is most commonly used. These approaches provide information about the sensory modality affected and is well suited for longitudinal studies of changing function in DN.

Intraepidermal nerve fibers (IENF) density is used to measure functional innervation in the epidermis of the foot pad. Quantitation of IENF is presented as the number of fibers per linear mm of epidermis in the fixed tissue. For unmyelinated fibers, antibodies such as protein gene product 9.5 (PGP 9.5) are used [209] among others [210], whereas neurofilament heavy chain (NFH) and myelin basic protein (MBP) are common markers used for analysis of myelinated fibers [211]. The human counterpart of this test is skin biopsies performed and analyzed under strict guidelines [68, 212, 213]. Other immunohistochemistry approaches have been used to evaluate end-organ innervations, such as heart [214, 215], gut [216] and motor end plate [138]. Other non-invasive but also less specific methods have been used with fluorescent transgenic animals [217, 218] to asses cutaneous innervation. Overall, these methods are end-stage analyses, because neuronal function may be affected well before evidence of distal nerve degeneration is apparent [219].

All of these methods have limitations. Morphometric studies and nerve conduction velocity decreases are not enough to demonstrate that the animal has DN. Large amounts of variability can be seen in these parameters in both animals and humans without overt signs of neuropathy. IENF density is sometimes difficult to

quantify and requires sacrificing the animal. For this reason, quantitative sensory testing is the most common method to assess the presence of sensory DN in animals. Because of its behavioral character, it is frequently used as a reference point in advanced characterization of the development and severity of DN.

C. Aim and rationale

The aim of this section was to establish and characterize a type 1 DM rat model that develops insensate neuropathy in a tractable time.

The prevalence of DN is higher in type 1 than in type 2 diabetic patients, probably indicating that the major contributing factor is insulin deficiency rather than hyperglycemia. To study the pathogenesis of DN in a type 1 model, DM was induced by injection of streptozotocin (STZ). STZ selectively kills beta cells of the pancreas rendering the animal hyperglycemic and hypoinsulinemic within days. However, the diabetogenic effect of STZ is highly dependent on species, strain and genetic background in addition to doses and route of administration. The degree of diabetogenic effect of STZ was assessed by measuring plasma glucose and body weight; common biomarkers of diabetes. After establishing the diabetic model, parameters relevant to sensory and autonomic neuropathy were evaluated. Signs of sensory neuropathy include progressive sensory loss with or without pain. Many already established animal models reproduce early painful symptoms, some of them were even able to reproduce both painful in the early stages and insensate in the later stages but no animal model has been able to reproduce pure insensate neuropathy. In this study, quantitative sensory testing was used to precisely evaluate the type of neuropathy present, sensate, insensate or both.

D. Materials and methods

1. Experimental scheme

The following table represents a summary of the set of variables measured at specific times post injections, citrate buffer for control and STZ for DM groups.

Weeks	0	1	2	3	4	5	6	7	8
Glucose	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X
Sensory deficits	X	X	X	X	X	X	X	X	X

2. Animals and experimental groups

Fischer 344 and Sprague-Dawley rats (16 weeks of age, 300 g) were purchased from Charles River Laboratory (Wilmington, MA) and housed two per cage in the Biological Resources Laboratory, University of Illinois at Chicago under pathogen-free conditions. Animals followed a 12:12-h light/dark cycle and had unlimited access to food and water using both automated and bottle systems. After two days of acclimatization, rats were divided into two groups, STZ DM (injected with STZ) and control (injected with STZ vehicle - 0.1 mM citrate buffer pH 4.5). As required by hazard control regulations, animals injected with STZ were housed individually in separate cages in hazard rooms for one week until complete elimination of the drug. After this time, diabetic animals were housed in the same rooms as control ones. Diabetic animals experience polyuria,

diarrhea, and polydipsia. Therefore, bedding was changed as necessary and water bottles replaced to provide comfortable dry housing, and prevent dehydration.

3. Confirmation of diabetic state

a) Glucose levels

Glucose levels were measured by a coulometric electrochemical method using the human blood glucose monitoring system Freestyle (Therasense ®, Alameda, CA) on a drop of tail blood. The meter was calibrated using a control solution and used and maintained following manual guidelines. The measurements were expressed in mg/dL units(U.S. Standard). Glucose levels were measured before injections and only animals with normal glucose values (around 100 mg/dL) were used for the experiments to avoid the inclusion of spontaneous dysglycemia. Because of normal glucose fluctuations and individual susceptibility to STZ and to avoid cases of transient euglycemia, animals injected with STZ were considered diabetic if glucose values were >300 mg/dL. Under this criteria, animals still fell under the diabetic label even if glucose fluctuations occurred. After confirmation of diabetes, glucose levels were monitored weekly until the time of sacrifice.

b) Body weight

Body weight was measured weekly to monitor health status. Empirically, DM animals lose up to 20% of initial body weight as a result of metabolic changes associated with DM. As per Animal Care guidelines, animals need to be euthanized if they lose >20% of their baseline weight or show other signs of distress. STZ DM animals used in the present study remained within healthy limits, thus no animals were

euthanized within this study. Body weight was measured before injections to adjust STZ dose and weekly until time of sacrifice to monitor healthy progress of diabetes Other parameters characteristic of the DM state were monitored, such as polydipsia (increased necessity for fluids intake), polyuria (increased urination) and diarrhea. All STZ DM animals showed the above mentioned signs of DM

4. Quantitative sensory testing

Sensory deficits were measured in collaboration with Dr. Wang's laboratory in College of Pharmacy, UIC. Stress can modulate sensory responses, therefore an acclimation period of 30 min or more was allowed before the start of testing. Thermal responses were measured using the Hargreaves method [220] and sensory responses using von Frey filaments [221]. Both tests were performed before injections for baseline measurements and weekly thereafter until 8 weeks post-injection. Von Frey filament test was performed first, to avoid any paw desensitization caused by heat, and allowed 30 min between tests. In order to avoid any circadian variation in the responses, all tests were performed commencing at 9 AM.

a) Von Frey Filaments

A floorless fiber glass box with separators for four animals (18 x 8 x 8 cm) was placed on top of an elevated mesh floor for paw access. Rats were placed in the individual cages and behavioral accommodation was allowed until cage exploration and major grooming activities ceased. The mid-plantar of both hind paws was tested. Using the up and down method of Dixon [222, 223], 50 % threshold, meaning the average force in grams needed to elicit a positive response, was calculated for left and right paw and then averaged for each animal.

Stimulus presentation

The paw was touched with 1 of a series of 8 von Frey hairs with logarithmically incremental stiffness (0.4, 0.6, 1, 2, 4, 6, 8 and 15 g) (Stoelting). The Von Frey hair was presented perpendicular to the plantar surface with sufficient force to cause slight buckling against the paw and sustained for 5 s. A positive response was recorded if the animal withdrew the paw. The cut-off of a 15 g hair was selected as the upper limit for testing, since higher filaments could raise the limb by themselves independently of the type of response

Fifty percent paw withdrawal threshold testing

The 50% withdrawal threshold was determined using the up and down method of Dixon (1980) [222]. In this paradigm, testing was initiated with the 2.0 g hair, in the middle of the series. Following, if the response was a)negative, the subsequent stronger stimulus was presented ;b) positive, the following weaker filament was used. Optimal threshold calculation by this method requires 6 responses in the immediate vicinity of the first positive response at which time the 2 responses straddling the threshold were retrospectively designated as the first 2 responses of the series of 6. Four additional responses to the continued presentation of stimuli that were varied sequentially up or down, based on the rat's response, constituted the remainder of the series. Thus, the number of actual responses collected using this paradigm can vary from a minimum of 4 (in the case of paw withdrawal sequentially to the 4 hairs in the descending range 2.0-0.4 g) to a maximum of 9 (in the case of the first withdrawal occurring on the fifth

ascending stimulus). The pattern of positive and negative responses was tabulated using the convention, X= withdrawal; 0 = no withdrawal, (see example in Appendix A)

and the 50% response threshold was calculated using the formula:

$$50\%\ g\ threshold = \frac{10^{[\mathrm{Xf}+\mathrm{k}\delta]}}{10{,}000}$$

Xf = final von Frey filament used (in log units)

k= tabular value for the pattern of positive/negative responses see Appendix B

δ= mean difference (in log units) between stimuli

b) Hargreaves method

Thermal paw-withdrawal threshold was quantified by the method of Hargreaves [220]. Rats were placed 4 at a time under an inverted clear plastic box (18x8x8 cm, L x× W x H) on top of an elevated glass floor and allowed to acclimate. Frequently, individual cages needed to be cleaned of urine to keep constant heat conductance. After the acclimation period, the radiant heat source was positioned under the glass floor directly beneath the hind paw. A trial was started by a switch, which activated the radiant heat source and electronic timer. The radiant heat source consisted of a high intensity fiber optic lamp (250 W) (Hugo Basile Biological Research Apparatus Model 737150), powered by a variable voltage AC power supply, located 40 mm below the glass floor and projecting through a 5 mm x 10 mm aperture from the top of a movable case. A photoelectric cell aimed at the aperture detected light reflected from the paw and turned off the lamp and the electronic clock when paw movement interrupted the reflected light.

In the absence of withdrawal, both timer and lamp were set to shut off after 25 sec to prevent damage to sensory fibers. In pilot experiments, the infrared beam intensity directed on the hindpaw was adjusted to give a response latency of less than 15 seconds in control animals by lowering the power supply voltage to 50V.

E. Results

1. Protocol of diabetes mellitus induction

Initial studies on SD rats (data not shown), showed a marked difference in weight between control and diabetic. Diabetic animals were losing more than 50% of their weight when compared with their age control group. At the beginning of the studies, control groups weighed 300g. After two months, their weight averaged 500 g, their glucose levels were variable and in several measurements plasma glucose was above normal. The SD control group showed some characteristics of metabolic syndrome/pre-diabetes (obesity, higher than normal glucose levels), which are known to be risk factors for peripheral neuropathies. To avoid these types of issues, Fischer 344 rats were chosen to continue the studies, since they represent a better control than other species that become obese.

The optimal route of administration was established based on different criteria shown in TABLE VII. Both femoral and tail vein injections were performed. Tail vein injection was chosen due to its easy access, more accuracy on final STZ concentration and less chance of infections. Figure 6 shows the protocol generated for induction of DM. DM 0-12 indicates the number of weeks post-DM induction.

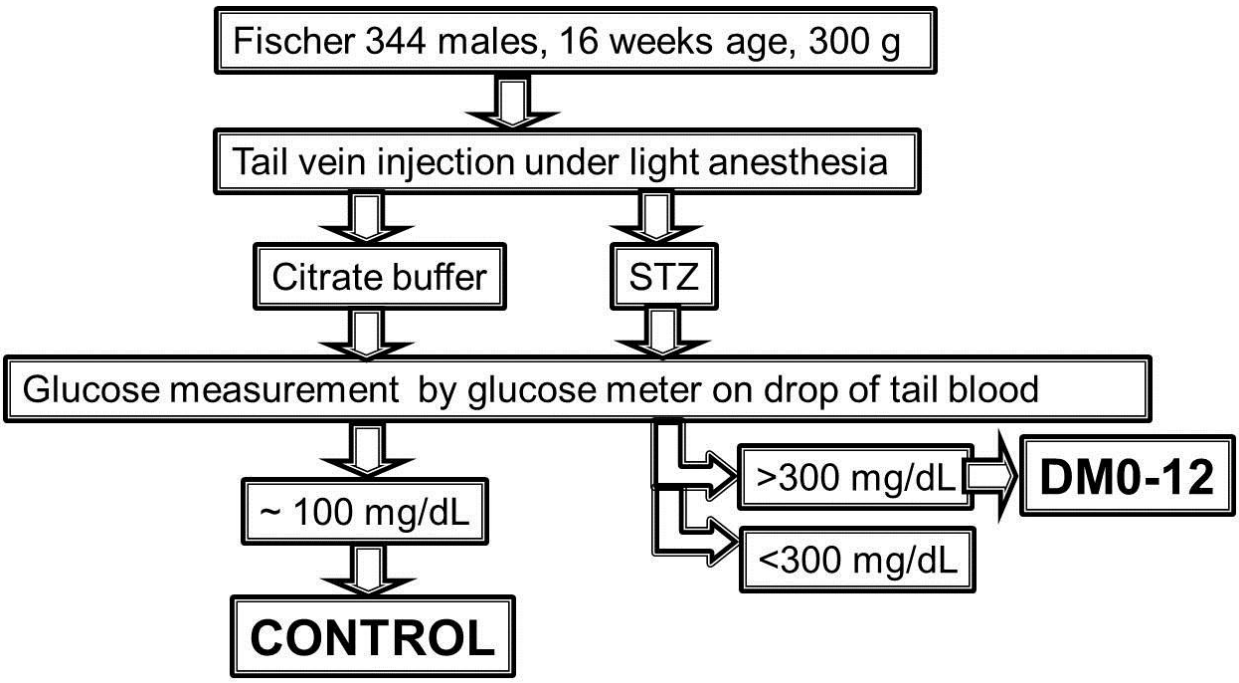


Figure 6. Protocol for DM induction

2. Single i.v. injection of 35mg/kg was the optimal concentration to generate DM in Fischer 344 rats

STZ produces diabetes of dose-dependent severity in relationship to the strain used. Thus, several STZ concentrations were tested in Fischer 344 via tail vein injection, ranging from 25 to 60 mg/kg (TABLE IX). Age-matched control animals were injected with saline used to dilute STZ (0.1 M citrate buffer, pH 4.5). Glucose was measured before (data not shown) and after one week of STZ injection for all the animals. The initial protocol used for SD rats required fasting before STZ injection to increase drug toxicity (data not shown). For Fischer 344, fasting increased the sensitivity for STZ (60 mg/kg), resulting in high mortality, probably due to hyperglycemic shock. Therefore, fasting was excluded from the protocol for subsequent injections. After several trials with varying concentrations, the optimal STZ dose was determined at 35 mg/kg yielding the maximum number of diabetic animals with the lowest mortality.

3. Fischer 344 rats showed sustained hyperglycemia and inability to gain weight typical of diabetic state

Some animals are reported to need several STZ injections to maintain hyperglycemia for long periods. To test if a single STZ injection of 35 mg/kg was sufficient to maintain diabetic levels throughout the experimental timeline of 8 weeks, glucose levels were measured weekly. As shown in Figure 7, diabetes was evident as early as 1 week and glucose values remained above the cut off value of 300 mg/dL for the entire length of the experiment.

TABLE IX

OPTIMAL STZ DIABETOGENIC DOSE OF 35MG/KG FOR FISCHER 344 RATS

STZ (mg/kg)	Fasting before STZ injection	Dead	Diabetic	Non Diabetic
60	yes	100%	-	-
45	no	83%	17%	-
35	no	13%	74%	13%
30	no	-	13%	87%
25	no	-	33%	67%

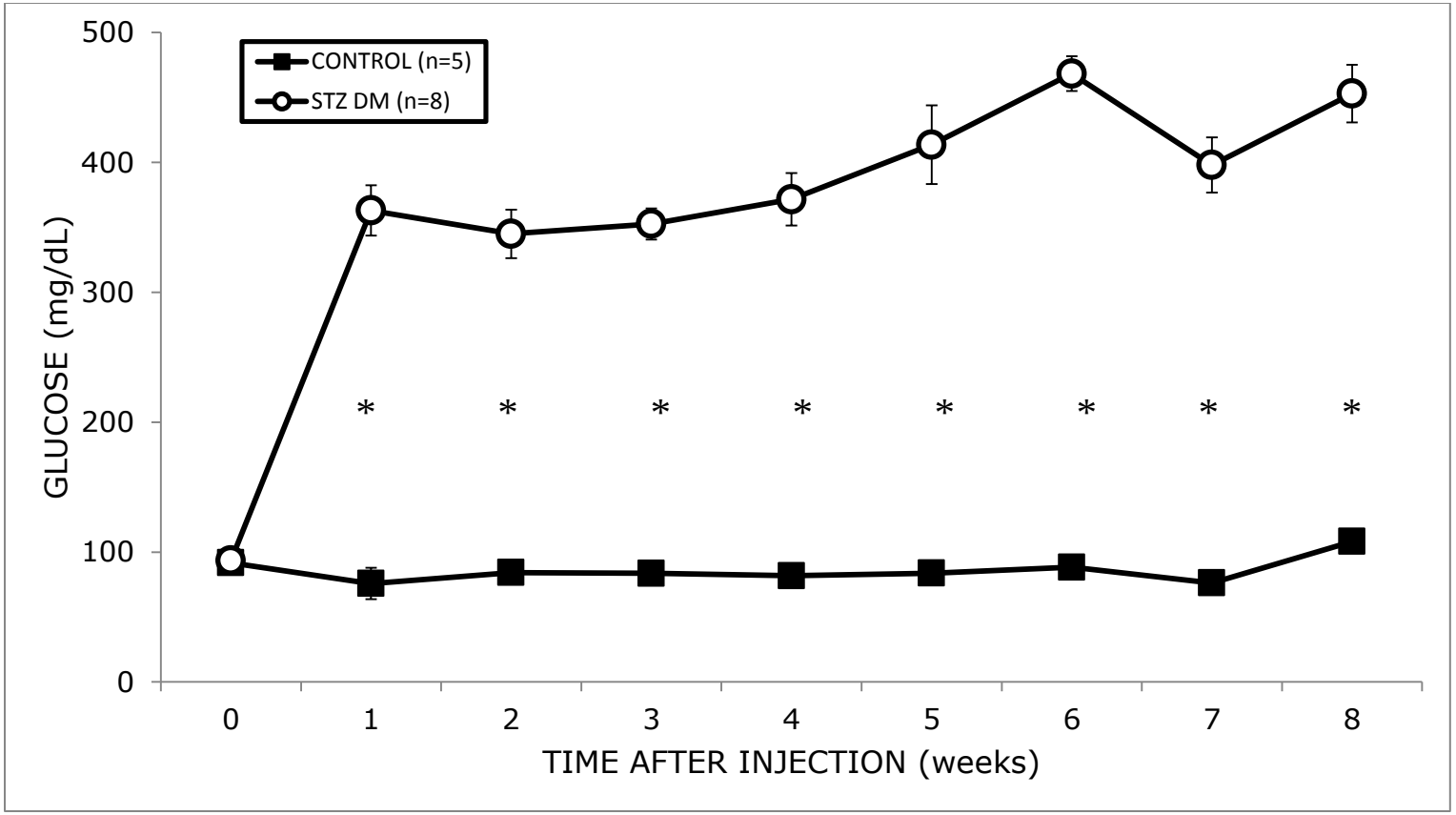


Figure 7. Sustained hyperglycemia obtained by single i.v. injection of 35mg/kg in Fischer 344 rats

Data is plotted as mean±SEM

ANOVA * $p<0.001$

Diabetic animals can lose up to 15%-20% of their weight as a result of the common metabolic changes in diabetes. For this reason, along with weekly glucose measurements, animal weights were also obtained. As shown in

Figure **8** diabetic animals lost 10-20% of their initial weight soon after DM induction throughout the experiments when compared with age-matched control animals and only 10% of their weight from the beginning of the studies. When compared to controls, diabetic animals were unable to gain weight, a typical sign of type 1 diabetes. Within 3 days of STZ injection, animals also developed other typical signs of diabetes including polydipsia, polyuria and diarrhea (data not shown).

4. STZ DM Fischer 344 rats developed reduced sensitivity to tactile stimuli and thermal hypoalgesia

Behavioral responses of control and DM animals to non-noxious tactile stimuli were evaluated using Von Frey filaments of calibrated forces using the up and down method of Dixon (1980) before injections (data not shown) and after injection, weekly for the length of 8 weeks (Figure 9). This method estimates the average force in grams that is needed to elicit paw withdrawal. As early as 5 weeks post STZ injection, DM animals developed decreased sensitivity to tactile stimuli as exemplified by an increased threshold, meaning a greater force was needed to elicit a positive response. The tactile insensitivity of DM animals progressively worsened throughout the testing period, reaching its maximal difference when compared with control animals at 8 weeks. Although there was no statistical difference at week 6 and 7, it still showed a tendency towards a less sensitive pattern, which became evident at 8 weeks.

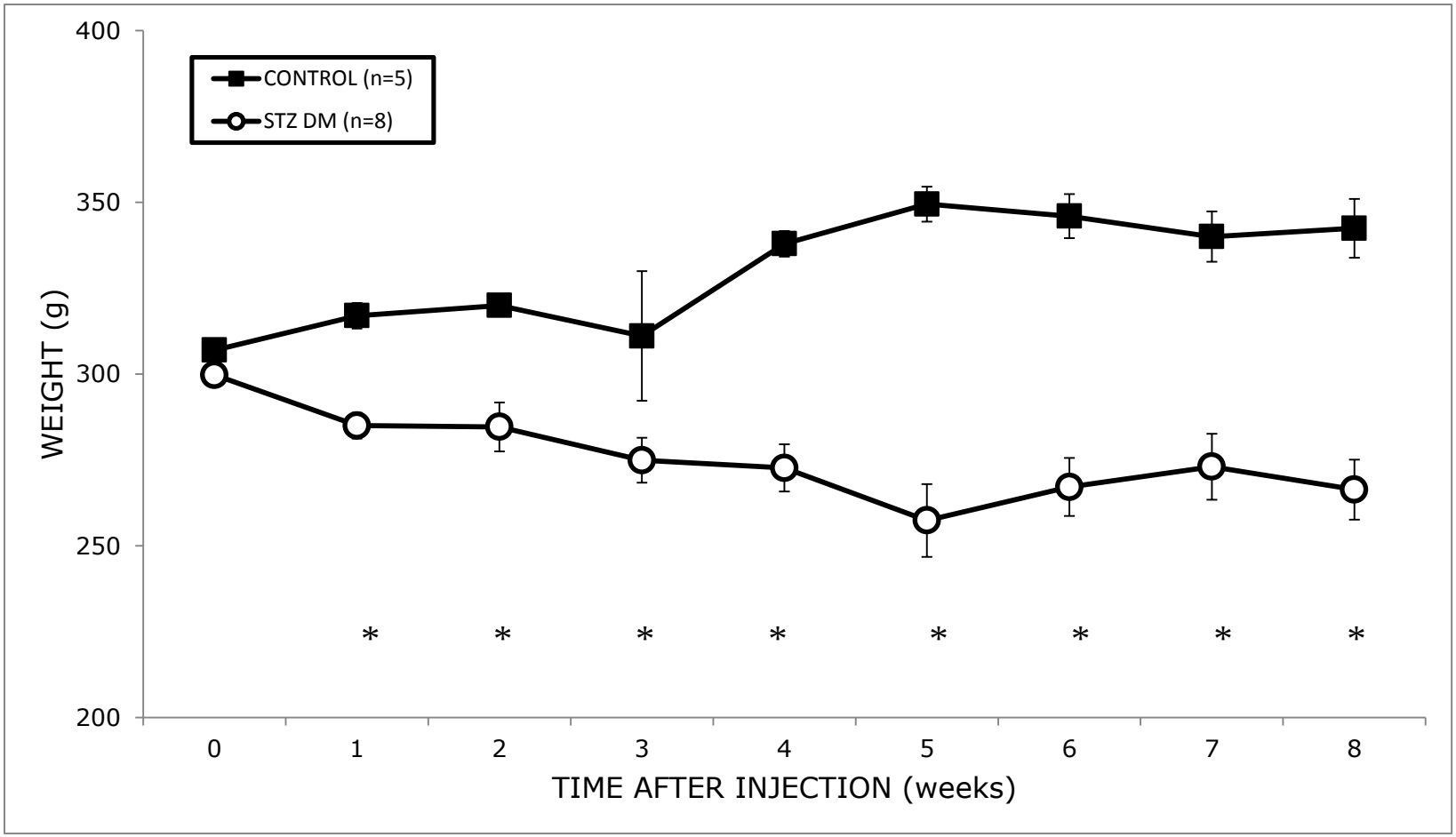


Figure 8. Inability to gain weight in STZ DM Fischer 344 rats

Data is plotted as mean±SEM
ANOVA *p< 0.001

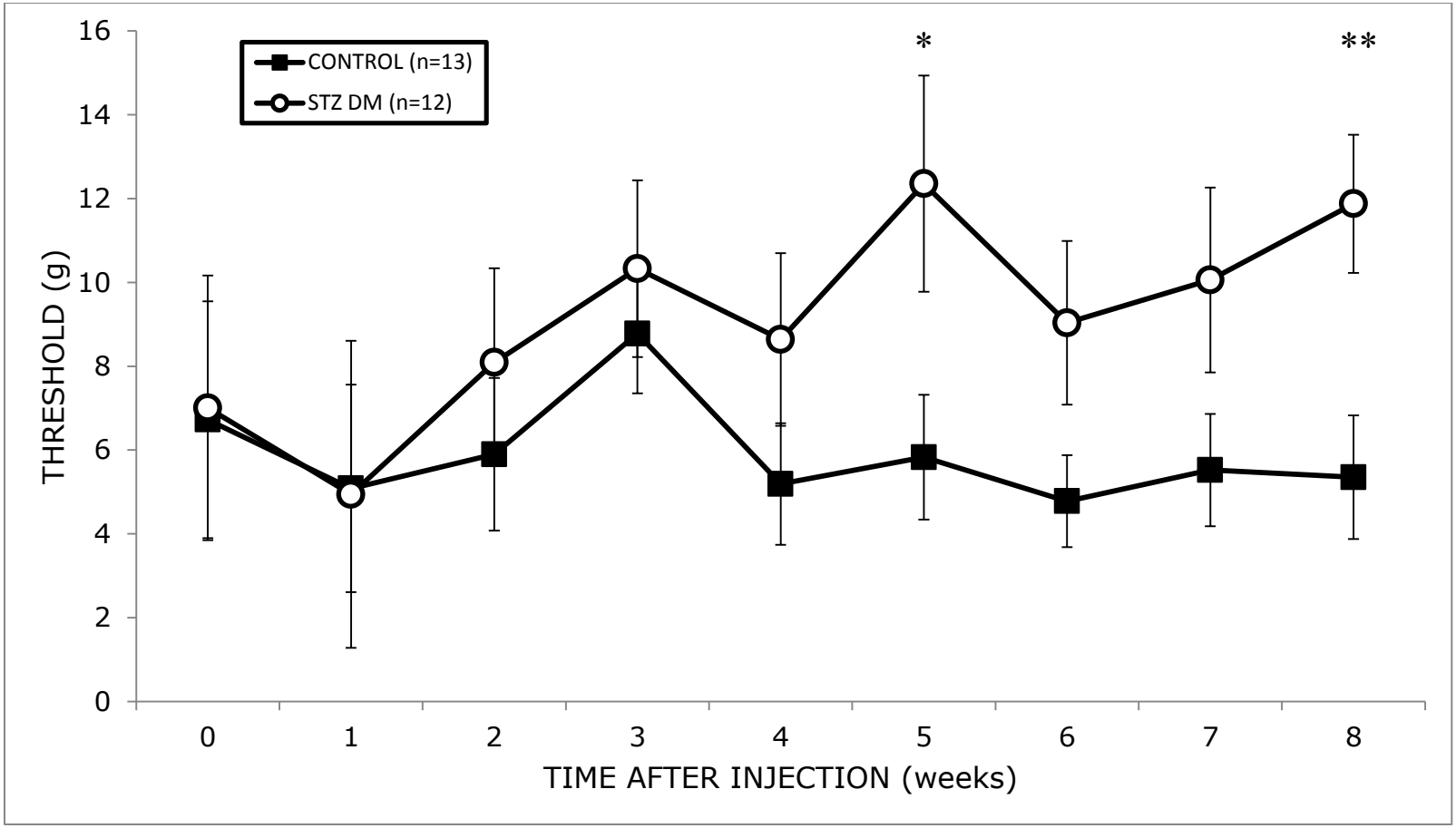


Figure 9.STZ DM Fischer 344 rats developed decreased sensitivity to tactile stimuli

Data is plotted as mean±SEM

Mann-Whitney Rank Sum Test: control vs STZ DM * $p < 0.05$; ** $p < 0.005$

Behavioral responses of control and DM animals to noxious thermal stimuli were assessed by the Hargreaves method before injections (data not shown) and after injection, weekly, for the length of 8 weeks. This method measures the time in seconds (latency) from the start of the thermal stimuli to the paw withdrawal. As shown in Figure 10, when compared to control animals, DM animals developed hypoalgesia (decreased sensitivity to painful stimuli) to thermal stimuli starting at week 4 post injection ($p<0.05$). DM animals progressively lost thermal sensitivity from week 5 until the last measurement in week 8 ($p<0.001$).

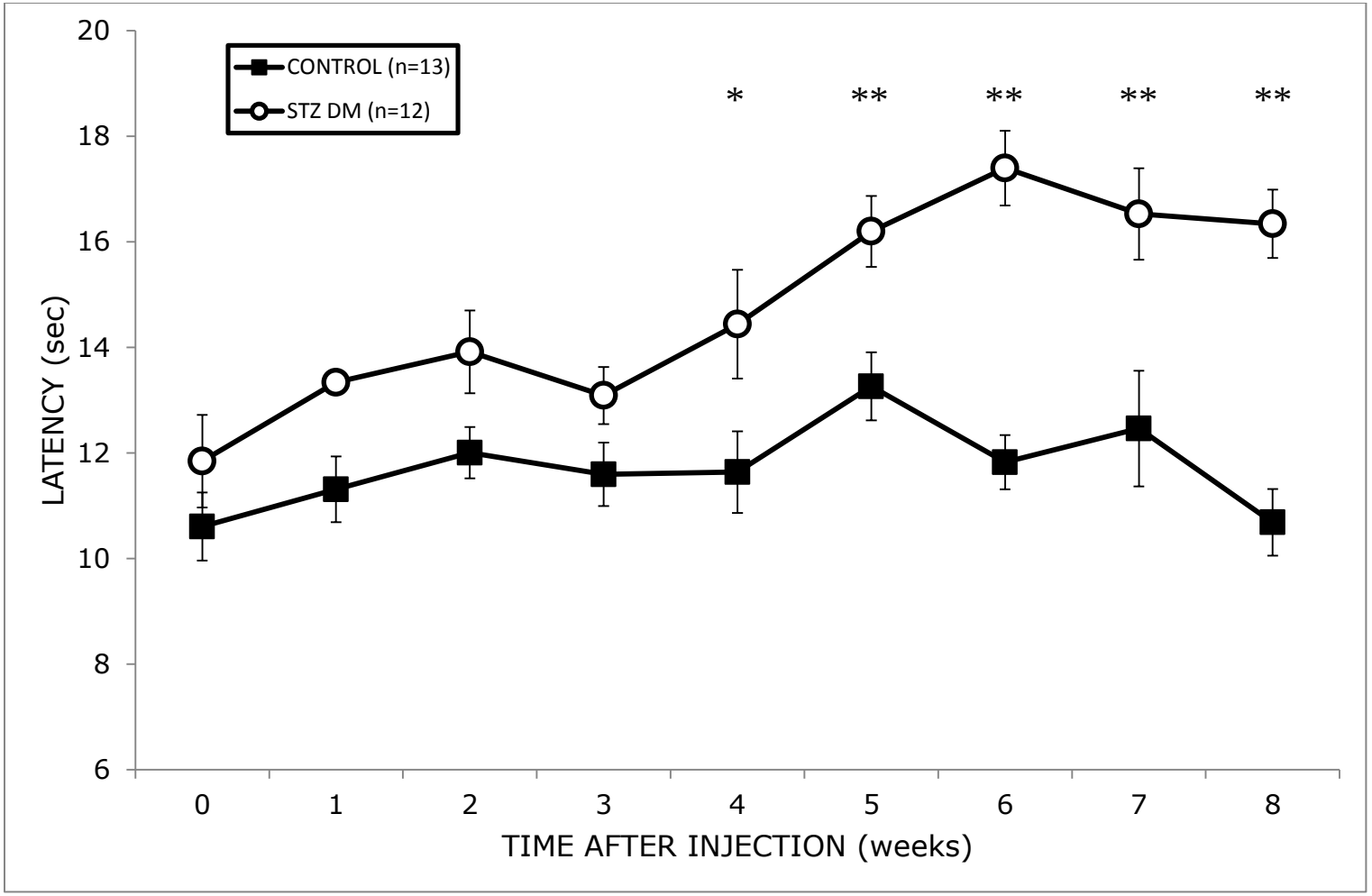


Figure 10.STZ DM Fischer 344 rats developed thermal hypoalgesia

Data is plotted as mean±SEM
ANOVA control vs STZ DM: * $p<0.05$; ** $p<0.0001$

F. Discussion

Several combinations of STZ doses, routes of administration and initial weight and age of animals have been used to generate diabetes in Fischer 344 rats. These include: 25mg/kg for 270-300g animals via i.v. [224], 35mg/kg at 2 months old via i.p. [225], 4 months old [226] and 8 weeks old [227] animals, 45mg/kg for 250-300g via i.p. [228], 3-4 month old males via tail vein [229], 55mg/kg via i.v. [230], 70mg/kg via i.p. [231], 75 mg/kg via i.v. [232]. In the present study the dose of STZ of 35 mg/kg of STZ was used and was sufficient to produce DM. Moreover, a single dose of 35mg/kg via tail vail injection was sufficient to maintain a steady diabetic state, as measured by sustained hyperglycemia during the length of the studies (8 weeks post STZ injection).

Some STZ models require insulin for survival if healthy parameters, such as adequate weight, are not reached. STZ rodents may only live up to a year with the disease but require insulin therapy to avoid excessive weight loss after 12 weeks of diabetes [233]. In the present study, diabetic animals remained within healthy weight limits and insulin was not required for survival. This model represents several advantages, which in addition to simplifying the protocol did not required the use of insulin, a key variable important to control when studying the pathogenesis of DN. Acute insulin treatment may lead to some form of neuropathy and prolonged used of insulin may alleviate some of the symptoms .

Several groups have reported both sensate and insensate neuropathy in diabetic animal models. In particular, STZ models show mechanical hyperalgesia with thermal hypoalgesia only [234] or early thermal hyperalgesia that evolves into hypoalgesia at later stages [235, 236]. In this study, STZ DM Fischer 344 rats showed both reduced

tactile sensation and thermal hypoalgesia with no signs of hyperalgesia or allodynia. This pattern of response is in accordance to recent studies that show deficits in sensory perception in STZ mice for tactile stimuli [211], indicative of myelinated fiber dysfunction, and thermal stimuli, indicative of unmyelinated C-fiber dysfunction [217]. In the present study, thermal hypoalgesia preceded signs of sensory loss, indicating that smaller fibers may be more vulnerable to damage than larger ones. The longitudinal study allowed weekly observations for both types of tests, and unless pain developed in a transient way in between weeks, no indication of pain development was observed. In other animal models where hyperalgesia develops transiently in the early stages, pain usually appears acutely after onset of diabetes and remains for a maximum of 4 to 12 weeks before disappearing or progressing into a hypoalgesic state. Nevertheless, for a more complete study of behavioral symptoms, responses to other types of tests may be measured in future studies including: testing monofilaments using methods other than up and down (i.e. average response to single monofilaments), pressure stimulation (i.e. Randal-Sellito) [237], noxious mechanical stimuli (i.e. rigid monofilaments, needle pinprick), noxious cold temperature (i.e. acetone) and chemogenic pain (i.e. acute and late phase of formalin injection, bradykinin, carrageenan, complete Freund's adjuvant) [238].

Moreover, for drug testing purposes and to better complement behavioral symptoms with physiological and anatomical aspects of degeneration, a careful and longitudinal study on those parameters should be also performed in the future. For physiological parameters, electrophysiology studies for motor and sensory nerve conduction velocities can be measured [239]. Early anatomical events, such as skin

denervation, could be measured by histological studies for myelinated [211] and unmyelinated fibers [240, 241].

The diabetic model described in this study represents the only rat model described to date that shows thermal hypoalgesia and reduced tactile sensitivity without signs of pain. Mechanical hypoalgesia was only described for diabetic mice. Differences in sensitivity with other rat strains could be attributed to inherited genetics of the Fischer strain. Normally, Fischer rats appear to be more sensitive to thermal and tactile stimuli than SD rats [242]. Moreover, Fischer rats are believed to be inflammation-resistant due to heightened HPA axis activity and elevated corticosteroid levels [243, 244], probably the reason behind using this strain for transplant studies.

In conclusion, the STZ Fischer 344 rat model characterized in this aim showed for the first time a STZ rat animal model capable of reproducing both thermal and tactile hyposensitivity independent of pain, despite its short life.

III. CHARACTERIZE THE DEVELOPMENT OF CARDIOVASCULAR AUTONOMIC NEUROPATHY AND ASSOCIATED COMPLICATIONS

A. Summary

Cardiovascular autonomic neuropathy (CAN) is a frequent but usually neglected aspect of DN. In particular, in diabetic patients, CAN strongly associates with sensory neuropathy; however, it is not clear if CAN correlates better with painful or insensate neuropathy. In addition, increasing evidence exists showing that CAN may be a key contributor to cardiomyopathy, in particular left ventricular dysfunction, in the absence of atherosclerosis and hypertension, especially in type 1 patients, but may also contribute to overall cardiac pathology in type 2 patients. Furthermore, cardiac sensory afferents are also affected, as indicated by the high rate of painless myocardial infarction in people with DM.

Results obtained from this study revealed presence of CAN exemplified by reduced cardiac norepinephrine. In addition, cardiac sensory markers were altered in DM animals suggesting early dysfunction of cardiac afferent fibers Moreover, echocardiographic measurements revealed early left ventricular dysfunction with cardiac dilation and myocardial stiffness. Presence of CAN co-presented with insensate neuropathy and the timely analysis of the presentation may indicate that autonomic fibers may be affected before sensory ones.

B. Introduction

1. Peripheral nervous system

The main function of the peripheral nervous system (PNS) is to convey information from the periphery to the central nervous system (brain and spinal cord) via afferent fibers and from the central nervous system to the periphery via efferent fibers. The PNS is a collection of spinal and cranial nerves with different combinations of sensory and motor neurons. The organization of PNS and interconnections between somatic and visceral nerves are of extreme relevance to understand disease processes involving the peripheral nervous system [245].

a) Somatic nervous system

The somatic part of nervous system consists of afferent sensory nerves that carry conscious sensory perception (somatic sensory) and efferent nerves that innervate voluntary muscles (somatic motor)

Somatic sensory neurons are responsible for transmitting sensory information (position, temperature, vibration, pressure, touch and pain) from the periphery to the CNS. Sensory neurons are pseudo-unipolar cells, whose cell bodies reside in the dorsal root ganglia with two distinct processes, one reaching the periphery and the other one extending into the CNS. Damage to somatic sensory neurons can elicit loss of sensory perception and/or pain.

Somatic motor neurons are efferent neurons, namely lower motor neurons, whose cell bodies reside in the spinal cord and brainstem leaving the CNS through motor roots and branching out to innervate neuromuscular junctions. The activity of lower motor neurons are controlled by built-in patterns of neural connections (reflexes),

descending pathways via upper motor neurons, whose cell bodies reside in the motor cortex, and higher centers that influence upper motor neurons (association cortex, basal ganglia, cerebellum). Damage to lower motor neurons causes weakness and muscle wasting [245].

b) Visceral nervous system

Similarly to the somatic, the visceral part of the nervous system consists of afferent sensory fibers that carry unconscious sensory perception from the viscera (visceral sensory) and motor efferent fibers that innervate involuntary muscles (Autonomic Nervous system (ANS)). The ANS can be further subdivided into three major categories: sympathetic (Sy), parasympathetic (PS) and enteric nervous system [246]. The enteric nervous system is composed of neurons that reside within the gut wall. It contains intrinsic primary afferent neurons, interneurons and motor neurons responsible of coordinating gut motility independent of CNS. And modulation of gut motility is performed through connections with CNS via Sy and PS neurons [247]. Damage to these neurons may cause gastrointestinal problems in many disease states [248], such as in diabetes [249]. Similarly to the enteric nervous system, other sets of intrinsic neurons that do not project to the CNS were found in the cardiac [250], bronchial and tracheal ganglia.

Just like somatic neurons, Sy and PS neurons reside in both the CNS and PNS, orchestrating the activities of tissues and organs through sensory afferents and motor efferents. However, motor efferents follow a two-neuron system with specific characteristics, namely preganglionic and post-ganglionic neurons. All preganglionic neurons originate in the CNS, are finely myelinated and use acetylcholine as

neurotransmitter. They synapse with post-ganglionic neurons in pre-vertebral or paravertebral ganglia, in the case of Sy, or in scattered ganglia located near the end organ, in the case of PS. Post ganglionic Sy and PS neurons differ in their neurotransmitter, namely acetylcholine for PS and norepinephrine for Sy. Both types of neurons can also use small peptides as co-transmitters, such as vasoactive intestinal peptide (VIP) [251], calcitonin gene-related peptide (CGRP) [252] and substance P, as well as gaseous neurotransmitters, such as nitric oxide (NO) [253]. Another difference between Sy and PS nervous systems is the origin of nerves, subject of the traditional division of ANS. The Sy nervous system is composed of thoraco-lumbar outflows that innervate visceral organs and pathways that supply head, neck and intra-cranial arteries. The PS is composed of cranial-sacral outflows that innervate via post-ganglionic neurons, structures in the head, neck, abdomen and pelvis. Damage to autonomic neurons, independent of their nature, can cause a wide range of symptoms and loss of coordination of basic organ functions, common in DM [254, 255].

Visceral afferent neurons usually run in sets of nerves together with Sy and/or PS nerves or alone in a pattern that is particular to the species, sex and organ innervated. One example is the vagus nerve in which 25% of its fibers are PS efferent and the rest are afferent fibers with different degrees of myelination that monitor mechanical and chemical events in the respiratory tract, cardiovascular organs and gastrointestinal tract. Furthermore, visceral sensory and somatic sensory neurons are known to interconnect at several levels and monitor certain organ functions. One example is cardiac pain (e.g. during ischemic heart disease). Cardiac pain is thought to be mediated by spinal visceral afferents and some involvement of vagus nerve that in turns activate certain

spinothalamic tract neurons in the superficial dorsal horn (somatic sensory pathway) responsible for sensations in neck, shoulder and jaw. Also, some phrenic (diaphragm) afferents that innervate the pericardia project directly to cervical segments which also contain neurons with similar convergent synaptic inputs that form vagal, spinal visceral and somatic afferents projecting to more caudal spinal thoracic, lumbar and sacral regions [256]. The latter pathway and others, not well described in the literature, may be more relevant in women, who often lack typical signs of heart attack and may often experience milder, more flu-like symptoms instead.

2. Diabetic autonomic neuropathy

Diabetic autonomic neuropathy (DAN) is frequent and co-presents at different degrees with either painful or insensate sensory neuropathy. Although DAN is not traditionally considered part of DPN definition, many experts feel a need to include DAN as a part of a larger category of DPN, because of their high degree of co-occurrence and possible common pathogenic mechanism. DAN is a frequent and serious complication of diabetes affecting patients at subclinical and clinical levels. It manifests with a wide array of symptoms involving nearly all the systems in the human body, such as cardiovascular, gastrointestinal (gastroparesis, diarrhea, constipation), genitourinary (bladder and erectile dysfunction), metabolic (hypoglycemia unawareness), sudomotor (anhidrosis, heat intolerance, dry skin), pupillary (pupillomotor function impairment) and peripheral vascular [257]. Among all the different types of DAN, presence of cardiovascular autonomic neuropathy (CAN) can lead to the most serious complications of DN increasing overall risk for cardiovascular morbidity and mortality [258].

Diabetic cardiovascular autonomic neuropathy (abbreviated DCAN or CADN) is the result of damage to autonomic nerve fibers that innervate the heart and blood vessels resulting in abnormalities in heart rate control and vascular dynamics manifesting clinically as exercise intolerance, resting tachycardia, intraoperative cardiovascular instability, orthostatic hypotension, abnormal blood pressure regulation and decreased heart rate variability [70, 259]. Exercise intolerance is manifested as reduced heart rate, blood pressure, and cardiac output in response to exercise. Resting tachycardia may reflect a relative increase in sympathetic tone associated with vagal impairment with heart beats around 100 bpm (beats per minute) with occasional 130 bpm. Intraoperative cardiovascular instability is evidenced by extra vasopressor support needed to compensate for vasodilation of anesthesia, presence of excessive hypothermia and reduced hypoxic-induced ventilation drive. Orthostatic hypotension is manifested by light headiness, weakness, faintness, visual impairment, syncope on standing caused by reduced vasoconstriction of the vasculature as a result of efferent sympathetic vasomotor denervation. Abnormal blood pressure regulation resulting in sympathetic predominance during sleep and subsequent nocturnal hypertension, these are associated with a higher frequency of left ventricular hypertrophy and both fatal and non-fatal cardiovascular disease. Impaired heart rate variability can be detected as early as one year after the onset of diabetes and is characterized by decreased variability in response to different metabolic functions, typical of parasympathetic dysfunction. In addition, damage to visceral sensory afferents results in decreased sensation of chest pain (i.e.: silent ischemia), a key contributor of delayed treatment and sudden death [260, 261]. Recently, it has also been suggested that diabetic CAN may directly

contribute to the development of left ventricular dysfunction and cardiomyopathy [262] independent of hypertension and coronary heart disease with an overall risk for cardiovascular disease (CVD) [263, 264].

In patients, commonly used tests to assess CAN include testing for PS (heart rate response to deep breathing, heart rate response to standing, Valsalva manuever) and Sy (systolic blood pressure in response to standing and diastolic blood pressure in response to sustained handgrip and response to tilting) function [265]. Although these tests are relatively simple, they are not particularly sensitive to detect early autonomic dysfunction. Heart rate variability (HRV) has been widely used to detect early deficits in PS function. Moreover, recent imaging techniques have been developed to provide earlier markers of neuronal degeneration, such as myocardial denervation. Myocardial Sy denervation can be measured by cardiac scanning with radiotracers such ^{123}I-metaiodobenzylguanidine (MIBG) [266, 267] and C-11 hydroxyephedrine ([^{11}C] HED) [268], which can show denervation even with normal cardiovascular reflex testing. These radiotracers are radiolabeled analogs of norepinephrine and can be actively taken up by the sympathetic nerve terminals in the heart. One limitation of these studies, although sensitive enough to show early DCAN even in patients with normal cardiovascular reflex testing [76], is that active transport may be limited to local distribution of receptors [269] and axonal transport deficits.

3. Association of diabetic cardiovascular disease with cardiomyopathy and cardiovascular autonomic neuropathy

Cardiovascular diseases (CVD) in diabetes [270] results from a multitude of vascular and myocardial changes. The most prominent myocardial effect is the

development of diabetic cardiomyopathy (DCM) [29, 271], characterized by ventricular dysfunction, metabolic derangements, intrinsic myocyte dysfunction, as well as abnormal neural regulation, namely DCAN [259]. In particular, diastolic dysfunction seems to precede systolic dysfunction in the evolution of DCM, as measured by echocardiograms [272, 273]. The exact pathogenesis of DCM remains unknown; however, there is evidence that DCAN may play a an important role. Specifically, Sacre *et. al.* recently found an association between regional cardiac denervation (determined by Iodine 123-metaiodobenzylguanidine imaging) and diastolic dysfunction in asymptomatic patients with type 2 DM [274]. Similarly, DAN correlates with DCM, in particular diastolic dysfunction, in the absence of other known CVD risk factors, such as hypertension [275-277]. Generally, a clear association seems to occur between DCAN and left ventricular dysfunction [264, 278] in particular in the absence of atherogenesis [279]. Interestingly, a recent study from Moon et al, showed that CAN, as measured by the Ewing's method, precedes coronary artery calcification and suggest that CAN may be a predictor for development of atherosclerosis and increased mortality in type 2 patients [77]. In addition, CAN may directly influence the plasticity of cardiac vasculature needed to appropriately respond to changes in blood flow required under certain activities such as standing, running, and other stress tests and may be particular important in non-ischemic DCM [75]. Finally, direct autonomic cardiac dysfunction and denervation also seems to be associated with ventricular function [74, 280, 281]. In general, although the role of CAN in CVD has been under appreciated, it seems that CAN may have silent and cumulative effects on heart function before CVD and coronary arterial disease are evident [73].

4. Association of DCAN with painful vs insensate DPN

There is increasing amount of evidence that DCAN correlates with DPN [69, 282]. In particular, emerging data from the DCCT and Epidemiology of diabetes Interventions and Complications (EDIC) studies show that both DPN and DCAN still develop under tight glycemic control, suggesting a common pathogenic mechanism independent of sustained hyperglycemia [72]. In terms of mechanisms and neuronal susceptibility to damage it would be interesting to determine if different modalities of DCAN correlates better certain modalities of DPN. Although there is some evidence that DCAN may better correlate with painful DSPN [71, 283, 284], there is also evidence that DCAN may be equally or more associated with insensate neuropathy [285, 286].

5. Autonomic testing in animal models

Some of the above non-invasive tests used for patients have also been used in animal models [287, 288], such as HRV [289-291], radiotracers [292] including ^{3}H-NE [293]. and NE levels in plasma[294] and urine [295-297]. Other invasive tests that can only be performed in animal models have served to study more in detail autonomic dysfunction such as myocardial catecholamines (usually dopamine (precursor of NE) or NE release [298] and content [299-301]. Moreover, cardiac denervation can be studied in details by immunohistochemistry analysis of efferent and afferent fibers, using specific types of markers, such as CGRP [302, 303] and PGP 9.5 [304], NPY and nNOS[305], and tyrosine hydroxylase and choline acetyl-transferase [306], among others [215, 261, 307].

C. Aim and rationale

The main goal of this section was to characterize the development of cardiovascular autonomic neuropathy along with cardiomyopathy and evaluation of lipids.

Cardiovascular autonomic neuropathy is found frequently in association with sensory neuropathy in diabetic patients. However, this association is not very well described in animal models of DPN. In particular, association of CAN with either painful or insensate neuropathy is not clear. Thus, different measurements of CAN were evaluated, such as cardiac and urinary norepinephrine, in the insensate animal model described for Chapter II. In addition, another aspect of cardiac pathology in diabetes is the loss of cardiac sensory perception, thus sensory markers in cardiac tissue were also evaluated. Moreover, there is increasing evidence that CAN may be a major contributing factor in the development of cardiomyopathy in type 1 diabetic patients. Therefore, along with the other parameters, development of cardiomyopathy was measured by echocardiograms. Finally, hypertriglyceridemia was shown to be a risk factor not only for CVD, but for sensory and autonomic neuropathies as well. Therefore, in order to complement the characterization of metabolic changes occurring in the present animal model, lipid panel in plasma was evaluated before signs of neuropathy appeared.

D. Materials and methods

1. Experimental scheme

The following table represents a summary of the set of variables measured at specific times post injection of citrate buffer for control groups and STZ for DM groups.

Weeks	0 (STZ inj)	2	4	8	12
Glucose	X		X	X	X
Weight	X		X	X	X
Lipids		X			
Urinary NE				X	
Echos			X	X	X
Cardiac NE				X	
Cardiac Western Blots				X	

2. Animals and experimental groups

Specific pathogen-free male Fisher 344 rats were used in all experiments. This strain was preferred because they are relatively inbred and do not become obese with age, like the related Sprague-Dawley rat strain. After arriving at the UIC animal facility and a brief acclimation period, animals were randomly assigned to STZ DM (STZ-injected) and control (CON) groups. Diabetic animals were generated by a single

injection of 35 mg/kg of STZ in the tail vein of 300 g males of 16 weeks of age as described in detail in chapter 2. Age-matched control animals were injected with saline (0.1 M citrate buffer pH 4.5). All animals were maintained on standard rat chow and water (both automatic and bottled).

3. Determination of diabetic state

Glucose levels and body weight were measured as described in detail in Chapter III. After confirmation of DM, glucose levels and body weight were monitored at 4, 8 and 12 weeks post injections in control and diabetic animals.

4. Lipid panel

Plasma was obtained from control and diabetic rats after 2 weeks post STZ injection. In order to avoid pain during blood extraction xylocaine was applied to rat tails. After 10 min, a small cut in the tail was made using a razor blade and blood was drawn into 0.5mL eppendorf chilled tubes previously filled with of 10 µl of NaEDTA (1mg/ml) for plasma preparation. Samples were immediately centrifuged at 2,000 x g for 15 minutes at 4°C, after which plasma was removed, aliquoted and kept frozen at -20°C until testing. Plasma aliquots were sent to the chemical pathology laboratory in the UIC Biologic Resources Laboratory for plasma lipids panel quantification, including cholesterol and triglycerides measured by compact chemistry analyzer and expressed in mg/dL.

5. Quantitation of urine norepinephrine

At 8 weeks post STZ injection, 24 h urine was collected from control and diabetic animals. Animals were placed in individual bottom wired cages, previously cleaned,

without bedding with plenty of food and water for 24 hrs. To minimize oxidation of NE, exposure of urine to direct light was avoided by covering tubes with aluminium foil. After 24 hrs, urine was collected and added to tubes containing 6M hydrochloric acid (1.2 mL HCl: 100mL urine) for preservation and separated from feces by centrifugation. Then, urine volume was measured and stored in aliquots at -20C. Aliquots were used to measure norepinephrine by ELISA. Before addition of hydrochloric acid, a small aliquot was taken for creatinine measurements so that urinary NE could be normalized to creatinine.

The enzyme immunoassay for the quantitative determination of norepinephrine (Noradrenaline EIA, ALPCO diagnostics 17-EA610-96) was used to quantify noradrenaline in control and diabetic urine samples. Norepinephrine was extracted from the sample using a cis-diol-specific affinity gel, acylated to N-acylnoradrenaline, and then converted enzymatically during the detection procedure into N-acylnormetanephrine. Subsequently, N acylnormetanephrine was bound to the surface of microtiter plates. Acylated metanephrines from the sample and solid phase bound N-acylmetanephrines competed for a fixed number of specific rabbit antibody binding sites. When the system was in equilibrium, free antigen and free antigen antiserum complexes were removed by washing. The antibody bound to the respective solid phase N-acylmetanephrine was detected by a mouse anti-rabbit IgG-peroxidase conjugate using TMB (3, 3´, 5, 5´-tetramethylbenzidene) as a substrate. The absorbance was read within 10 minutes, using a microplate reader set to 450 nm and a reference wavelength between 620 nm and 650 nm. Control and diabetic urine samples were processed along with a series of norepinephrine standards and two positive

controls of known concentrations. The standard curve constructed by plotting the linear mean absorbance of the standards vs the log of the concentrations in ng/mL in the x axis. The results of the unknown samples were calculated from the fitted curve and expressed in Millimoles. Finally NE was expressed as 24h NE/creatinine.

6. Cardiac norepinephrine quantitation by HPLC/MS MS

a) Norepineprhine (NE) and d-NE standtards

NE (Fisher #50-230-6498) and deuterium-NE (d-NE) (Fisher #NC9750934) stock solutions were prepared at 1mg/mL in 1M HCl and stored at -20°C until use. Working standards were diluted from stock solutions in 0.1 M formic acid. Typically, d-NE working solution was prepared at 10 µg/mL and NE solutions at 50 and 5 µg/ml.

b) Tissue harvesting and homogenization

Control and STZ DM animals after 8 weeks post injection, were pre anesthetized with CO_2 and euthanized by cervical dislocation. The heart was removed, rinsed in PBS solution, pH 7.4, blotted dry, weighted, then covered in aluminum foil and snap frozen in liquid nitrogen. Catecholamine levels in biological samples can change significantly over time due to auto-oxidation, exposure to light, air oxidation and/or exposure to high pH and temperature [308, 309]. Therefore, special care was taken when handling, storing and preparing samples to avoid degradation of NE. Hearts were kept at -80°C until time of homogenization.

Tissue homogenization was performed as previously published with modifications [310]. Tissue was pulverized in liquid nitrogen and homogenized in glass homogenizer (metal homogenizers can oxidize NE) in buffer (2mL/g tissue) containing:

0.1 N perchloric acid ($HClO_4$), 0.02 mM EDTA. Right after homogenization, homogenates were centrifuged at 15,000g for 20 min at 4°C and supernatants used for NE extraction.

c) Norepinephrine extraction by alumina

Extraction by alumina was performed as previously described [311]. A 500 µl aliquot of pre-cleared heart homogenate was spiked with 500 ng of deuterium (d)-NE (50 µl) used as internal standard. Activated alumina was then added to each sample (100mg). One milliliter of 1M Tris buffer (pH 7.8) was added to each vial to raise the pH. Each tube was then capped and gently rocked for 10 minutes, allowing NE to adsorb to the alumina at room temperature (RT). Then, tubes were centrifuged at 5,000 rpm for 5 minutes and supernatant discarded. Following, 5 ml of deionized water was added to each tube to wash, tubes vortexed and centrifuged again at 5,000 rpm for 5 minutes and supernatant discarded. To desorb NE, 1ml of 1M formic acid was added to each tube and agitated for 10 minutes and centrifuged twice at 5,000 rpm for 5 minutes. Recovered supernatant was filtered and stored at -80°C until HPLC procedure.

d) HPLC-MS-MS conditions and calibrations

High-Performance Liquid Cromatography (HPLC) was preferred for its specificity in separation of metabolites and other compounds [312]. Tandem mass spectrometry (MS-MS) was preferred for its high sensitivity and specificity of distribution of similar compounds with different mass [313]. Methods used were based on previously published protocol for NE quantitation in urine samples [311] Liquid Chromatography (LC) was performed on NE using an Agilent 1200 series HPLC system. An Agilent Zorbax R_x-C_8 (150 x 2.1mm I.D., 5µm) column along with a pre-column filter was utilized

in the isocratic, reverse-phase, ion-paring mode. The column temperature was set at 30^{o}C to avoid any fluctuating room temperatures. The mobile phase consisted of water-methanol-HFBA (Heptafluorobutyric Acid) (85/15/.13, V/V/V), with a flow rate of .2 mL min^{-1}. Sensitivity was such that only 10 µL of each sample was injected for analysis, representing only 1% of the final solution. The entire chromatographic effluent was passed through the mass spectrometer interface for subsequent detection. Under these conditions, NE retention time was about 4.4 min resulting in a total time (injection-to-injection) of 6 min (Appendix C)

The mass spectrometer employed was an Agilent 6410 Triple Quadrupole LC/MS. The mass spectrometer's temperature and nitrogen gas flow rate were 300^{o}C and 10 L min^{-1}, and the nebulizer gas pressure was 40 PSI (pound per square inch). Protonated analyte ions were formed using electrospray ionization (ESI), with the source oriented perpendicular to the spray and orifice potentials of 4kV positive and 4kV negative. The MS-MS detection scheme utilized SRM (selective reaction monitoring)-CID (collision induced dissociation assays). CID was completed using nitrogen as the collision gas, ion energy of 5eV for NE and 10eV for d-NE. The SRM transitions and their respective collision energies, m/z 152-135:5, and m/z 158-141:10, were sequentially monitored for detection of NE and d-NE. The dwell time for each transition was 200 ms.

NE/d-NE area ratios were determined for the SRM chromotagraphic peaks using Agilent's Mass Hunter software. Calibration curves were generated in Microsoft Excel by plotting peak area ratios (NE/d-NE) obtained for working standard versus NE

concentrations and fitting these data to a weighted (1/x) linear regression curve. NE concentrations in test samples were then interpolated from this line.

Dopamine is a precursor of NE. Thus to test for possible dopamine contamination in the signal, several concentrations of dopamine standards were tested. Dopamine runs in very different spectrum and therefore does not represent contamination in the NE signal even if co-eluted with NE (data not shown).

Several buffers containing different types of antioxidants to prevent NE oxidation are reported in the literature [314]. Ethylenediaminetetraacetic acid (EDTA) is used as an antioxidant with no interference with HPLC MSMS. Other antioxidants, such as sodium metabisulfite (Na_2 (SO_3)), have also been used in combination with EDTA to minimize oxidation. Two homogenization buffers were tested (0.1 N perchloric acid ($HClO_4$), 0.02 mM EDTA) with and without sodium metabisulfite for possible interferences with the method used. As shown in (Appendix D), sodium metabisulfite quenches the NE signal, and was not used for the homogenization procedure

7. Analysis of neuronal markers in heart tissue by western blots

a) Tissue harvesting and sample preparation

Control and STZ DM animals after 8 weeks post injection, were anesthetized with CO_2 and euthanized by cervical dislocation. Their heart was removed, rinsed in PBS solution, pH 7.4, blotted dry and snap frozen in liquid nitrogen. Tissue was weighted, cut in small pieces on frozen metal block, pulverized in ceramic grinder using liquid nitrogen and then homogenized in glass homogenizer with cold buffer (10mL/g tissue) containing: 50 mM HEPES pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1X Phosphatase inhibitor cocktail set II (Calbiochem # 524625), 1X protease inhibitor

cocktail (Sigma #P8340), 50 nM okadaic acid. Homogenates were then centrifuged at 14,000 rpm for 15 min at 4°C. Supernatant was aliquoted and stored at -80°C until time of electrophoresis. A small aliquot was reserved for protein quantification by BCA using BCA kit

b) Quantification of proteins by BCA

Frozen aliquots were thawed the day of the assay and protein concentration measured by bicinchoninic acid (BCA) assay kit (Pierce #23225, Thermo Scientific) using the microplate procedure. Basically, same volume (25 µl) of standards (0-2mg/ml) (triplicates) made of bovine serum albumin (BSA) and several dilutions of samples of unknown concentrations (duplicates) were loaded into a 96 well plate. Then, 200 µl of working reagent containing a 50:1 mixture of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and Reaction B (4% cupric sulfate) added and incubated for 30 min at 37°C. During this time, peptide bonds and four particular amino acids (cysteine, cystine, tryptophan and tyrosine) reduce Cu^{+2} to Cu^{+1} in an alkaline medium producing a purple- colored reaction that can be measured by absorbance. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/mL). The BCA method is not a true end-point method; that is, the final color continues to develop. However, following incubation, the rate of continued color development is sufficiently slow to allow large numbers of samples to be assayed together. Therefore, after incubation the plate was allowed to return to RT to slow the reaction and was read

in ELISA plate reader at 562nm. Blank absorbance was subtracted from all readings and standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL and fitted using a linear function. Protein concentration of each unknown sample was determined based on the standard curve.

c) Western blots

Protein samples with sample buffer containing: 300mM Tris HCl pH6.8, 10% SDS (denaturalizing agent), 50% Glycerol (reduces hydrophobic interactions and for adding density), 0.005% Bromophenol Blue (front of gel), 25% Betamercaptoethanol (reducing agent added it fresh to each sample), were loaded onto a 4-12% Bis/Tris gradient gel (Invitrogen) and separated at 150 Volts (V) constant in MOPS SDS NuPage running buffer (Invitrogen #NP0001). Molecular weight ladder was used as reference for molecular weights (See Blue Plus 2 Standard, Invitrogen #LC5925). Proteins were transferred to Immobilon-P transfer membranes (PVDF, Millipore 1PVH00010, 0.45µm pore) at 0.4 Amps for 2 hours in wet transfer system (TE 22, Hoefer) with cooling system at 4 ºC (Ecoline RE 106, Landa Brinkmann) in (25mM Tris Base, 192mM Glycine) plus 10% methanol with gentle stirring. To test correct transfer, membranes were stained with Ponceau solution (5% Ponceau, 0.1% Acetic Acid) and visualized bands served as reference to cut membranes as needed. Membranes were blocked at RT 60 minutes with 5% milk in PBS. Primary antibodies in 1% BSA in PBS were added overnight at 4ºC with gentle rocking. The next day, primary antibodies were washed for 10 minutes (3 times) with PBST (0.25% Tween), and corresponding secondary antibodies conjugated with HRP (horseradish peroxidase) enzyme Goat anti Rabbit:

Jackson#111-035-045 and Goat anti Mouse:Jackson#115-035) diluted 1:50,000 in 5% milk PBS Tween 0.1%, were added for 1 hour at 4ºC with gentle rocking. Membranes were washed of unbound secondary antibodies for 10 minutes (five times), incubated with ECL Plus western detection system for 5 minutes (GE Healthcare # RPN2132) and laid onto plastic wrap in radiographic cassette. Membranes were exposed on autoradiography film (HyBlot CL, Denville Scientific #E3018) for different time intervals until clear definition of signal and developed using film Processor (Kodak X-OMAT 1000A). Auto fluorescent tags were used to identify the position of bands on the film relative to the membranes.

The antibodies used are listed in the table below.

Primary antibody	Company Catalog #	Molecular Weight (KDa)	Dilution	Species Host
CGRP	Millipore AB15360	14	1:5,000	Rabbit
PGP 9.5	Millipore AB1761	27	1:2,000	Rabbit
GAPDH	abcam 341048	36	1:5,000	Mouse

Relative optical density was obtained using Image J (NIH) software. Optical density (OD) from neuronal markers was normalized by GAPDH OD and expressed as percentages.

8. Determination of structural and functional changes in the myocardium using echocardiography (echo)

a) Method conditions and parameters

In collaboration with the laboratories of Drs. Geneen and Piano and the expert echocardiographist Shamim Chowdury, echos were obtained using a research

dedicated echocardiography machine and high resolution 15 MHz transducer (Sequoia C256 Echocardiography System, Acuson, Mountain View, CA) as previously published [315]. Echos were performed in control and DM animals at 4, 8 and 12 weeks after injection in STZ injections. Immediately before echos animals were anesthetized with methoxyflurane inhaled in a closed chamber and intubated with a 20-gauge angiocath tube. Surgical anesthesia was maintained using 1.0-1.5% isoflurane delivered through a vaporizer with a mixture of 100% oxygen connected in series to a rodent ventilator with the tidal volume set at 3.0 to 4.0 ml/min. Animals maintained a respiration rate of 90 breaths/min (this anesthetic has been shown not to produce myocardial depression and is associated with minimal changes in heart rate). The transthoracic transducer was placed on the left hemithorax and targeted M-mode recordings were made by directing the beam toward the mid papillary level. Electrocardiographic electrodes were attached to the animal's paws and a single lead electrocardiogram was recorded on the imaging system. Measurements were obtained after a well-defined continuous interface of the anterior and posterior walls visualization. After good visualization and according to the methods forwarded by the American Society of Echocardiography, end-diastolic left ventricular dimension (LVDD), end-systolic left ventricular dimension (LVSD), posterior wall (PW) (for echo pilot study) and interventricular septal thickness in diastole (IVSD) and in systole (IVSS) were measured by the leading edge method [315]. In addition, to heart rate (HR) and left ventricular mass (LV mass). As indirect measures of contractility other parameters were also measured, such as fractional shortening (FS) and ejection fraction (EF). Rat heart rates under anesthesia vary between 250–320 bpm, hand tracings of the

LV endocardial contours of at least 3 frames were made and EDD and ESD were measured and then used to determine LV ejection fraction (EF). Relative wall thickness (RWT) was calculated based on the following formula: (LVDD-LVSD/LVDD) x 100. Moreover, signals from the ventricular inflow and outflow tracks were measured as indices of systolic and diastolic dysfunction. Indices of diastolic function include: isovolumetric relaxation time (IVRT) (time between the closure of the aortic valve [S_2] to opening of the mitral value), deceleration time (DecT), and E/A ratio (the E wave represents early rapid filling of the ventricle, the A wave represents late filling of the ventricle).

b) Pilot experiment

A pilot study was performed after 16 weeks of post injection in one STZ-DM and one control rat, to test feasibility of measurements and to identify possible outcomes (Appendix E).

E. Results

1. Hyperglycemia and reduced weight typical of diabetes in STZ animals

STZ-DM animals exhibited a typical diabetogenic state as identified by hyperglycemia and impaired ability of gaining body weight. Glucose levels and body weight measured at at 4, 8 and 12 weeks post injections for control and diabetic animals (TABLE X). These results were similar to the ones obtained in Chapter II and were maintained for 12 weeks post-injections as well.

TABLE X

GLUCOSE AND BODY WEIGHT

	4 WEEKS	**8 WEEKS**	**12 WEEKS**
	Body weight, g (mean ± SEM)		
CONTROL (n=6)	340 ± 5	378 ± 7	396 ± 10
STZ DM (n=7)	255 ± 9	256 ± 13	239 ± 13
	Glucose, mg/dL (mean ± SEM)		
CONTROL (n=6)	87 ± 7	68 ± 4	76 ± 4
STZ DM (n=7)	370 ± 22	373 ± 28	398 ± 30

2. Elevated triglycerides but normal cholesterol in STZ DM Fischer 344 rats

At 2 weeks post DM-induction a marked increase in triglycerides levels were found in DM animals compared to controls. However, no difference in total cholesterol levels were found between groups at 2 weeks post DM induction (Figure 11)

3. Increased levels of urine NE in STZ DM Fischer 344 rats

Eight weeks post DM-induction, 24h urine was collected from control and diabetic animals and levels of NE were measured by ELISA. Urine creatinine levels were also measured to adjust for variable urine values. As shown in (Figure 12), diabetic animals have a higher NE urine excretion compared controls ($p<0.005$).

4. Decreased cardiac norepinephrine in STZ DM Fischer 344 rats

Cardiac NE levels measured by HPLC/MS-MS were reduced in STZ-DM animals at 8 weeks post STZ injection when compared with control groups, indicating cardiac sympathetic dysfunction (Figure 13).

5. Altered neuronal sensory cardiac markers in STZ DM Fischer 344 rats

Markers of sensory innervation (CGRP and PGP 9.5) were measured on heart tissue by western blots. As shown in Figure 14 at 8 weeks post DM induction, an increase in CGRP was found in cardiac tissue of DM animals compared to controls, whereas no difference was found in PGP 9.5 between groups. GAPDH expression, used to normalized the signals, was constant and no significant difference was observed between control and DM groups.

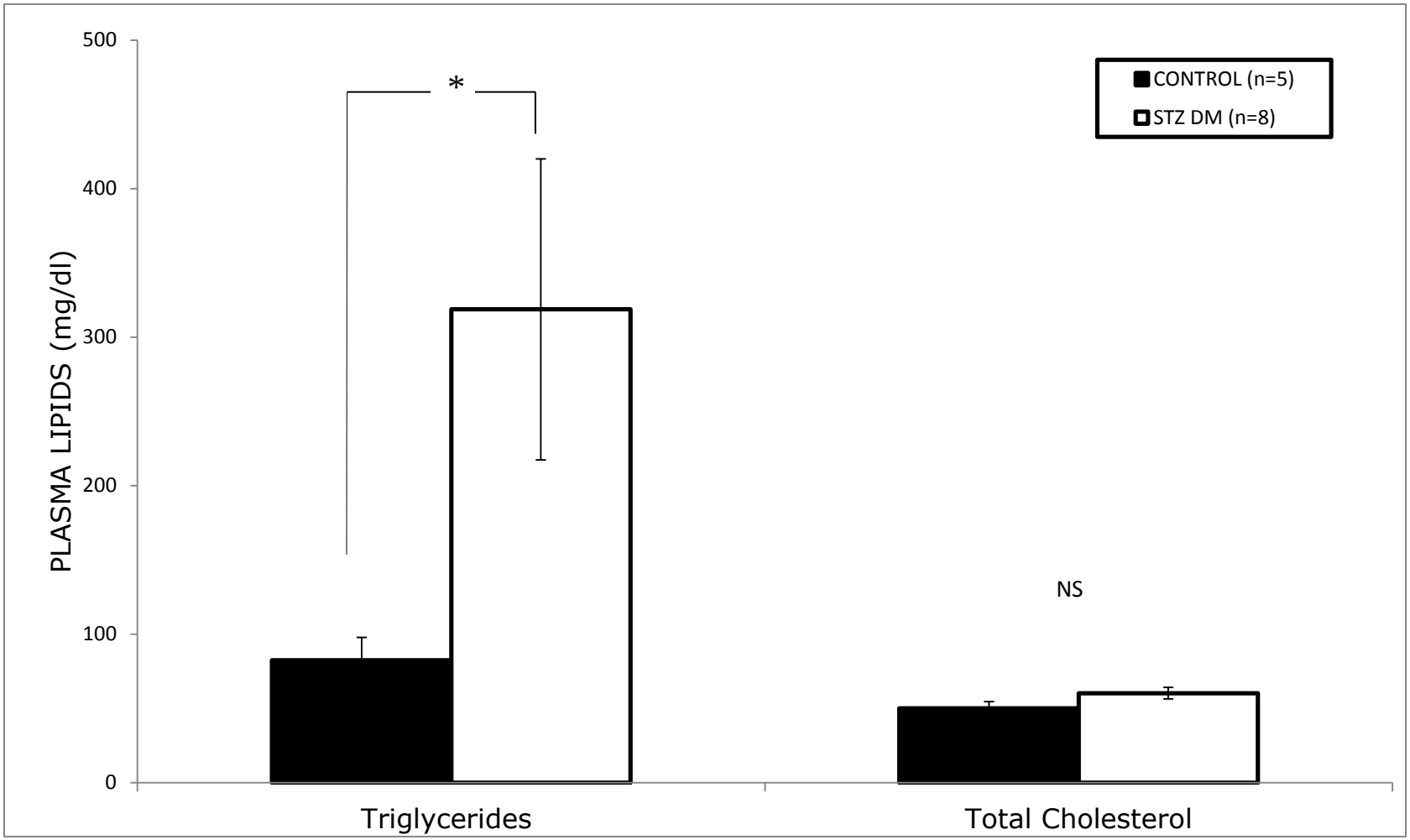


Figure 11. Elevated triglycerides in STZ DM Fischer 344 rats

Data is plotted as mean±SEM
T-test * p< 0.05; NS: not significant

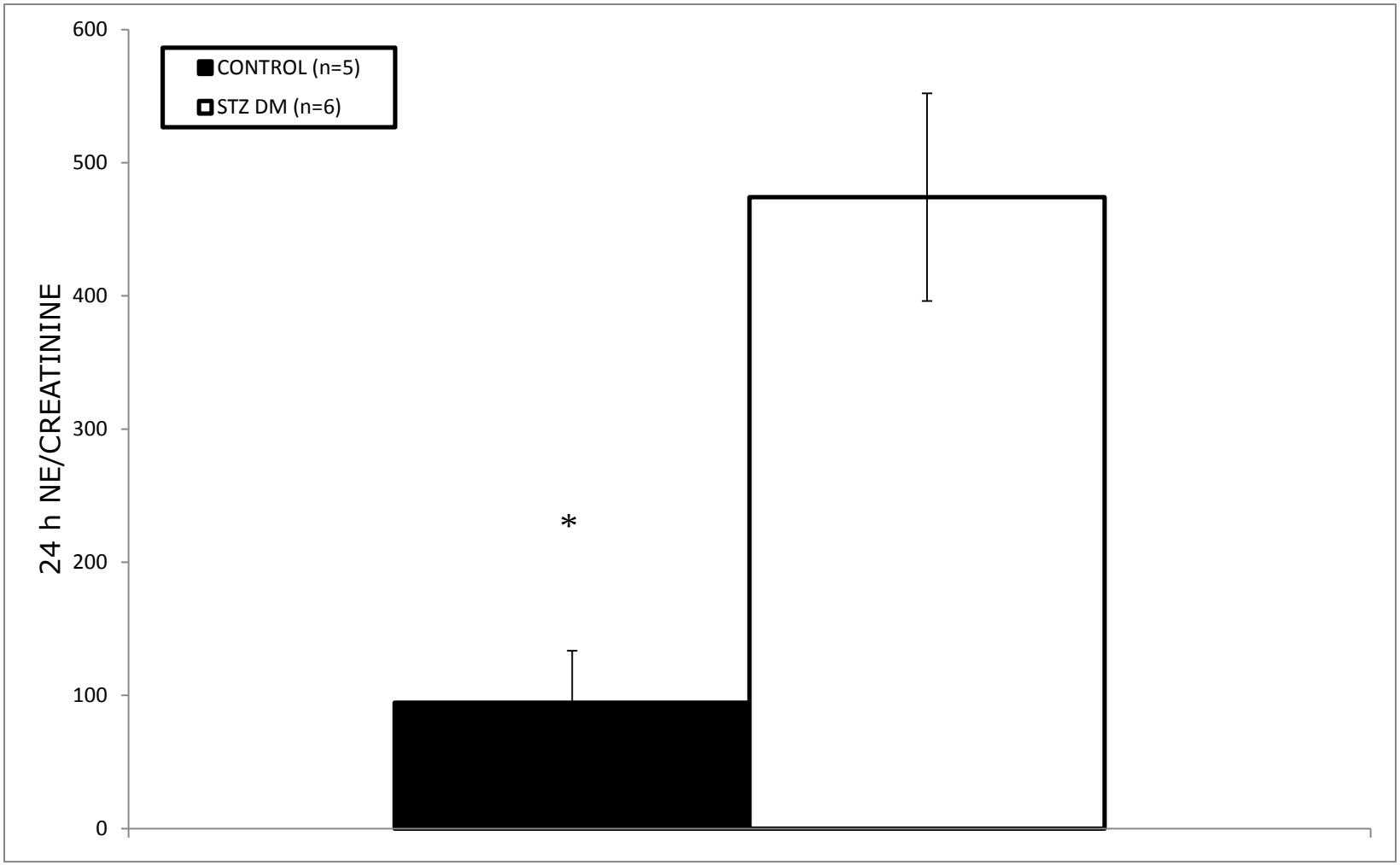


Figure 12. Increased urine NE in STZ DM Fischer 344 rats

Data is plotted as mean±SEM

T-test * $p<0.01$

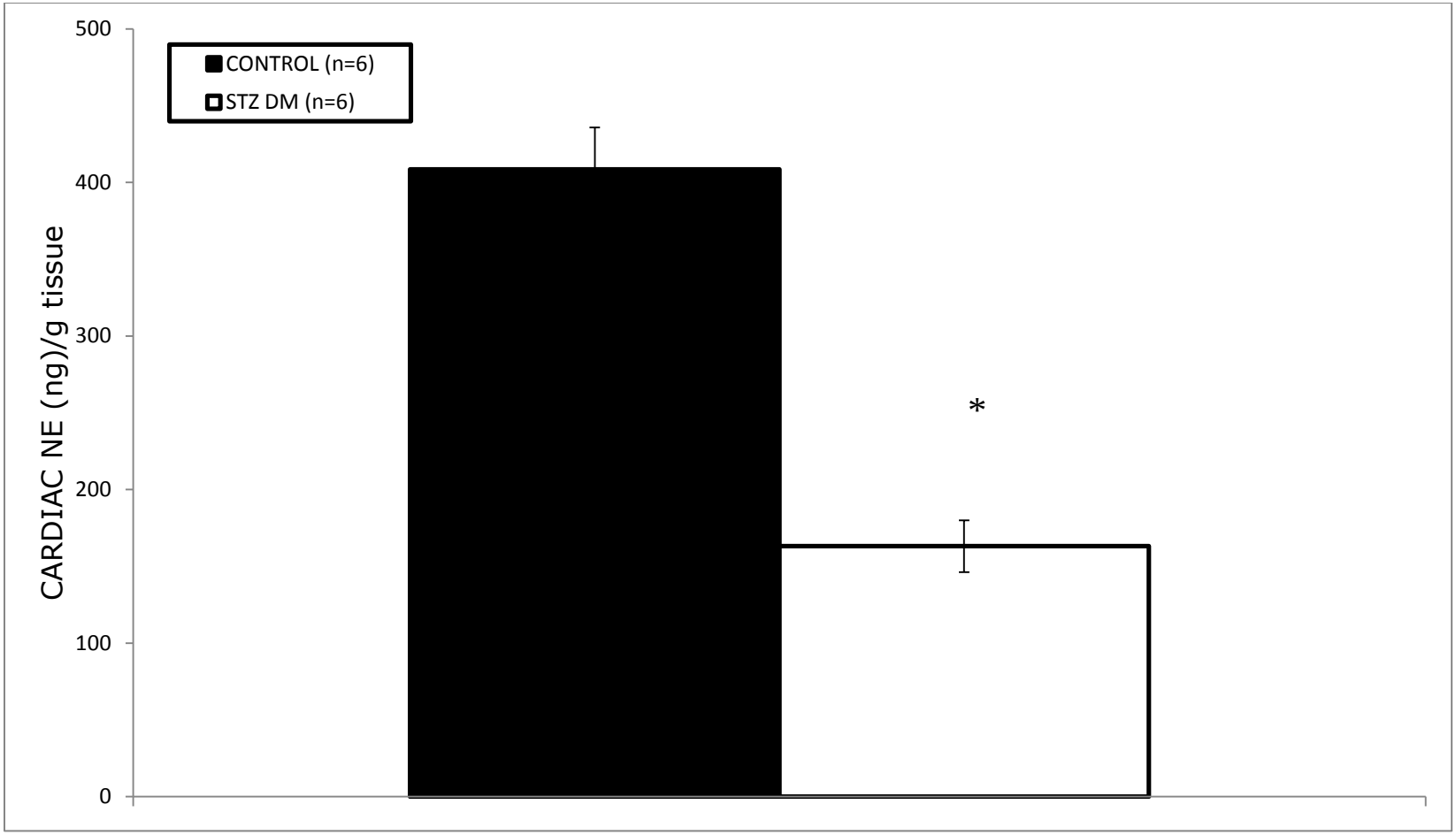
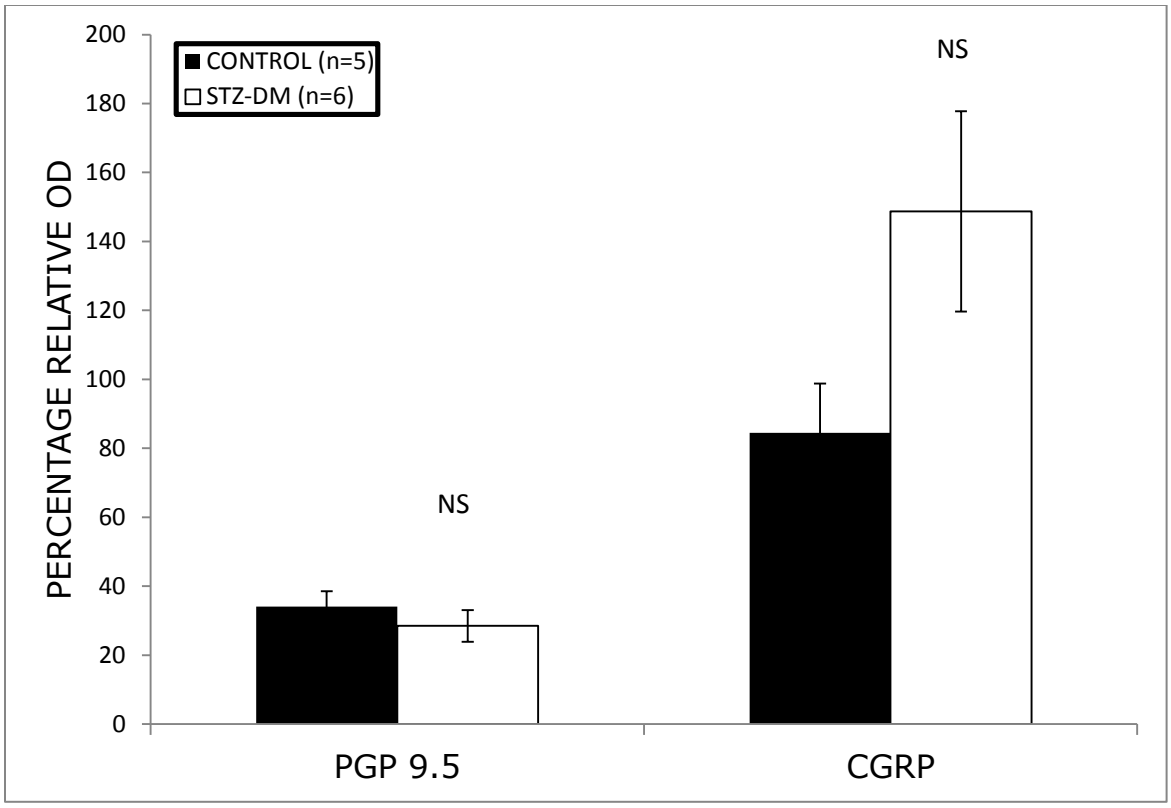


Figure 13. Decreased cardiac norepinephrine in STZ DM Fischer 344 rats

Data is plotted as mean ± SEM

T-test: $p<0.0001$



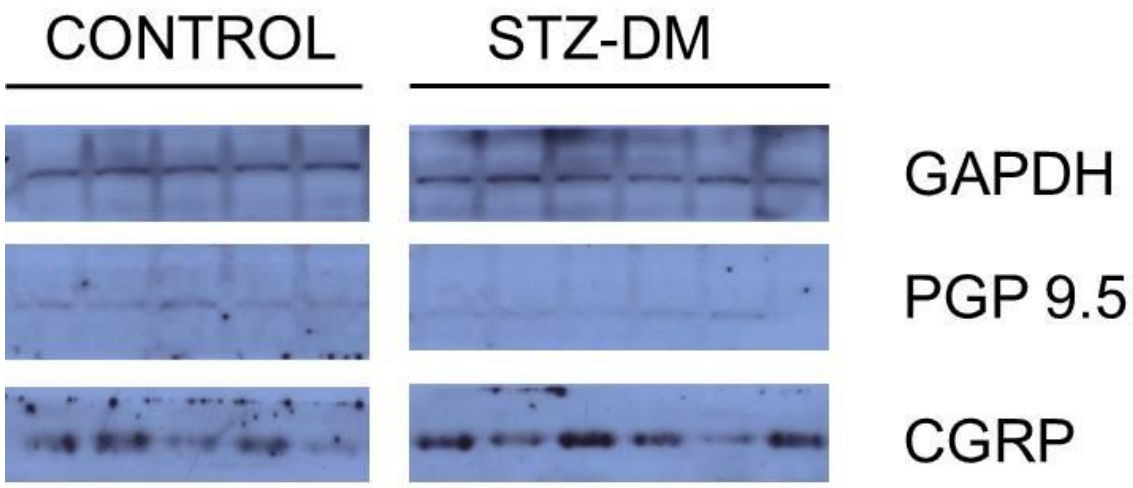


Figure 14.Sensory markers in whole cardiac tissue homogenates

Data is plotted as mean ± SEM

T-test NS between CON and STZ DM for PGP 9.5 and CGRP

6. Left ventricular dysfunction in STZ DM animals

Structural echo parameters were normalized to body weight (BW), due to the difference in weight in control and DM animals (TABLE XI). Significant increases in LVSD/BW and LVDD/BW were found in the DM group compared to control group, indicating that DM hearts were dilated. No significant differences were found in RWT/BW between groups. Except for a difference at 4 weeks, no significant difference in LV mass/BW was found between groups at 8 and 12 weeks post-DM induction. Except for a significant increase in IVSS/BW in DM compared to control at 4 weeks post DM-induction, no differences in IVSD or IVSS/BW were found between groups at other time points.

Bradycardia (reduced heart rate) was found in STZ DM animals at 8 and 12 weeks post DM-induction (TABLE XII). Moreover, a significant reduction in EF was found in DM animals compared to their age-matched controls at 4, 8 and 12 weeks. In addition, a significant reduction in E/A ratio was found in DM animals at 4, 8, and 12 weeks post DM-induction compared to controls.

TABLE XI

STRUCTURAL ECHO PARAMETERS

Weeks after STZ injection	4		8		12	
Groups	CON	STZ DM	CON	STZ DM	CON	STZ DM
LV mass/BW (g/kg)	1.21 ±0.03	0.80 ±0.06 **	1.20 ±0.07	1.45 ±0.08 *	1.12 ±0.06	1.25 ±0.1
IVSD/BW (mm/kg))	2.68 ±0.13	3.08 ±0.21	2.63 ±0.14	2.99 ±0.15	2.24 ±0.11	2.50 ±0.21
IVSS/BW (mm/kg)	5.35 ±0.32	5.98 ±0.47 *	5.19 ±0.28	5.55 ±0.34	4.24 ±0.25	4.47 ±0.32
LVSD/BW (mm/kg)	12.40 ±0.5	19.50 ±1.3 **	11.20 ±0.3	21.20 ±1.3 **	12.21 ±0.39	25.64 ±1.5**
LVDD/BW (mm/kg)	21.90 ±0.4	30.90 ±1.1 **	19.70 ±0.4	32.20 ±1.5 **	19.91 ±0.63	35.25 ±1.9**
RWT/BW (mm/kg)	7.71 ±0.62	8.03 ±0.61	7.42 ±0.54	7.89 ±0.48	6.05 ±0.33	6.04 ±0.39

LV: Left Ventricle; BW: Body Weight; IVSD: Interventricular Septum in diastole, IVSS: Interventricular septum in Systole; LVSD: Left Ventricular Diameter in systole; LVSD: Left Ventricular diameter in Dyastole; RWT: Relative Wall thickness

Data is expressed as mean ± SEM.

Two way RM ANOVA Holm-Sidak test control vs STZ DM * $p<0.05$; ** $p<0.0001$

TABLE XII

FUNCTIONAL ECHO PARAMETERS

Weeks after STZ injection	4		8		12	
Groups	CON	STZ DM	CON	STZ DM	CON	STZ DM
HR	330.7±	314.4±	349.7±	297.9±	328.3	277.7±
(bpm)	13.6	26.4	8.5	8 *	±18.7	9.3*
FS(%)	43.7±	37.1±	43.2±	32.6±	38.8±	27.4±
	2.2	2.4 *	1.4	1.3*	1.1	0.7*
E/A	1.98±	1.5±	1.98±	1.55±	1.83±	1.35±
	0.07	0.05 *	0.13	0.12	0.15	0.02*
IVRT	26.3±	34.4±	25.3±	32.9±	27.7±	38.1±
(msec)	2	3 *	1.7	2.8 *	1.8	2.9*
DecT	45.7±	41.6±	41.2±	47.9±	44.2±	43.3±
(msec)	0.8	2.6	2.9	3.2	2.8	2.5

HR: heart rate; R-R: time between two consecutive R waves, FS: fractional shortening; IVRT: interventricular relative wall thickness; DecT: deceleration time

Data is expressed as mean ± SEM

Two way repeated measurements ANOVA. Holm Sidak test.* $p<0.05$

F. Discussion

Plasma triglycerides (TG) were elevated already at 2 weeks post DM induction in DM animals, while total cholesterol was unchanged. This finding is in agreement with others [316] that shows plasma elevation of TG as early as 1-3 days post STZ injection, while elevations of total cholesterol were only evident at 28-42 days post STZ injection. Elevated triglycerides and hyperglycemia are evident before signs of sensory neuropathy appear, suggesting that not only hyperglycemia but also elevated triglycerides may contribute to development of sensory neuropathies.

This STZ DM model also shows signs of autonomic neuropathy identified by increased NE levels in urine. Increased urine NE is indicative of increased plasma NE. NE increase in plasma or urine is a common phenotype observed in diabetic animal models [317]. This elevation could be a result of increased NE spill over into the blood due to either a) increased sympathetic activity, or alternatively, b) loss of sympathetic nerve terminal function as determined by reduction of NE transporters [318] or decrease in cardiac beta-adrenergic response [319].

Myocardial concentrations of norepinephrine have been reported to be reduced in long-term DM [320]. In animal models, cardiac NE concentrations are controversial. Some reports indicate that cardiac NE is increased at early times and decreased or normal late in the course of DM [295, 298, 317]. In the present study, cardiac NE levels are considerably reduced at 8 weeks post DM induction. This reduction of NE could be attributed to direct sympathetic denervation and/or altered sympathetic function [321].

Deficits in cardiac sensory afferents, another aspect of cardiac pathology that leads to silent cardiac infarction in DM patients, has been linked to changes in neuronal

sensory markers such as CGRP and PGP 9.5 [215]. In other studies, differences in CGRP levels have been reported. In some cases CGRP was elevated in cardiac atria as a result of accumulation at the dystrophic terminals [302]. In other cases that looked into long term DM, they found that cardiac CGRP levels were decreased [215]. These observations may reflect early dysfunction at the terminal with impaired release of neurotransmitters (elevated CGRP) and later events associated with cardiac denervation (decreased CGPR). In the present study, cardiac CGRP protein levels were elevated indicating early terminal dysfunction with impaired release. PGP 9.5 is a general marker for sensory fibers, usually found in peripheral tissues, such as the skin. Although there was no difference in PGP 9.5 immunoreactivity, the observation that the signal was present suggests that cardiac tissue also presents sensory innervation similar to the one observed in the periphery.

Functional parameters studied by echos to evaluate cardiac function, showed that DM animals develop bradycardia with early systolic and diastolic dysfunction already at 4 weeks post DM induction. This cardiac dysfunction co-presented with cardiac dilation at all-times. These results are similar those reported by Mihm *et al*, that shows in a Sprague-Dawley STZ model of DM developments of bradycardia, reduced cardiac performance and cardiac dilation, but at later stages. [322]. The systolic and diastolic dysfunction developed early in the present animal model, since parameters reflecting the latter were altered at 4 weeks post-DM induction, which was the earliest point evaluated. To further understand the natural evolution of cardiac dysfunction, future studies should address the latter parameters at earlier time points.

The observed bradycardia correlated with reduced NE cardiac levels, suggesting that reduction in heart rate may be related to autonomic dysfunction. Since NE levels were measured only at 8 week post-DM induction it is difficult to conclude any cause and effect situation, thus, measurement of NE cardiac levels along with echos measurements earlier in the progression of DM are needed. Diabetic patients with CAN usually show tachycardia instead of bradycardia, although there are some reports of reduced mean heart rate under Valsalva manoeuvre [323]. The basis for the difference observed in heart rate between DM patients and animal models is not clear, but one possibility is that in humans, arrhythmias may result from heterogeneous sympathetic denervation and subsequent hyperinnervation [324] as a response to regeneration. This latter process may be absent or different in animal models. Moreover, many patients with DCAN also present with severe cardiovascular dysfunction with cardiac hypertrophy, hypertension, vascular disease, all of them leading to different patterns of arrhythmias [325]. another possibility is that bradycardia might be happening in humans but had never been captured. The tachycardia may be a compensatory mechanism for the decrease in fractional shortening or systolic dysfunction.

IV. ELUCIDATE THE PATTERN OF KINASE ACTIVATION PERTINENT TO AXONAL TRANSPORT REGULATION AND EVALUATE THE FUNCTION OF MOLECULAR MOTORS IN DIABETIC POLYNEUROPATHY

A. Summary

Among the many abnormalities described in diabetic nerves, axonal transport deficits may be sufficient to explain a dying back phenotype characteristic of diabetic neuropathies. Although fast axonal transport (FAT) deficits have been vastly described in DN, a pathogenic mechanism has yet to be identified. Kinases that alter FAT in other neurological diseases have been identified and include JNK and GSK3β among others. These kinases are also dysregulated in DM nerves due to changes in insulin signaling and hyperglycemia, having the potential of affecting FAT as well.

In the present animal model of insensate neuropathy, the pattern of kinase activation was consistent with lack of insulin/growth factor deficiency, rather than hyperglycemia, in particular reduced JNK and p38 activation and increased activation of GSK3β. Using squid axoplasm, our laboratory has shown that JNK and GSK3β directly alter FAT. In order to understand the role that p38 may have on FAT regulation in diabetic nerves, p38 isoforms were tested for their ability to affect FAT in the axoplasm. The results showed that p38 was also able to alter axonal transport and that this effect was isoform specific. Thus, altered activation of any of these three kinases (JNK, p38 and GSK3β) in diabetic nerves also may lead to changes in FAT.

In order to assess how this particular pattern of kinase activation can affect specific functions of molecular motors, the ability of kinesin to bind to microtubules was tested in both sciatic and vagusnerves of diabetic rats. Significant reduction of kinesin binding to MT was observed in DM vagus nerves. In sciatic nerves, although no significant difference, a trend towards decreased binding in DM animals observed.

In conclusion, although there are some reports of disruption on insulin/growth factor signaling, this is the first report to study JNK, p38 and GSK3β in both vagus and sciatic nerves of DM animals with insensate neuropathy. Moreover, this study is the first one to look into the mechanism of FAT through kinase activation and molecular motors activity.

B. Introduction

1. Axonal transport

Intracellular neuronal transport was originally described in sciatic nerve regeneration studies, suggesting the idea of intracellular transport of proteins along the axon, therefore named axonal transport, and implicating the cell body as the source of that material [326]. Subsequently, many of the same constituents (molecular motors and associated proteins) involved in axonal transport were described for other structures in the neuron, such as dendrites [327], dendritic spines, nerve terminals, monocilium and locally in the cell soma involved in mitotic processes [156]. Similarly, constituents of axonal transport were also described for other non-neuronal cell types, such as the ones involved in transport of glucose transporters in pancreatic cells [328], and in accessory structures, such as flagella in several organisms [329]. Although the axonal

transport machinery may be shared among all cells, it is particularly essential for cells with high complexity and elaborate structures, and multiple functional domains with local regulation, as is the case of neurons.

In neurons, active transport occurs via three distinct molecular motors that travel along different components of the cytoskeleton: myosin V on actin filaments and dynein and kinesin on microtubules. Original description of axonal transport was based on the average transport rate calculated by accumulation of different materials under nerve ligation studies and pulse-labeled experiments with radioactive precursors. Two main velocities were observed, namely slow and fast. Slow axonal transport has two components, slow component a (SCa) and b (SCb) usually transporting material distally to the cell body on MT tracks. SCa transports mainly structural proteins (neurofilaments and microtubules) distally to the cell body at a rate of 0.2-1 mm/day, whereas SCb transports microfilaments, metabolic enzymes and clathrin complexes at a rate of 2-8 mm/day. Studies on the rate of transport reflects the average material transported a certain distance in a specific time. Fast axonal transport includes the transport of vesicles in anterograde direction by kinesin at a rate of 200-400 mm/day and dynein, the retrograde motor at rates of 200-300. Transport of mitochondria is in the order of 50-100 mm/day[326]. Myosin V moves in actin filaments and myosin-based vesicular transport may occur in places with fewer MT, such as growth cones. Evidence of motor-cross talk also exist and may reflect the high complexity of this system to ensure proper delivery of materials necessary for neuronal function and structural maintenance [160].

Slow axonal transport deficits have been linked to lack of regeneration, whereas deficits in movement of membrane-bound organelles (MBOs) by fast axonal transport

(FAT) has been linked to impairment of a variety of neuronal functions that include synaptic transmission, action potentials and neurotrophin supply [326]. Intracellular transport involves several distinct steps, all of which represent points of regulation, and include at least a) selection of motors, b) selective loading of cargo, c) control of directionality and targeting, d) movement and velocity, e) unloading and release of cargo at its destination. All these steps are tightly linked to regulation of molecular motors (Figure 15).

a) Molecular motors

Molecular motors generate force from ATP hydrolysis to move cargoes along cytoskeleton tracks. The most studied microtubule-based motors are members of the kinesin superfamily and cytoplasmic dynein.

Kinesin represents a super family of motor proteins with many subfamilies with specific functions [153]. So many variants were discovered in such a short time that the same variant would have several different names. Thus, a new standardized nomenclature was designed based on 14 family designations [330]. Kinesin-1 (also known as KIF5 or conventional kinesin) was the first one identified and is the most abundant. It participates in transport at different neuronal locations such as axon, terminal, golgi/ER, endosomes and cell bodies and it is widely observed in anterograde axonal and dendritic transport. It consists of 3 isoforms, A, B and C, all of them form a heterotetramer of two kinesin heavy chains (KHC) and two kinesin light chains (KLC).

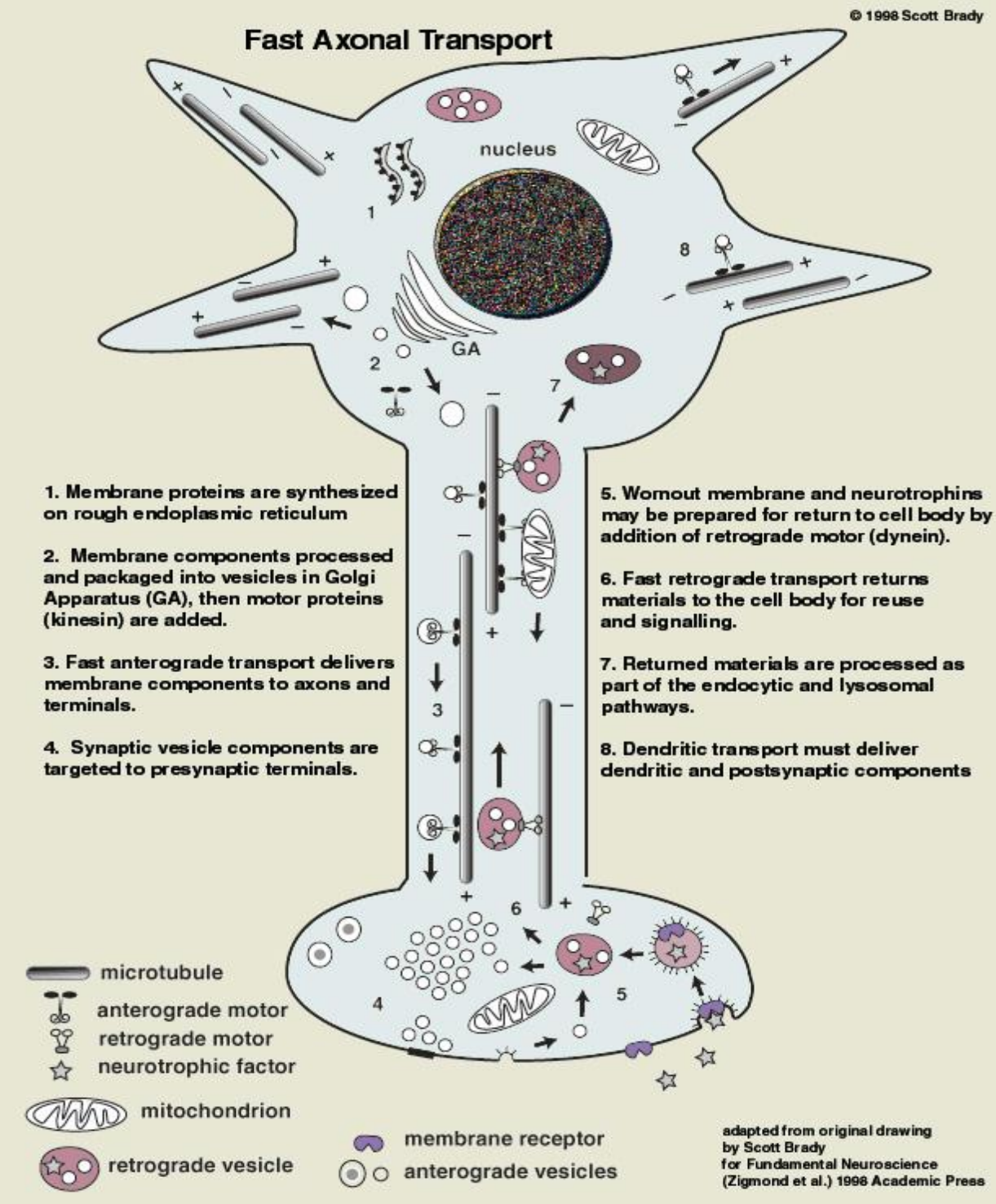


Figure 15. Fast Axonal Transport

KHC is composed of the catalytic motor domain, a short neck linker region, an α-helical coiled-coil stalk that is interrupted by two hinge regions, and the tail. The motor domain binds microtubules, contains the ATPase activity, and, together with the neck, confers processivity and directionality. The stalk is involved in dimerization and the tail, together with KLC, binds cargo and regulates motor activity [331]. KLCs also have several isoforms that give specificity to cargoes [332].

Cytoplasmic dynein (CDyn) is part of the dynein superfamily that consist of a multi subunit complex with two sub variants, CD1 and CD2, the latter one being exclusive of flagella. CD1 consists of two heavy chains that are associated with intermediate chains, light intermediate chains, and light chains. In mammals, all but one are encoded by more than two genes with differential cellular expression patterns. The heavy chains harbor a complex ATPase activity and bind to microtubules, whereas the other chains are involved in cargo binding and binding to dynactin. Dynactin is a protein complex of at least seven proteins (p150Glued, dynamitin, actin-related protein1- the most well-known) which can regulate the specificity of cargo as well as motor processivity. [154]. Similarly to kinesin a new standardized nomenclature was also needed due to the different variants discovered [333].

The complex and variable structure and organization of MT-based motors renders the possibility of a complex regulation of their function as well.

b) Molecular motor regulation in health and disease

Molecular motors are critical for cell function playing crucial roles in development, growth, and regeneration and dysfunction of molecular motors has been associated with several degenerative diseases [156, 334]. Interestingly, mutations in KCH isoforms can

cause inhibition of binding to vesicles and cause peripheral neuropathies. These include Charcot Marie Tooth disease 2A (KIF1Bbeta) [335], motor and sensory neuron degeneration in hereditary spastic paraplegia (KIF5) [164, 336] and denervation of oculomotor and/or trocheal nerves in congenital fibrosis of the extraocular muscles type 1 and 3 (KIF21A) [337]. Mutations in p150 glued have been identified in families with slowly progressive autosomal dominant forms of motor neuron disease with sensory deficits [338]. Other mutations in molecular motors or associated proteins using animal models were sufficient to cause neurodegeneration and resemble many of the neuronal specificity and phenotypes of human diseases [339]. These findings suggest that disruption of molecular motors function is sufficient to cause neurodegeneration.

Changes in FAT can also be a result, not only of molecular motor mutations but also regulation of their function by kinases and phosphatases [155]. Among some of them, cell-specific expression of isoforms with specific functional domains susceptible for post-translational modifications, such as phosphorylation, could in part explain neuronal vulnerability for different neurodegenerative diseases. These post-translational modifications can lead to motor conformational changes that can affect protein-protein interactions and change the affinities for binding to MT and vesicles or alter intrinsic ATPase activity locally at sites of selective delivery [157]. Initial studies on MT-based molecular motor functions have shown that phosphorylation of kinesin can affect its binding to vesicles [340]. GSK3 beta was shown to phosphorylate KLC leading to changes in transport via inhibition of binding to vesicles [158]. Later, it was discovered that this signaling pathway was also regulated by Cdk5 and PP1 [159]. Other sites of phosphorylation in KLC can also regulate binding to specific kind of vesicles [341]. Also,

phosphorylation of KHC adaptor proteins can regulate the binding of kinesin to specific cargoes, such as synaptic vesicles [342]. Moreover, phosphorylation of KHC itself can lead to dissociation to scaffolding proteins that bind to specific cargoes, like NMDA receptors [343]. Specific KHC phosphorylation by TNF-JNK signaling pathway can inhibit transport of specific vesicles by inhibiting kinesin binding to MT [344]. Phosphorylation of KLC can also lead to changes in protein-protein interactions, such as with the chaperone protein 14-3-3 [345]. Similarly, phosphorylation of DIC can regulate its binding to dynactin and affect cargo binding [346]. Moreover, phosphorylation of motor proteins may also serve as recognition sites for heat shock proteins and regulate kinesin binding to vesicles [347]. Heat shock proteins are elevated in diabetic neuropathies playing crucial roles as defense mechanisms. Recently it has been shown that inhibition of hsp reverses hypoalgesia in diabetic mice, representing a novel exciting target for drug treatment [348, 349].

A very tight regulation of kinases and phosphatases is needed for normal molecular motor function. Thus, disease-modifying signaling pathways, such as changes in kinase activities in diabetes, can alter the normal function of molecular motors leading to changes in FAT and subsequent degeneration. Specific kinases including GSK3 beta, p38 and JNK have been linked to changes in FAT in many neurodegenerative diseases [162] such as Alzheimer's [350-353], Parkinson's [168], Polyglutamine expansion diseases [165, 166, 354] and depression [355]. Changes in the activity of the aforementioned kinases also occur in diabetes, potentially modifying the normal pattern of motors phosphorylation and thereby causing the deficits in FAT observed in DN.

2. Axonal transport deficits in DN

A large amount of evidence has been published describing FAT deficits in diabetic nerves and its contribution to nerve dysfunction. Deficits in axonal transport in diabetic nerves can largely be divided into three categories: 1) Slow axonal transport and reduced transport of structural proteins leading to morphological changes to axons and reduce regeneration, 2) Fast axonal transport of neurotransmitters leading to decrease transmission 3) Fast axonal transport deficits involving signaling molecules and neurotrophins leading to altered connectivity and target withdrawal.

a) Structural and cytoskeleton proteins

Initial studies of axonal transport deficits have focused on the study of glycation of tubulin and its deficits in microtubule polymerization [356]. Decreased transport of structural proteins such as neurofilaments, tubulin and actin, have been shown in animal models of diabetes [170, 357-359]. Although these findings are relevant to the loss of normal nerve anatomy in DN , they may only reflect late events in nerve damage as exemplified by reduced axon caliber and reduced nerve regeneration occurring after several months of DM in animal models [360, 361].

b) Reduced transport of neurotransmitters

FAT deficits also occur early in the disease contributing to early nerve dysfunction and altered neurotransmission. Interestingly, early changes in the activity of diabetic nerves could be associated with disruption of catecholaminergic [362, 363] and neuropeptidergic neurotransmission, as a result of axonal transport deficits [364].

In STZ rats reduction in anterograde and retrograde transport of choline acetyltransferase in sciatic nerves correlated with deficits in motor nerve conduction velocities [171] and was prevented by aldose reductase inhibitor [365]. Moreover, reduced transport of substance P (SP), a molecule important for pain perception, has also been described in DM sensory nerves [366, 367]. SP has also been proposed to carry cardiovascular pressor responses via vagal afferent projections [368]. Aldose reductase inhibitor, statil, has shown partial recovery of SP transport in long term STZ-diabetes [369]. Furthermore, abnormal nitric oxide (NO) neurotransmission especially in the autonomic system was attributed to deficits in FAT [370]. In addition, impaired transneural retrograde trafficking, using wheat germ aglutinin labeled with horseradish peroxidase, was observed in vagus nerve of STZ animals [371]. Also, decreased retrograde of fluoro-gold labeling was observed in retina of STZ animals and this effect was reversed by aldose reductase inhibitor treatment [151]. These studies suggest an intrinsic neuronal transport deficit as a consequence of the metabolic disarrangements occurring in DM.

c) Altered transport of signaling molecules and growth factors/neurotrophins

Deficits in FAT can lead to reduction in neurotrophic factors altering trophic relationships between nerve and end tissues before signs of neuronal degeneration or death appear, leading to changes in innervation. Altered retrograde and anterograde transport of many of these factors have been shown in diabetic nerves such as NT-3, BDNF and NGF.

In the visceral sensory and autonomic system, alteration in NGF transport has been associated with neuropathy. A recent paper shows the dependency of NGF signaling in cardiac sensory function when rescuing normal cardiac sensory function by NGF gene transfer in an NGF -/- background [215]. Several reports show reduced axonal transport for both NGF and NT-3 in afferent and efferent vagus nerves [172, 173].

In the somatic sensory system, several groups have also shown reduction of FAT. In sciatic nerves of STZ rats, BDNF was shown be reduced due to FAT deficits [174]. Reduction of NGF without altering the expression of its receptor was also observed in STZ animals [175]. Reduced axonal transport of neurotrophic factors such as neurotrophin 3 (NT-3) and NGF has been described in sensory and vagus nerves [372-376].

There is also evidence of reduction of FAT in motor neurons of STZ animals [377]. This finding suggests that although motor neurons degenerate later than sensory ones, deficits in FAT could contribute to early changes in motor nerve conduction velocities. Altered transport of signaling molecules that carry information from the periphery to the cell soma has also been described. Retrograde transport of active JNK and p38 is increased in sciatic nerves of STZ animals [378].

Reduced neurotrophic signaling due to FAT deficiency, impairs the normal connectivity of the neuron with its target, eventually leading to nerve terminal withdrawal and target denervation. Thus, nerve terminals denervation from targets, a common consequence of FAT deficits have also been reported in DM for the heart [318, 379-381], skin [382-384], muscle [385], pancreas [386] and gut [387, 388].

C. Aim and rationale

The aim of this section was to elucidate the pattern of kinase activation pertinent to axonal transport regulation and evaluate the function of molecular motors as a pathogenic mechanism of FAT deficits in DPN.

Fast axonal transport deficits have been shown to play key roles in neurodegenerative diseases. In some cases, FAT deficits are sufficient to explain to whole process of neurodegeneration, especially for peripheral nerves due to their highly dependence on FAT to transport essential material along the extensive length of their axons. In diabetic neuropathies, FAT deficits were largely described for sensory and vagus nerves, however, the molecular mechanism underlying this deficit has never been explored. Mechanism for FAT deficits include activation of kinases capable of regulating FAT and changes in molecular motors function. Studies on squid axoplasm revealed that GSK3 beta and JNK can directly impair FAT. Thus, to complement the role of common disease-modifying kinases have on FAT, isoforms of p38 kinases were tested in squid axoplasm. Activation of JNK and p38 has been shown in diabetes as a result of hyperglycemia. However, these kinases were mostly associated with symptoms of hyperalgesia and were mostly activated in the cell soma, mechanism of which may be independent of the development of insensate neuropathy. Thus, to clarify the pattern of kinase activation in an animal model of insensate neuropathy, activation level of specific kinases, GSK3 beta, p38 and JNK, were studied in parallel in both sciatic and vagus nerves. Finally, phosphorylation of motor proteins can affect several of their functions, namely binding to MT, vesicles or associated proteins. Studies of phosphorylation of dynein are complex and less standardized as kinesin. Therefore, the

ability of kinesin to bind to MT was tested in both vagus and sciatic nerves. The study of kinase activation and ability of kinesin to bind to MT represents the first attempt ever described in the literature to elucidate the mechanism of FAT deficits in diabetic neuropathies.

D. Materials and methods

1. Kinase perfusion in squid axoplasm

a) Squid model

The squid, *Loligo peali,* was used as a model organism to test the effect of p38 active kinase on axonal transport. Squid giant axons lack myelin yet they are able to transmit fast action potential due to their large axons, 1mm wide. Brady lab and others have taken advantage of this natural phenomenon to study mechanisms of fast axonal transport regulation in a laboratory setting [389]. Squid's large axon is easy to dissect and visible at the naked eye, facilitating its handling and manipulation. Squid are extremely sensitive to captivity, only surviving a day or two after being caught in the wild and unhealthy squid often have reduced transport rates (Brady et al, unpublished). To overcome this difficulty, Brady lab spends the summer in The Marine Biological Lab (MBL) in Woods Hole, MA, which provides fresh caught squid every day (Monday-Saturday).

b) Motility studies in squid axoplasm

From the squid giant axon (*Loligo pealeii*, Marine Biological Laboratory), axoplasm was extruded onto a glass coverslip as described previously [390, 391]. Axons,400-600 μm in diameter, were tied on one end with black thread and white thread

on the other end to distinguish retrograde from anterograde positions. Axons were cleaned up and placed onto a coverslip. Slowly, axoplasm was extruded by gently and steadily rolling of a plastic tubing onto the giant axon while pulling the tread towards the opposite side. Axoplasm extruded this way maintains the proper cytoskeleton structure and activity needed to perform transport studies. After extrusion, a second coverslip was placed on top of the axoplasm, creating a chamber that allows perfusion of small volumes of solutions.

Recombinant phospho p38 isoforms (Upstate (p38 alpha #14-251, p38 beta #14-253)) were diluted in a buffer with a final concentration of 175 mM potassium aspartate, 65 mM $MgCl_2$, 5 mM EGTA, 1.5 mM $CaCl_2$, 0.5 mM glucose, pH 7.2 and supplemented with 2-5 mM ATP. chambers were perfused with 20µl of this mixture. Concentrations of each protein were adjusted based on IC50 values. Perfused axoplasms were analyzed on a Zeiss inverted Axiomat with a 100x, 1.3 NA objective, and DIC optics. Hamamatsu Argus 20 and Model 2400 CCD cameras were used for image processing and analysis. Organelle velocities were measured with a Photonics Microscopy C2117 video manipulator (Hamamatsu) by matching the speed of calibrated cursors to the speed of moving vesicles. Using this method the rate of transport is a combination of both the velocity and amount of vesicles in a population, since individual vesicles come in and out of the focal plane and cannot be individually tracked. Before perfusion, axoplasm was tested for proper structure and background velocities. If those were normal, then perfusion was allowed and transport for both retrograde (reverse arrow heads) and anterograde (forward arrow heads) directions were measured for each experiment for

50 minutes. After 50 min, ATP levels rapidly deplete and transport rates decline (Brady lab, unpublished)

2. Nerve homogenization

Control and STZ DM animals after 8 weeks post injection, were pre anesthetized with CO_2 and euthanized by cervical dislocation. Sciatic and vagus nerves were excised from control and diabetic animals, rinsed in PBS pH 7.4, snapped frozen in liquid nitrogen and kept at -80°C until time of processing. Tissue was weighed, cut in small pieces on frozen metal block, pulverized in ceramic grinder using liquid nitrogen and then homogenized in glass homogenizer with cold buffer (2ml/g tissue for sciatic nerves and 1ml/g tissue for vagus nerves). Since the same nerves were used for western blot studies and microtubule binding assays, homogenization buffer was adapted to be compatible for the binding experiments. The buffer was composed of: 80 mM BRB80 pH 7 (80 Mm PIPES, 5 mM $MgCl_2$,1mM EGTA), 0.1% CHAPS (zwitterionic detergent to preserve native state), 1X protease inhibitor cocktail (Sigma #P8340), 1X phosphatase inhibitor cocktail 3 for Ser-Thre phosphatases (Sigma #P0044), 1µM stausporine (wide spectrum protein kinase inhibitor) (Calbiochem #569396), 1µM microcystin R-R (inhibitor of PP2A) (Calbiochem #475816), 1µM K252a (protein kinase inhibitor). For microtubule binding optimization, different sets of inhibitors were used in addition to the already mentioned compounds: 1) With both phosphatase inhibitor cocktail 3 for Ser-Thre phosphatases (Sigma #P0044) and Phosphatase inhibitor cocktail set II (Calbiochem # 524625); 2) Without Phosphatase inhibitor cocktail set II (Calbiochem # 524625); 3) Without both Phosphatase inhibitor cocktail set II and phosphatase inhibitor cocktail 3. Homogenates were then centrifuged at 14,000 rpm for 15 min at 4°C. Fresh

cleared supernatant aliquots were used for microtubule binding assays and aliquots for western blots of kinases and protein quantification were kept frozen at -80°C until time of processing.

3. Protein quantification by BCA

Frozen aliquots were thawed the day of the assay and protein concentration measured by bicinchoninic acid (BCA) assay kit (Pierce #23225, Thermo Scientific) using the microplate procedure as explained in detail in Chapter III.

4. Western blots for phospho proteins

a) Characterization of p38 antibodies

Several activation-specific antibodies against p38 were evaluated for their ability to distinguish specific p38 isoforms.Equal amounts of GST-tagged, recombinant (human) p38 alpha, beta and gamma proteins were run on SDS/PAGE, transferred to nitrocellulose membranes and immunoblotted with the following antibodies and developed by ECL. *A*, anti-Glutathione –S-Transferase (GST) antibody *B*, anti-p38 MAPK antibody #9217, *C*, anti-p38 MAPK antibody #9212, *D*, anti Phospho-p38 MAPK antibody #9215. *E*, anti Phospho-p38 MAPK antibody #9211, *F*, anti Phospho-p38 MAPK antibody #4631. *G*, anti Phospho-p38 MAPK antibody #9216. (GST) Shows equal levels of each p38 isoform. (A-B) Both #9218 and #9217 were able to specifically see p38 alpha (C) #9219 recognizes both alpha and beta isoforms but none of the total antibodies tested were able to specifically distinguish the beta isoform (A-C). As seen in panels D-G, none of the phospho-specific antibodies were capable of distinguishing between isoforms

b) Positive control for p38 activation

p38 is activated by dual phosphorylation in Thr180/Tyr182 in response to different stimuli such as stress, UV, inflammation and growth factors. For positive control, SHY5Y cells were treated with sorbitol acting as an osmotic stress for the cells.SHY5Y cells were grown in DMEM medium in 100mm dish previously coated with poly-lysine. When cells reached 90% confluence they were washed once and then treated with freshly made 0.5 M sorbitol in DMEM medium in 37°C. After incubation, dish was placed on ice, washed with cold PBS and 500ul of cold lysis buffer added. Lysis buffer was composed of: 50mM HEPES pH7.4 (Sigma #H4034), 1% Triton X-100 (Sigma #X100), 150mM NaCl (Sigma #S7653), 5mM EDTA (Sigma #ED), Phosphatase inhibitor cocktail set II 1/100 (Calbiochem #524625), 0.1uM Okadaic Acid (Calbiochem #495604)(1/1000), Mammalian Protease inhibitor 1/100 (Sigma #P8340). After 5 minutes, cells were scraped and transferred to centrifuge tubes for sonication (5 sec x3). Sonication is crucial to break up the nucleus and release active p38. Tubes were then centrifuged at 14,000 rpm for 15 minutes at 4°C. Supernatant was recovered and aliquots frozen at -80°C. A small aliquot was reserved for protein quantification. SDS electrophoresis was run with 40µg of protein in 4-12% Bis Tris 1.5mm Nupage gels (Invitrogen) in MOPS buffer in 0.05Amp /gel and transfered to nitrocellulose membranes at 0.4Amp for 2 hours in a small Hoeffer tank. Membranes were blocked in 5% milk in 1X PBS for 1 hour and primary antibodies added overnight. Antibodies:

p-38 (CS9212):rabbit polyclonal; 1:250 dilution

P-p38 (CS9215): rabbit monoclonal@Thr180/Tyr182(3D7), 1:250 dilution

The following day, primary antibodies were washed (10min X3) and incubated with secondary antibody HRP-goat @rabbit IgG(H+L) (Jackson 111-035-045) 1:15000 dilution for 1 hour at 4°C. Secondary was then washed (10min X3) and protein visualized by ECL.

c) SDS electrophoresis and western blot specific for phospho proteins

A modified western blot protocol for phospho-proteins was used to preserve the phosphorylation state of proteins as previously described [392]. Basic western blot protocol was performed as described in detail in Chapter III. Phosphatase inhibitors (200mM Sodium orthovanadate and 300mM sodium fluoride) were added to blocking buffers and primary and secondary antibodies solutions.

List of primary antibodies used:

Antibody	Company	Catalog	Dilution	Source	Blocking
Total p38	Cell signaling	9212	1:1,000	Rabbit	5% BSA in TBST
P-p38	Cell signaling	9215	1:1,000	Rabbit	5% BSA in TBST
Total JNK		9252	1:1,000	Rabbit	5% BSA in TBST
P-JNK	Cell signaling	9251	1:1,000	Rabbit	5% BSA in TBST
Total GSK3 beta	BD	610201	1:2,000	Mouse	5% milk TBST
P-GSK3 beta	Cell signaling	9336	1:1,000	Rabbit	5% BSA in TBST
H2	Brady lab		1:10,000	Mouse	5%milk in PBST

Membranes were first blotted with phospho specific antibodies, stripped and then blotted against total antibodies.

d) Stripping procedure

Stripping was performed following abcam guidelines. Briefly, stripping buffer (medium strength) was prepared the day of the experiment. For 1L of buffer, 15 g glycine, 1 g SDS, 10 ml Tween20, and water was added with final pH of 2.2. Membranes were first washed 5 min with PBS (2x), 10 min with stripping buffer (2x), 10 min with PBS (2x), 1 min methanol rehydration, 5 min TBST (1X), Followed by blocking stage.

5. Microtubule binding in sciatic and vagus nerves

Microtubule polymerization and microtubule binding was performed based on published protocols with modifications [393, 394].

a) Microtubule polymerization

Purified pig tubulin (2mg/ml) was obtained by two cycles of polymerization-depolymerization in high molarity buffer as previously described [395]. Microtubule polymerization was performed for 30 min at 37°C, by incubating freshly thawed cycled tubulin in BRB80 buffer pH 6.8 (80 mM PIPES, 1mM $MgCl_2$, 1mM EGTA) plus 1mM DTT, 1mM GTP and taxol added stepwise to a final concentration of 20µM. Microtubules were pelleted in ½ volume warm cushion (50% sucrose, 1mM DTT, 20 µM Taxol in BRB80 pH: 6.8) at 50,000 rpm for 30 min at 37°C. Then, pellet was washed and microtubules resuspended in BRB80 plus 1mM DTT, 20 µM Taxol. Microtubules made this way are stable up to one week at RT.

b) Microtubule binding

Nerve lysates were used fresh, precleared by centrifuging at 50,000 rpm for 20 min at 4°C. Then cleared supernatant was incubated with 30 µM taxol, 5mM of either ATP or AMP.PNP and taxol-stabilized microtubules to 0.2mg/ml in BRB80 High Mg (80mM PIPES, 5mM $MgCl_2$, 1mM EGTA) with protease inhibitors and 200 µM microcystin. After incubating at 10 min at RT with rotation, binding reaction was overlaid onto ½ volume of 20% sucrose in BRB80 plus 30 µM taxol and either 2.5mM ATP or AMP.PNP and centrifuged at 50,000 rpm for 20 min at RT. Supernatant was recovered and protein precipitated by methanol and resuspended in sample buffer in same volume as pellet. Pellet was washed and resuspended in sample buffer. Fractions were separated by SDS electrophoresis, transferred onto PVDF membranes and blotted against kinesin (H2 antibody) and alpha tubulin (DM1alpha antibody) as previously described in the Chapter III. For method calibration, different conditions were tested as described in the text.

E. Results

1. FAT reduction of phospho-p38 is isoform specific

To test the effect of p38 activation on FAT, members of active p38 isoforms were perfused in squid axoplasm (Figure 16). Under control buffer perfusion anterograde transport (blue arrow heads) was around 1.2 μm/sec and retrograde (green arrowheads) around 1.6 μm/sec (Panel A). Perfusion of active p38 alpha produced a reduction in anterograde transport, without affecting retrograde rates (Panel B). Perfusion of active p38 beta and gamma decreased both anterograde and retrograde transport (Panel C and D).

2. Increased GSK3 beta activation and reduced MAPK activation in DM nerves

To test the activation state of several kinases involved in diabetes, including GSK3 beta, JNK and p38, western blots against phospho specific kinases were performed for sciatic and vagus nerves in control and STZ DM animals at 8 weeks. In the following figures panel A) shows the ECL Western blot of phospho and total kinases in control and diabetic nerves and panel B) shows the quantification of phosphorylation by Image J.

In sciatic nerves, a significant increase in GSK3 beta activation was found in the DM group compared to the control group ($p<0.01$) (Figure 17). In vagus nerves, no difference was found in GSK3 beta activation between DM and control groups, however a trend towards an increase of GSK3 beta activation was observed (Figure 18)

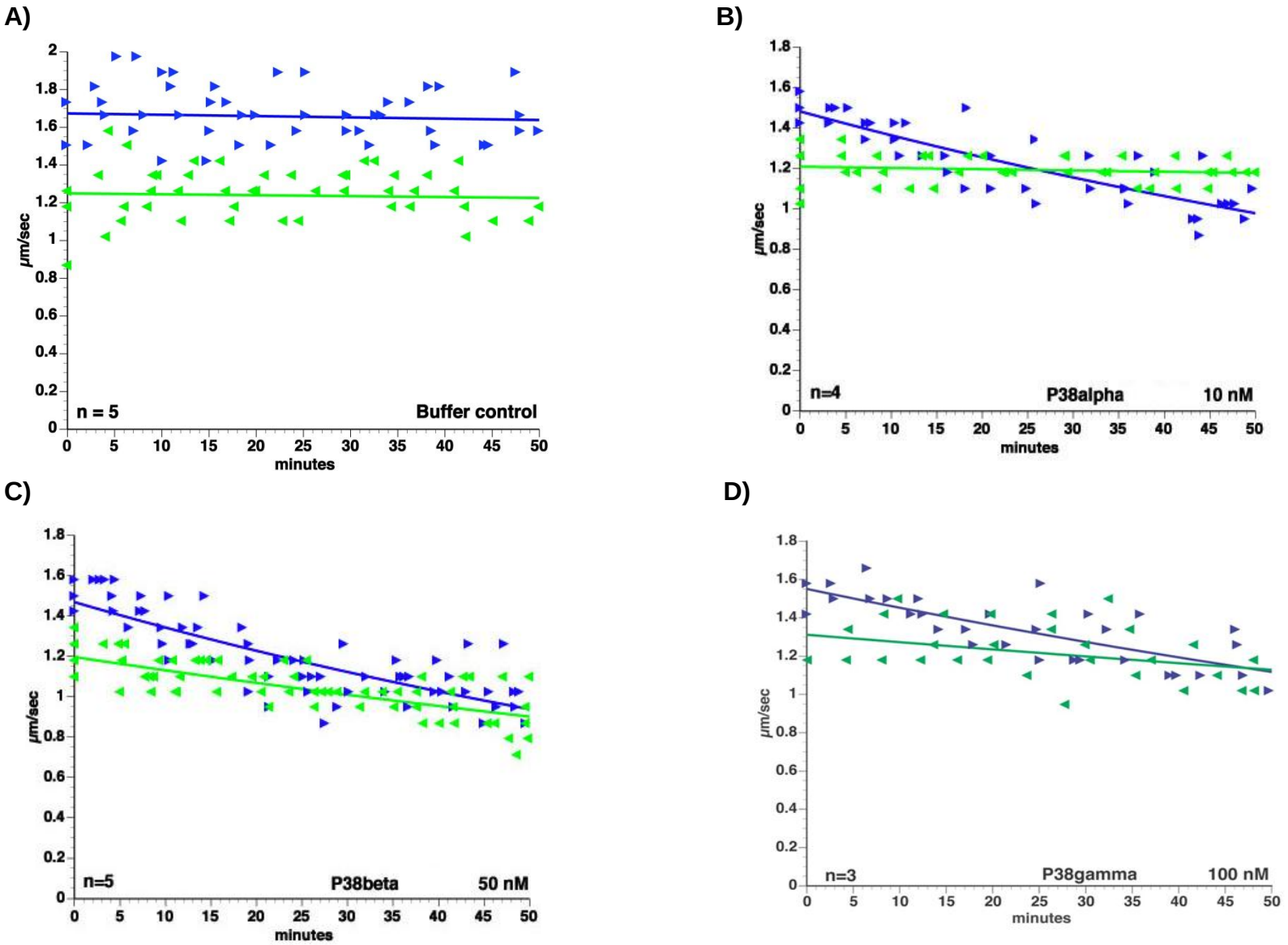
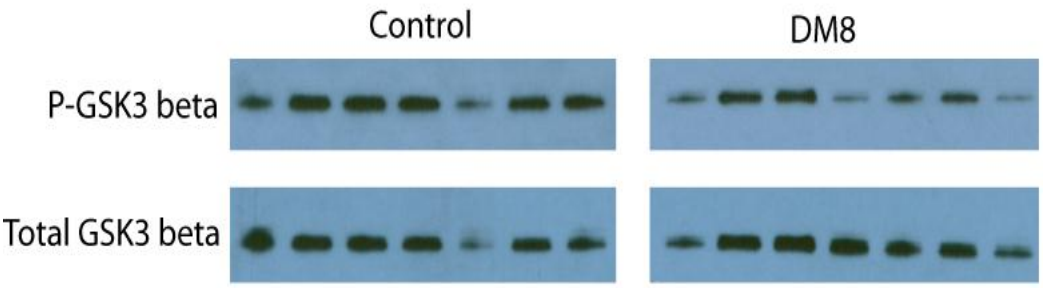


Figure 16. Effect of phospho-p38 isoforms perfusions on fast axonal transport

A)



B)

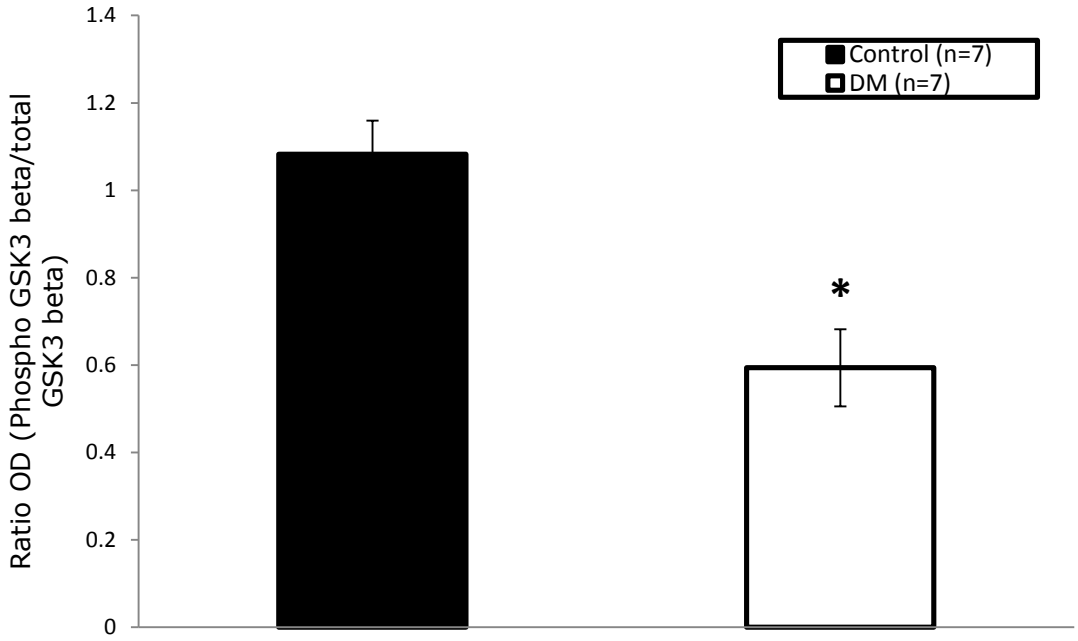
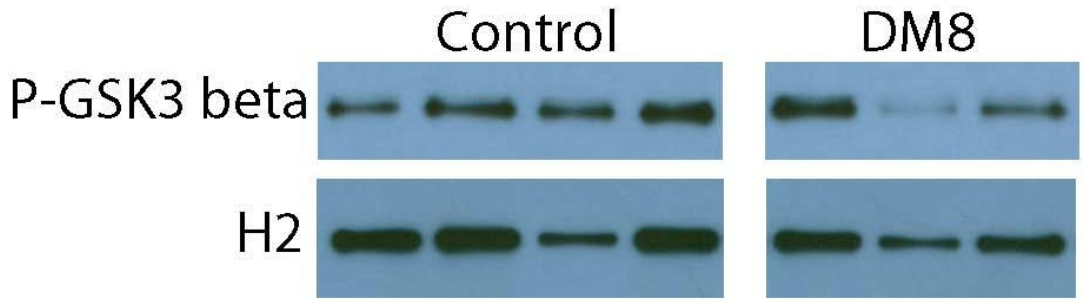


Figure 17. Increased GSK3 beta activation in DM sciatic nerves

T-test * $p<0.01$

A)



B)

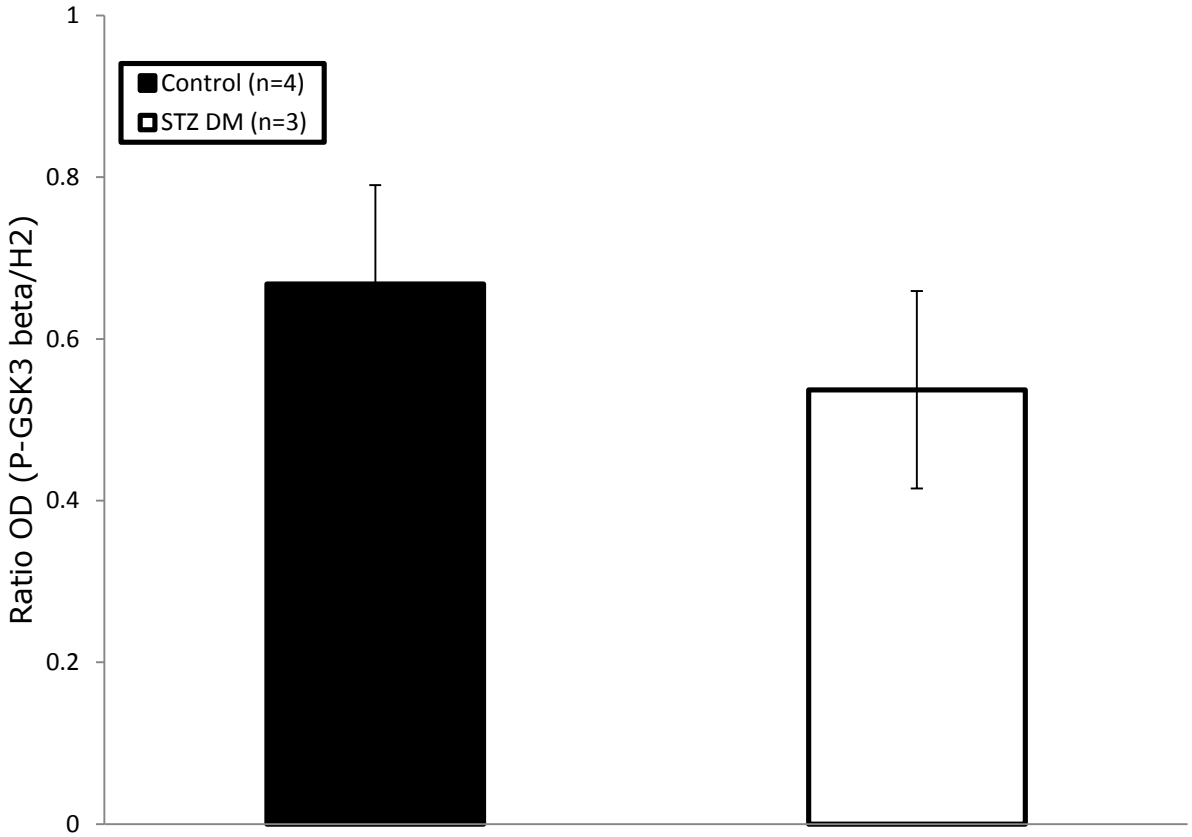
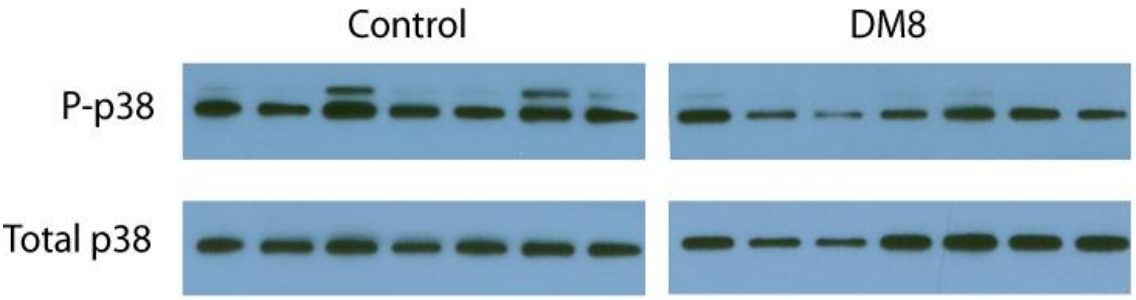


Figure 18. Study of GSK3 beta phosphorylation in vagus nerves

In sciatic nerves, a significant reduction in p38 activation was observed in DM group compared to control group ($p<0.01$) (Figure 19). In vagus nerves, p38 immunoreactivity was not sensitive enough to be picked up by the ECL signal (data not shown), thus the activity of p38 could not be studied. To test if the decreased activation observed in sciatic nerves could be isoform dependent, several isoform specific antibodies against p38 were evaluated (Figure 20). (GST) Showed equal levels of each p38 isoform. (A-B) Both #9218 and #9217 were able to specifically recognize p38 alpha (C) #9219 recognized mostly alpha and beta isoforms, and faintly p38 gamma. None of the total antibodies tested were able to specifically distinguish the beta or gamma isoform (A-C). As shown in panels D-G, none of the phospho-specific antibodies were capable of distinguishing between any of the isoforms. Thus, study of isoforms could not be readily distinguishable by simple western blotting.

In order to test that activation of p38 was occurring under typical diabetogenic state, such as increased sorbitol levels due to hyperglycemia, cultured cells were treated with sorbitol, a common p38 activator. Figure 21 panel A) showed the activation of p38 by treating SHY5Y cells with several concentrations of sorbitol, 0.1 M, 0.5 M and 0.8 M for 5 minutes. Activation of p38 was the highest when cells were treated with 0.5 M sorbitol. To test if increased incubation time increased p38 activation, cells were treated for 10 min with 0.5 M sorbitol. As shown in Figure 21 panel B), 10 minutes incubation produced a higher activation of p38 as indicated by increased immunoreactivity to phospho-specific antibody.

A)



B)

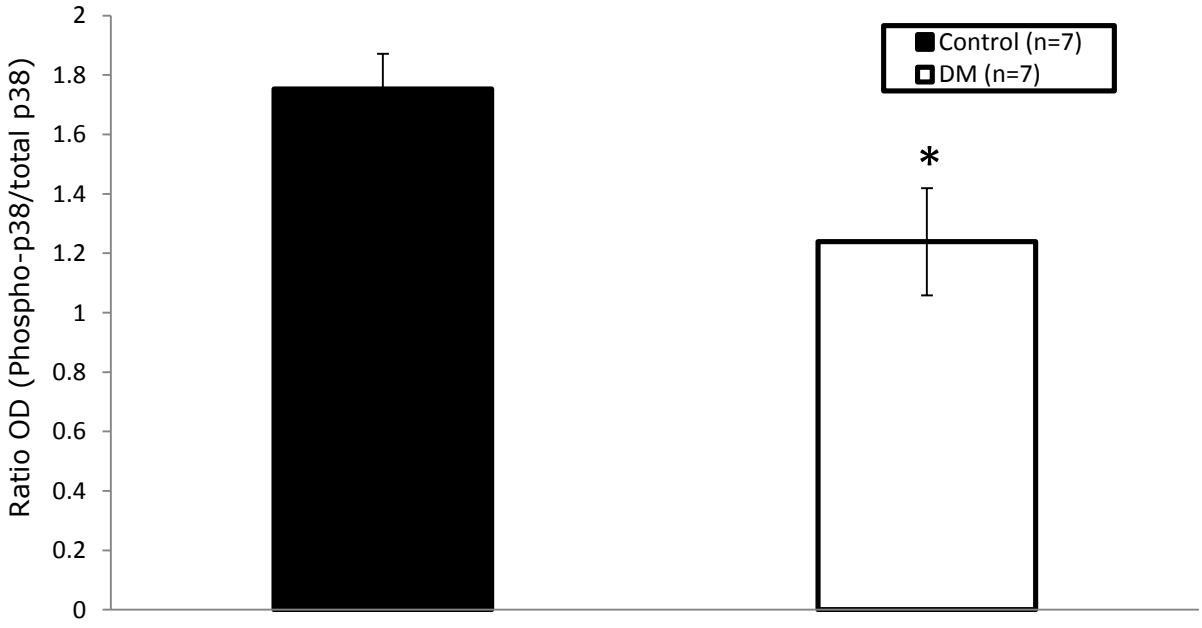


Figure 19. Decreased p38 activation in DM sciatic nerves

T-test * $p<0.05$

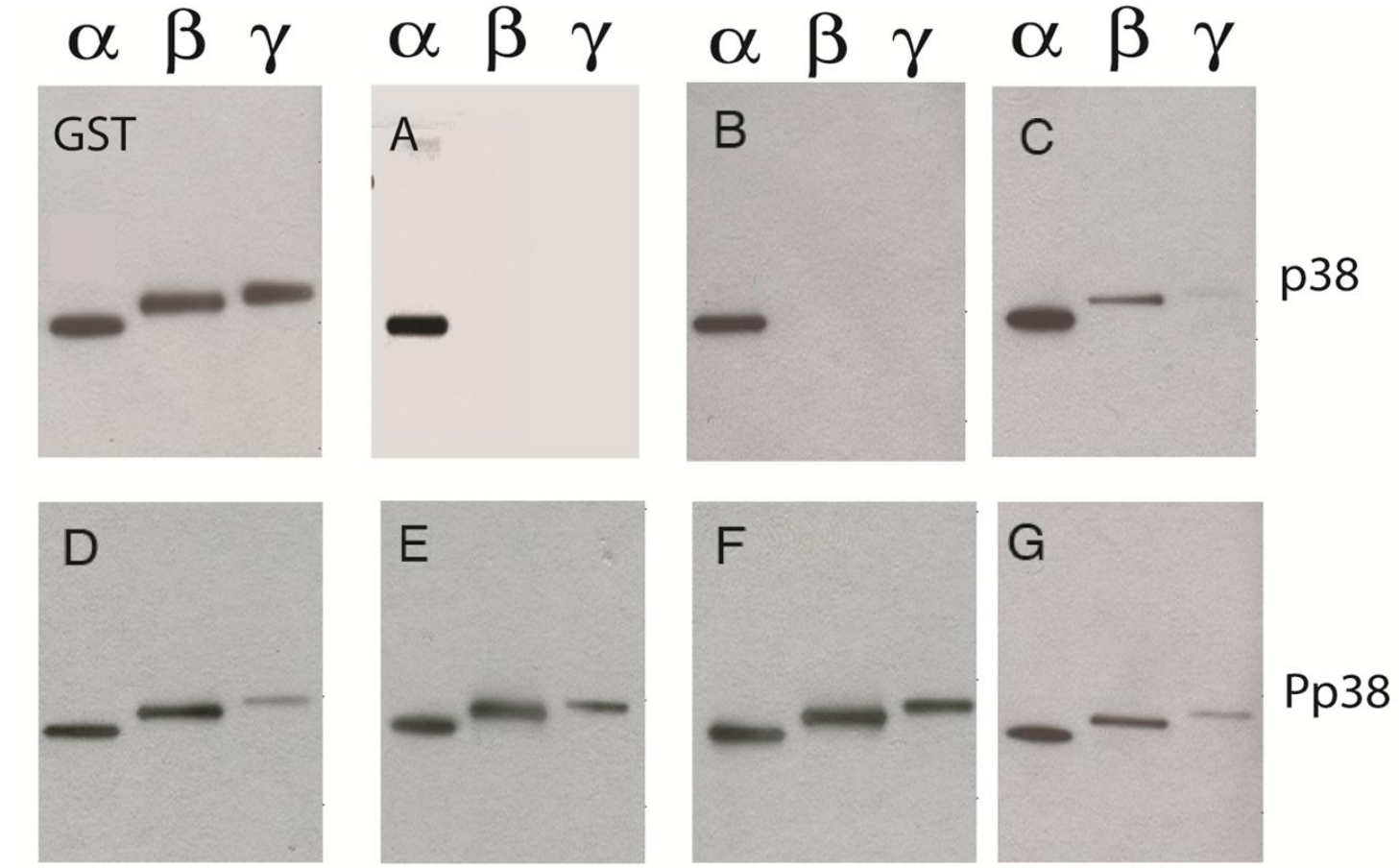
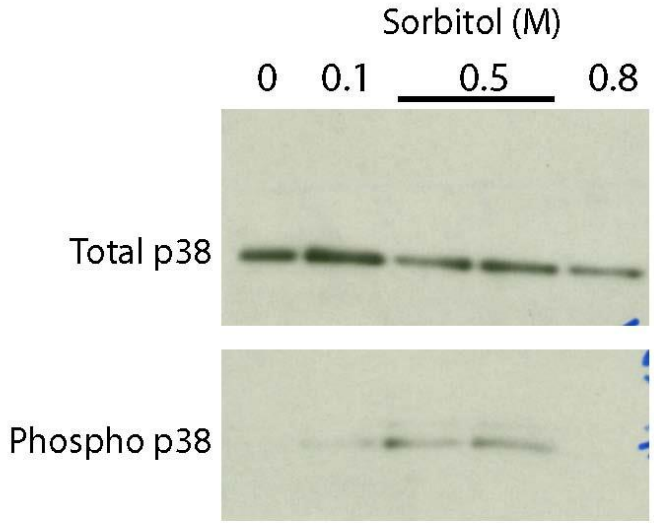


Figure 20. Characterization p38 antibodies

A)



B)

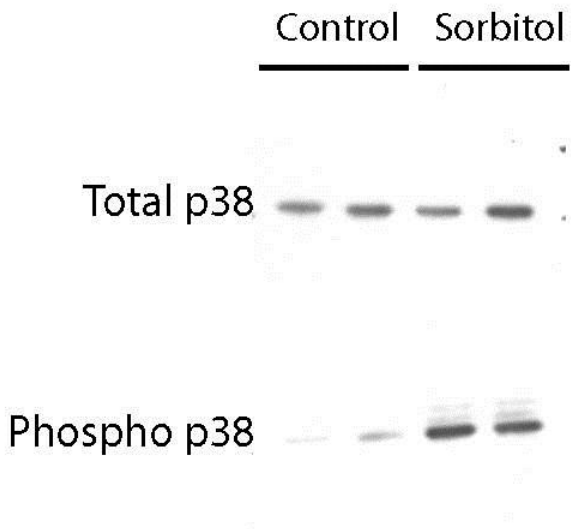
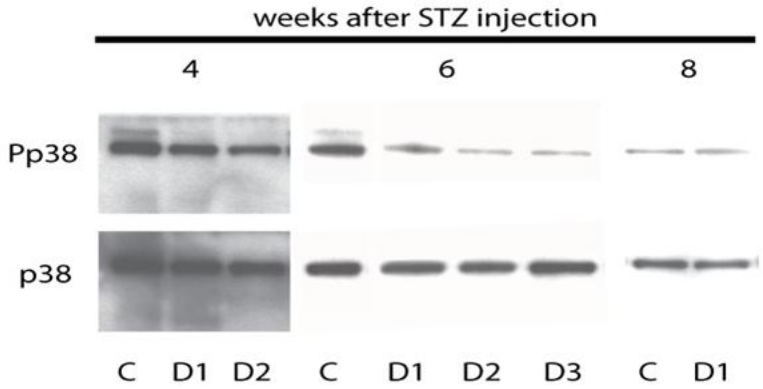


Figure 21. Activation of p38 under sorbitol conditions

To test if the decreased p38 activation observed in Fischer 344 DM rats was strain specific, activation of p38 was also tested in another strain, Sprague-Dawley rats, at 4, 6 and 8 weeks (Figure 22). For this experiment, male SD rats (300 g) were injected with 60 mg/kg of STZ. Glucose levels were >300mg/dl for all diabetic animals and weight loss was around 30% of controls. Two sets of control and diabetic animals were tested for different p38 antibodies. In DM animals, decreased of p38 activation was also evident at 4, 6 and 8 weeks. Because of the low number of animals, quantification and statistical analysis was not possible.

In sciatic nerves there was a significant decrease ($p<0.05$) in JNK p46 isoform activation at 8 weeks in DM group compared to control group (Figure 23). In vagus nerves, no significant difference was observed for JNK activation at 8 weeks in DM animals compared to controls. However, a trend towards a decreased activation of p54 isoform was observed (Figure 24).

A)



B)

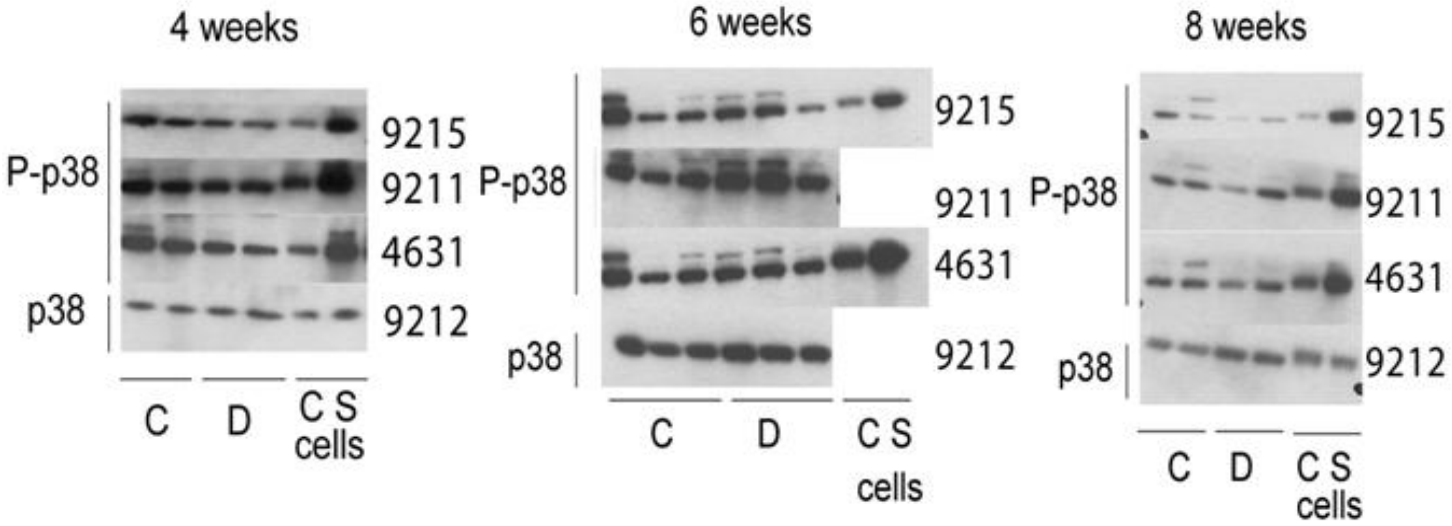
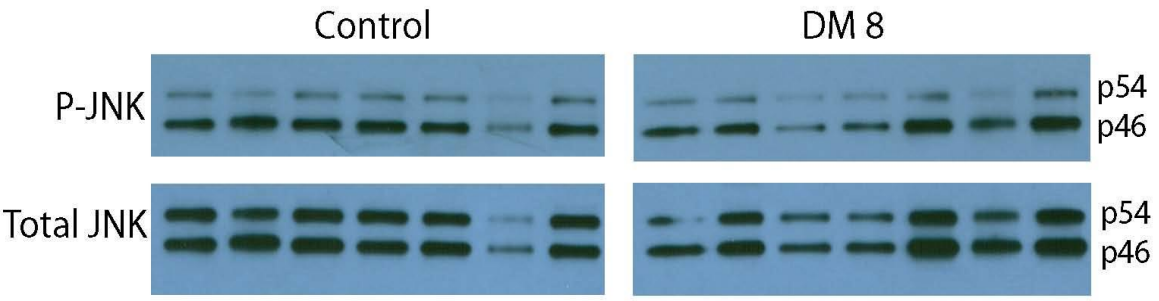


Figure 22.Time course study of p38 phoshorylation in Sprague-Dawley rats

A)



B)

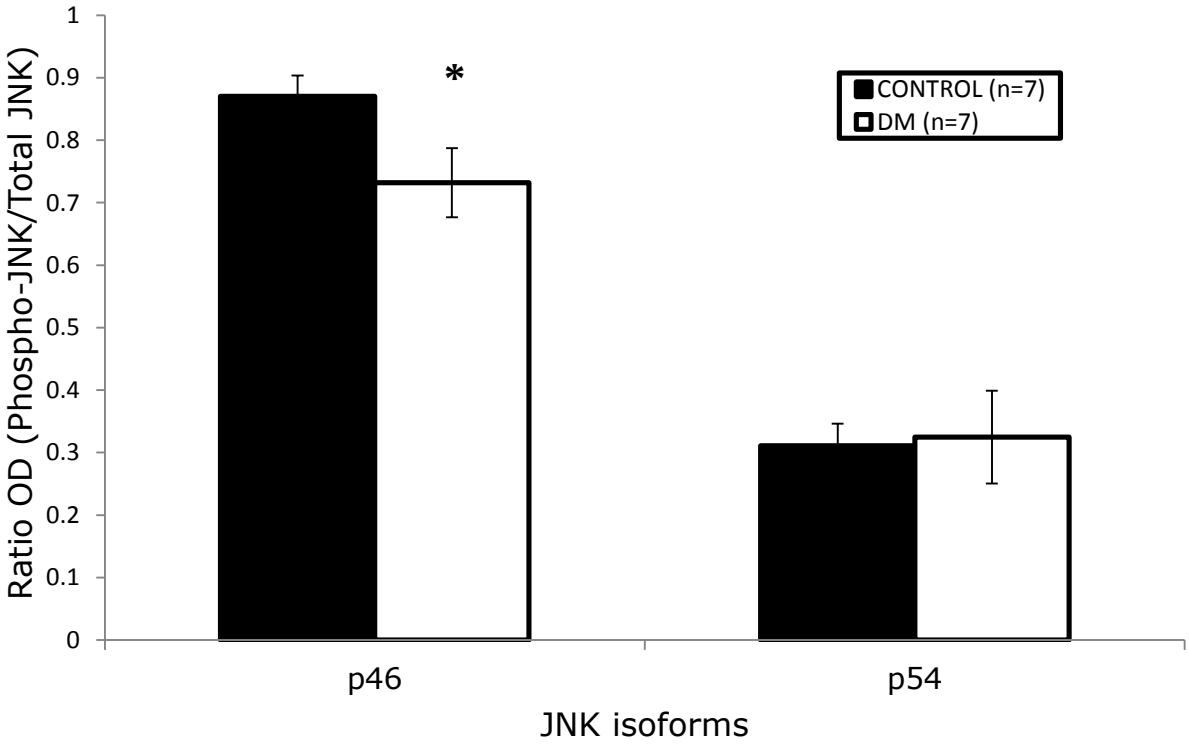
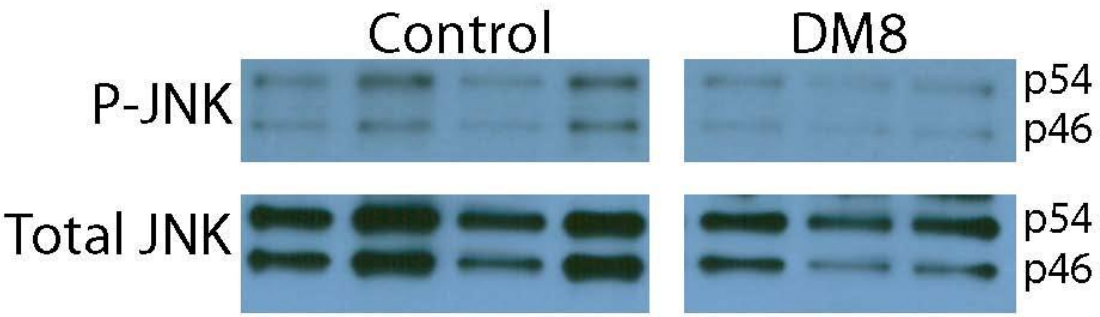


Figure 23. Decreased JNK activation in DM sciatic nerves

T-test * $p<0.05$

A)



B)

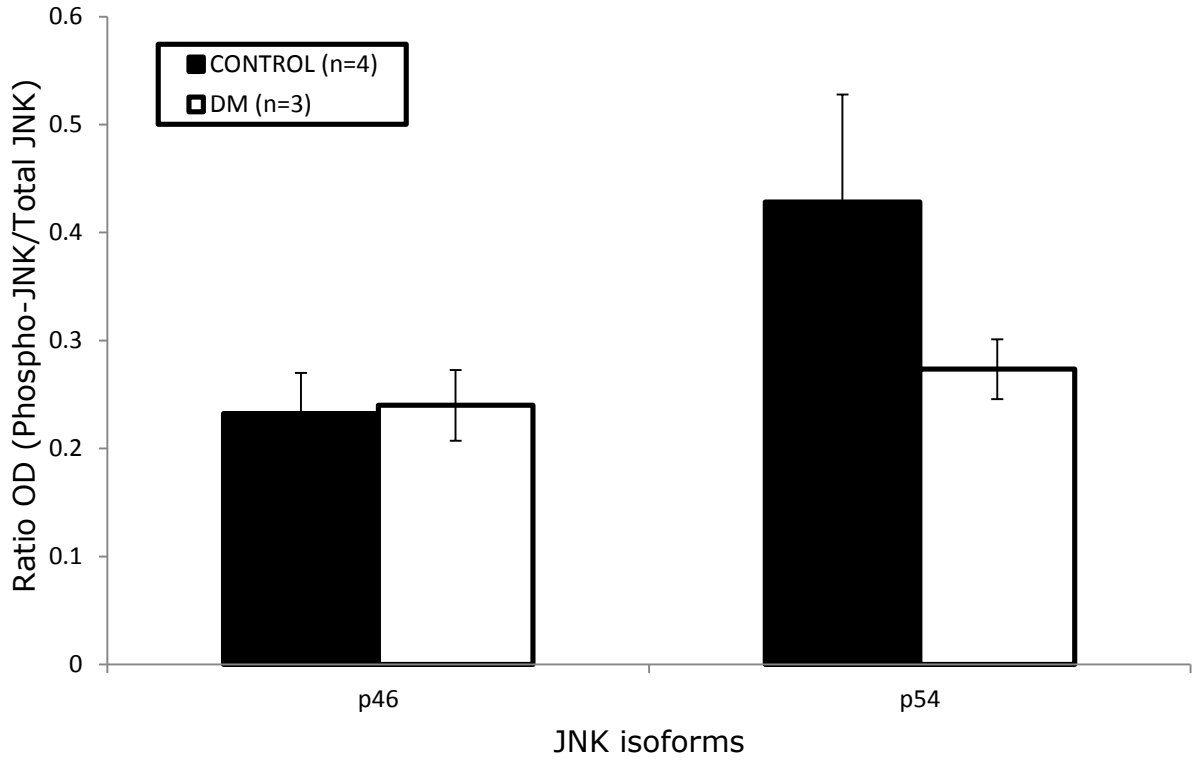


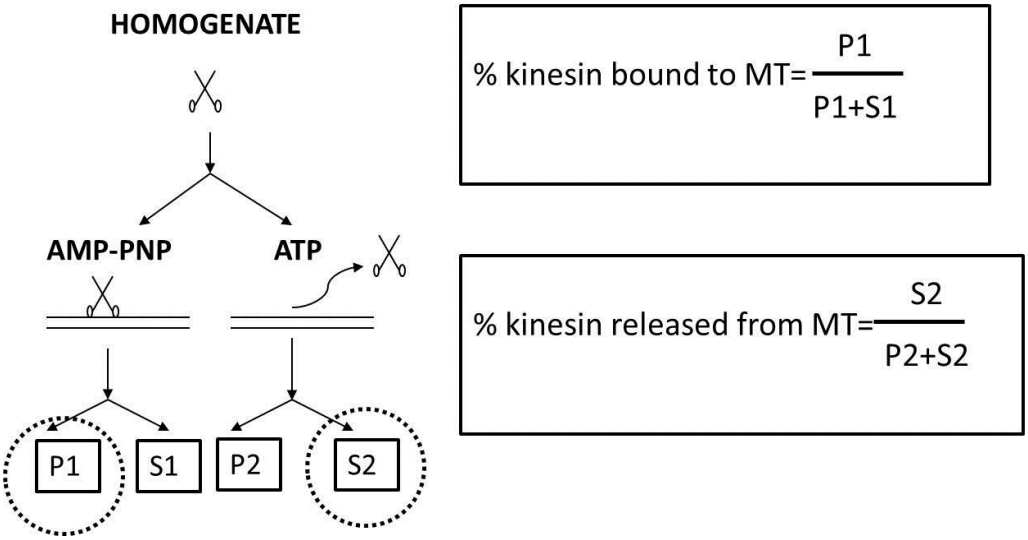
Figure 24. Study of JNK activation in DM vagus nerves

3. Microtubule binding optimization for frozen nerves

Microtubule (MT) binding assay is commonly performed for cell culture and fresh tissue. For this project, frozen nerves were used and therefore, different parameters needed to be adjusted from the basic protocol (Figure 25 and Figure 26). ATP hydrolyzation causes kinesin to be released from MT, allowing its movement. When kinesin is unbound to ATP, or bound to ATP but cannot hydrolyze it, it remains bound to MT. Thus, when endogenous kinesin from tissue of interest is subjected to binding to microtubules in presence of a non-hydrolyzable form of ATP, AMP.PNP, kinesin remains bound to MT and can be pelleted with MT. On the other hand, in presence of ATP, kinesin remains mostly unbound to MT and it is mostly found in the supernatant under MT pull down centrifugation process (Figure 25 panel A).

Nerves have a large amount of tubulin and to test possible endogenous tubulin polymerization, tubulin polymerization conditions and binding were performed at the same time with and without adding previously polymerized MT. As shown in Figure 25 panel B, without adding previously polymerized MT, no tubulin was observed in the pellets, indicating that endogenous tubulin was not able to polymerize. In accordance, kinesin was not observed in the pellet under AMP.PNP condition. This situation was recovered when MT were added exogenously. Under this condition, kinesin was found in the pellet in AMP.PNP conditions, and in the supernatant in ATP conditions. Confirmation of MT pelleting was observed by presence of tubulin in the pellets.This figure also showed that under this conditions dynein was AMP.PNP independent, and was always found in the supernatant under AMP.PNP and ATP conditions.

A)



B)

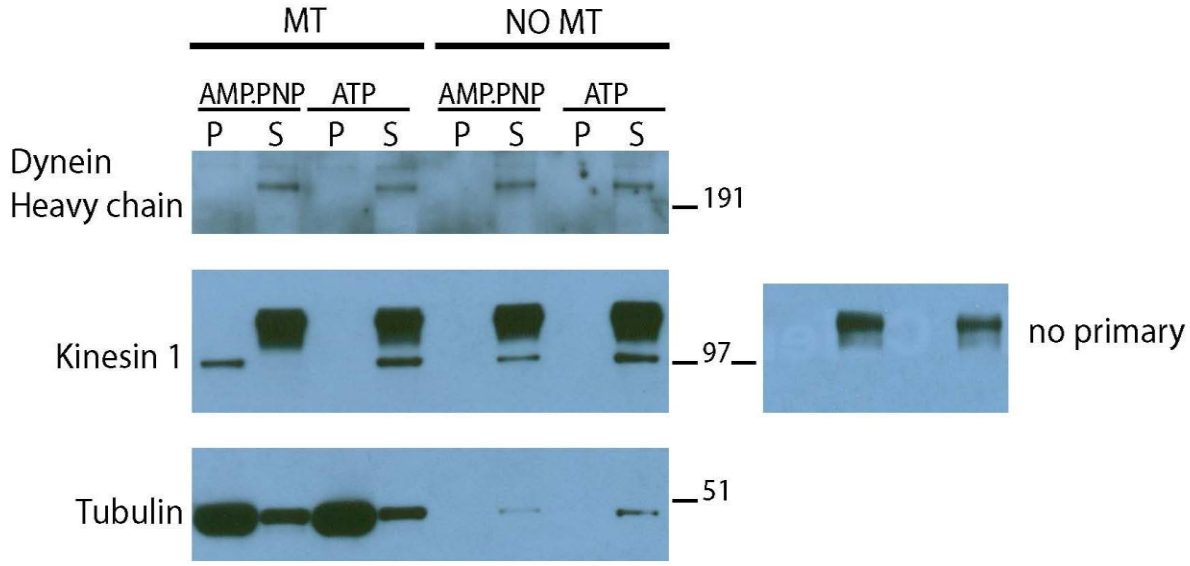


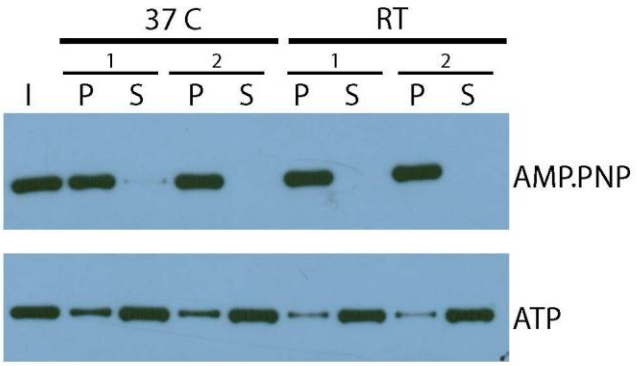
Figure 25. Microtubule binding optimization in frozen sciatic nerves

P: Pellet; s:Supernatant; MT: Microtubules; AMP.PNP: Adenosyl mono phosphate; ATP: adenosine tri phosphate

Frequently, kinesin was found in the pellet under ATP conditions when binding reaction was performed at 37°C. ATP is highly degradable, and binding incubation for long times at 37°C could be affecting ATP stability. To test this hypothesis, binding reactions were performed at 37°C and room temperature (RT) for 30 min. As shown in Figure 26 panel A), at 37°C under ATP conditions, more kinesin was found in the pellets when compared to RT conditions, indicating that higher temperatures may compromise ATP utilization, but even at RT kinesin was found in the pellet. To test if this could be a result of long incubation times, binding was performed at RT for 10, 20 and 30 min. As shown in Figure 26 panel B), kinesin was reduced in the pellets of ATP conditions as binding times were reduced.

Original protocols suggested to discard the cushion and only use supernatants to test for unbound proteins. However, it was noted that the amount of kinesin in the supernatants plus pellet did not match input levels, suggesting that some kinesin was missing. To test if the rest of kinesin was staying in the cushions, cushions were saved and blotted against kinesin along with the supernatants and pellets. As shown in Figure 26 panel B**,** cushions had a large amount of kinesin, thus both cushion and supernatants were taken together to assess the amount of kinesin unbound to MT.

A)



B)

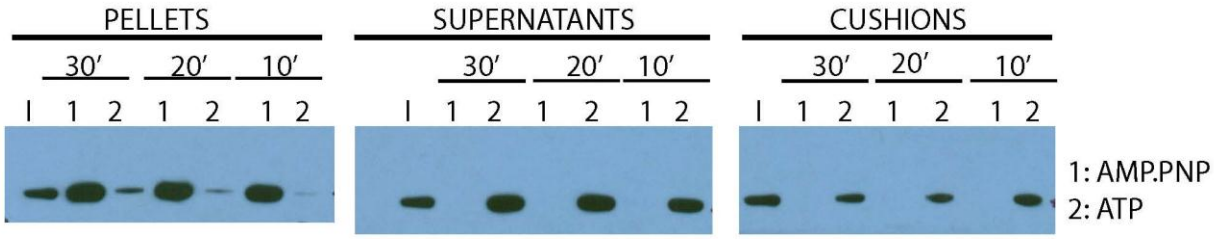


Figure 26. Effect of temperature and binding time on microtubule binding

Another factor that can affect ATP utilization by kinesin is the use of phosphatase inhibitors normally included during nerve homogenization, cocktail 2 from Calbiochem and cocktail 3 from Sigma. Cocktail 2 from Calbiochem had the potential of inhibiting ATPases, thus to test if any of those inhibitors could affect ATPase activity of kinesin, three MT binding conditions were tested: 1) with all inhibitors, 2) with only cocktail 3, 3) with no inhibitors. As shown in Figure 27, the inclusion of cocktail 2 alone in the homogenization procedure, was sufficient to reduce ATPase activity of kinesin as indicated by increased kinesin immunoreactivity in ATP pellets. Thus, cocktail 2 was excluded from homogenization procedures.

To test the ability of different isoforms to pellet with MT, both sciatic (Figure 28 panel A) and vagus nerves (Figure 28 panel B) were tested for kinesin 1 isoforms after microtubule binding assays. As shown in Figure 28, kinesin 1 A, B, and C, were able to pellet with MT under AMP.PNP conditions for both sciatic and vagus nerves. Kinesin 1A was less efficient in ATP utilization and a small percentage was independent of AMP.PNP for both sciatic and vagus nerves. Kinesin 1 B was less efficient in ATP utilization in sciatic nerves and a small percentage was AMP.PNP independent in vagus nerves. Kinesin 1 C behaved as expected in the ATP and AMP.PNP conditions for both sciatic and vagus nerves.

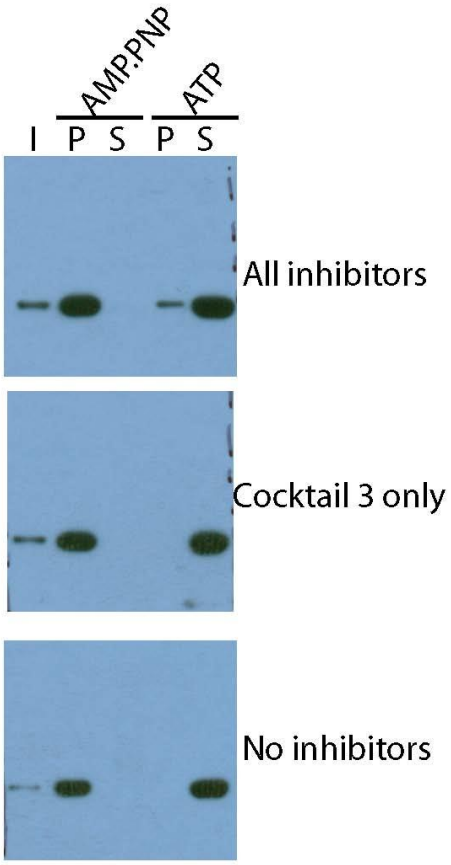


Figure 27. Effect of different phosphatase inhibitors on microtubule binding

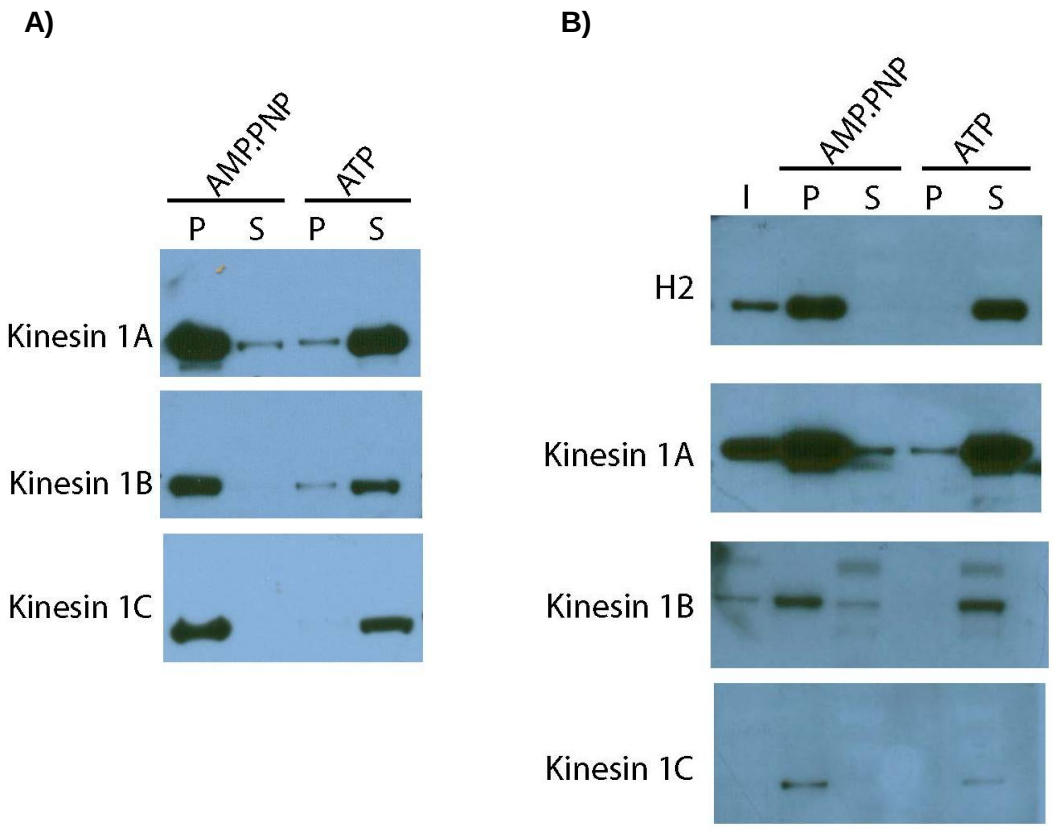


Figure 28. Microtubule binding of Kinesin 1 isoforms in sciatic and vagus nerves

4. Decreased microtubule binding in DM nerves

Microtubule binding was performed for sciatic (and vagus nerves in control and DM animals at 8 weeks. Kinesin immunoreactivity was analyzed under AMP.PNP conditions using H2 antibody. In vagus nerves MT binding was significantly reduced in DM animals compared to controls ($p=0.02$) as indicated by increased kinesin immunoreactivity in the supernatant and decreased immunoreactivity in the pellets (Figure 29). In sciatic nerves there was not significantly reduction in MT binding in DM nerves compared to controls, however a trend towards decrease kinesin binding to MT is apparent (Figure 30).

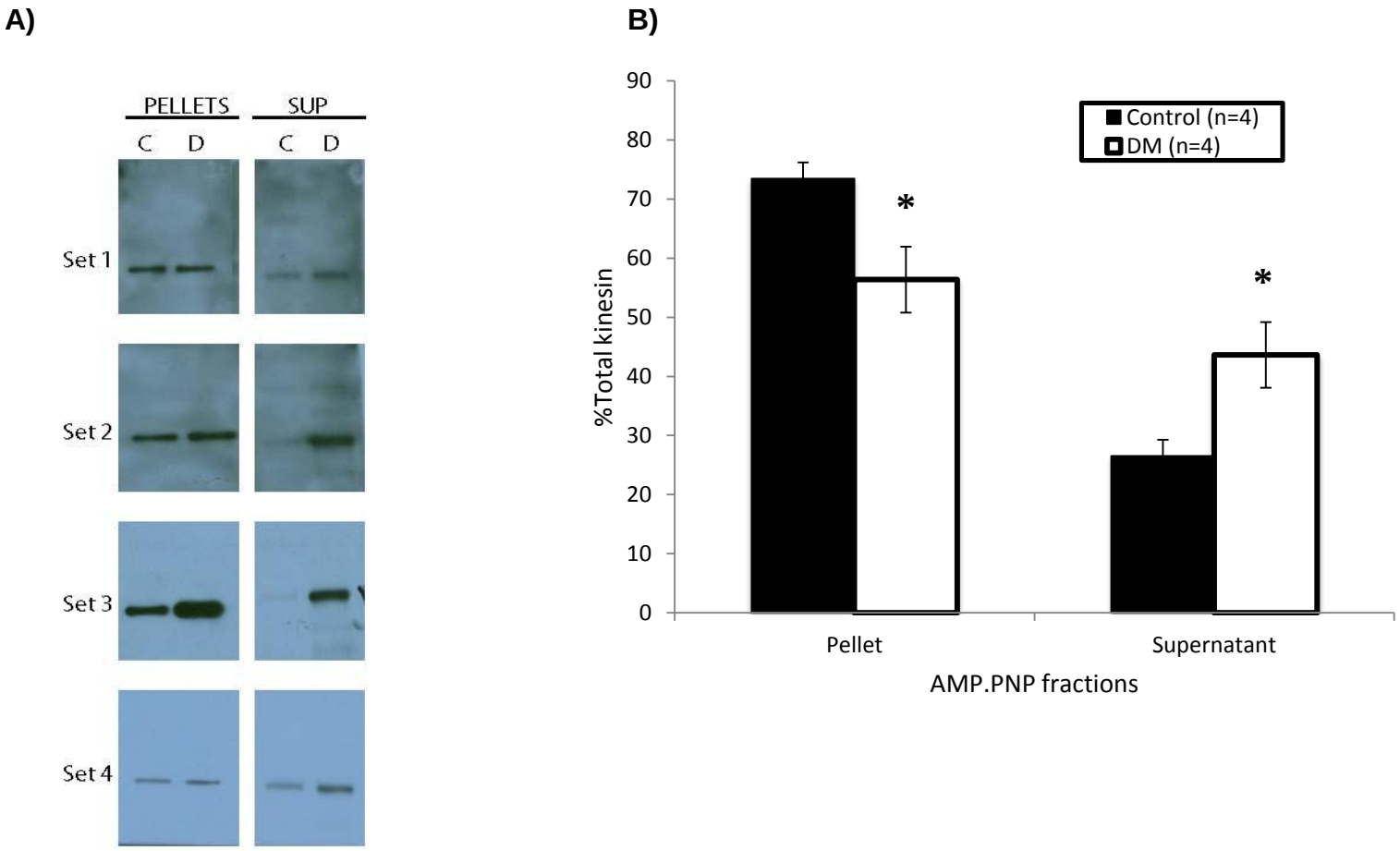


Figure 29. Decreased microtubule binding in DM vagus nerves

T-test * p=0.02

SUP: supernatant; C: Control; D: diabetic

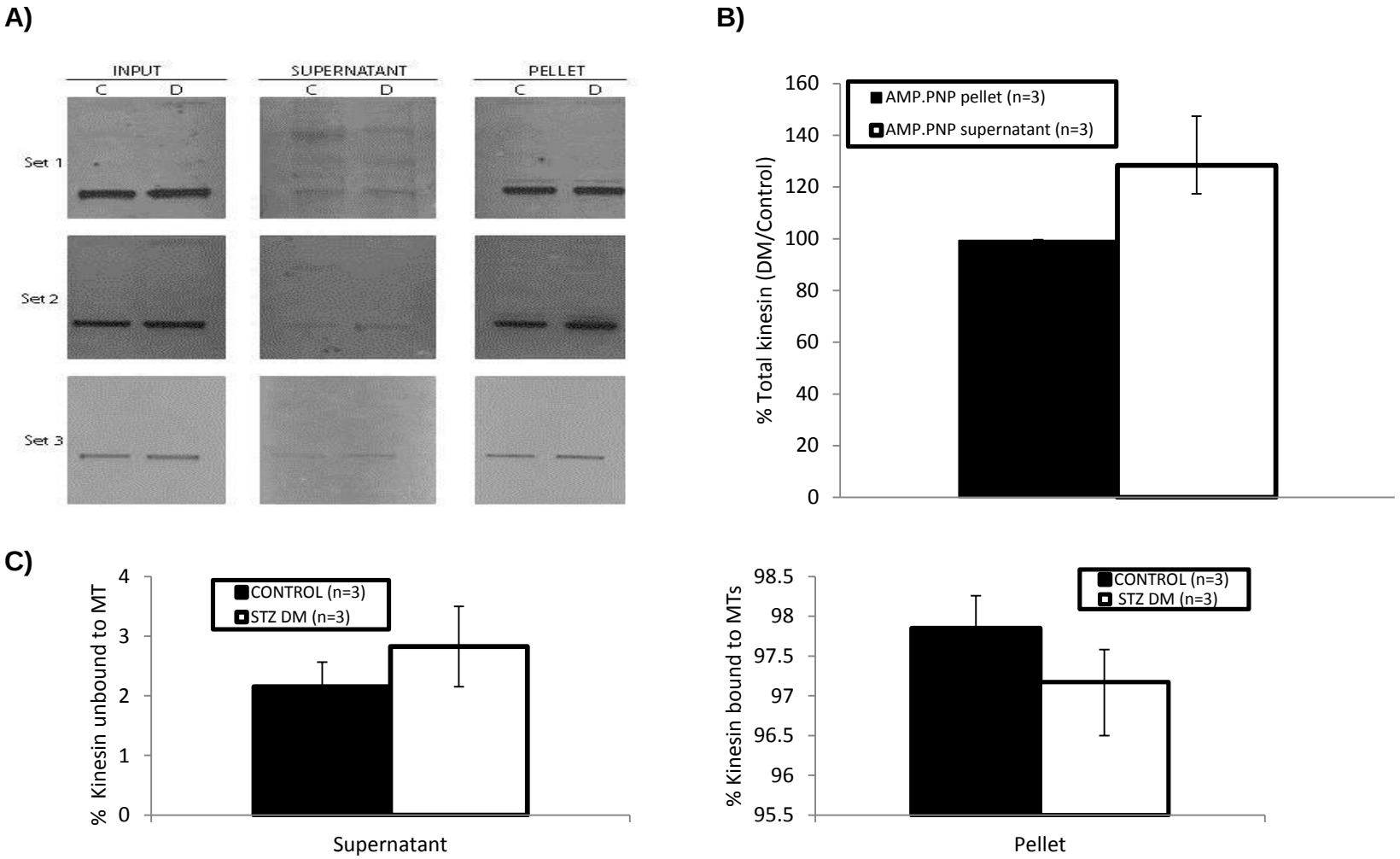


Figure 30. Study of microtubule binding in DM sciatic nerves

F. Discussion

1. Kinase activation in DM nerves

The pattern of kinase activation in both vagus and sciatic nerves, namely reduced activation of p38 and JNK and increased activation of GSK3 beta, is consistent with growth factor signaling deficiency. In the literature, several reports showed activation of MAPK. The differences in this finding may be due to several reasons including differences in: glucose vs insulin signaling pathways, time course, in vitro vs in vivo, painful vs insensate neuropathy, role of inflammation and local activation of signaling.

a) Glucose vs growth factor signaling

With normal insulin signaling, GSK3 beta is inactive (phosphorylated), facilitating glycogen synthesis. When insulin signaling is disrupted, GSK3 beta becomes active (dephosphorylated), stimulating glycogenolysis and contributing to decreased glucose utilization. Active GSK3 beta has been linked to disease-modifying pathways leading to neurodegeneration [396]. In the present study, there was an increase in GSK3 beta activation in DM sciatic and vagus nerves at 8 weeks compared to controls. Other groups have shown dysregulation of insulin receptor signaling in diabetic nerves [234]. The present study extended those results, showing for the first time disruption in GSK3 beta signaling in peripheral diabetic nerves at the same time of signs of insensate diabetic neuropathy.

In addition to the canonical insulin pathway via GSK3 beta, MAPKs are also activated under different signaling pathways by insulin, acting as positive and negative

regulators of insulin receptor substrate (IRS) function [397]. JNK is activated by insulin promoting a negative feedback loop, reducing IRS1 activation [398]. Insulin can also activate p38 through several different pathways [399-401]. Many of these pathways have been extensively studied in insulin-dependent organs; however, signaling cascades involving insulin-derived MAPK activation in noninsulin-dependent tissues, such as peripheral nerves, are less clear. IRS function and downstream signaling are also regulated by proinflammatory cytokines and derived signaling pathways, which could further complicate the analysis of kinase activation in DM nerves [402]. This is the first time that decreased activation of MAPK was found in diabetic nerves. Several reports suggested that lack of growth factor signaling may be more important than hyperglycemia in preventing nerve regeneration in DN [127]. This observation could possibly explain the more severe DN phenotype observed in type 1 patients.

Moreover, reduced levels of IGF, C-peptide, and neurotrophins, were found in DM patients and have been suggested to play a role in the pathogenesis of DN [104, 123, 128]. Signaling cascades derived from these components are similar to that of insulin, having the potential to affect the activation state of MAPK and GSK3 beta as well. Furthermore, there is also evidence that trace amounts of insulin can ameliorate mitochondrial dysfunction in sensory neurons, myelinated sensory atrophy and altered nociceptor responses without altering glucose levels, suggesting that the action of insulin in peripheral tissues can occur independent of insulin actions on glucose regulation [234].

b) Inflammation

Another aspect of the differences found with published literature is the role of inflammation in activation of signaling cascades. Hyperglycemia and oxidative stress lead to a wide range of signaling pathways, which upregulate pro-inflammatory components activating MAPKs [106-110]. Increasing evidence exists that these inflammatory pathways may not be selective to neurons and may equally affect glia [403-405] and vasculature [25, 112, 113]. In this sense, animal models with enhanced inflammation due to obesity [406] or autoimmune factors [407] may be more susceptible of activation of common inflammatory responses that involve MAPKs. In addition, the rat strain used in the present study, Fischer 344, is less prone to inflammation due to a heightened HPA axis and elevated levels of circulating corticosteroids, which may reduce inflammatory signals in peripheral tissues [408, 409].

c) Painful vs insensate neuropathy

Another possible explanation of the differences observed may be dependent on the type of phenotype observed in the different DM animal models used. For the most part, animals models that report activation of MAPKs do so in a background of hyperalgesia. Hyperglycemia and activation of MAPKs may play critical roles in central [410]and peripheral pain pathways [405, 411] and signaling pathways that lead to insensate neuropathy are still understudied [148]. Moreover, a few aldose reductase inhibitors [412], p38 inhibitors [413] and antioxidants [414] were minimally effective in treating symptoms of pain but no records exist for treating or preventing sensory loss.

d) In vitro vs in vivo

There is one report that showed increased JNK activation in sciatic nerves of STZ mice after 4 weeks of diabetes, which was inhibited in an AR (aldose reductase) knock out background [415]. In dorsal root ganglion neurons, JNK and p38 are activated under high glucose conditions and p38 activation was found in sural nerves of diabetic patients derived from lower limb amputations [416]; with the caveat that these tissues were end-stage and presenting together with inflammation, gangrene and vascular dysfunction. It was also shown in STZ Wistar rats after 12 weeks of diabetes that AR inhibitors (ARI) and insulin can inhibit p38 activation and nerve conduction velocities [417]. However, although they showed some activation of p38 in sciatic nerves, the most robust changes were seen in the cell bodies, suggesting a role of p38 in gene activation. Other studies with DM animals showed activation of p38 in spinal cord [405, 413] and DRG [417, 418] but not as consistent in the distal nerve. This is the first time that the activation of kinases is studied in vagus nerve, which in our case showed a pattern of kinase activation similar to sciatic nerves, suggesting that lack of growth factor signaling may be shared by different types of nerves but are specific of more distal portions.

e) Local activation

Local activation of signaling cascades may also contribute to the differences observed in kinase activation. In this sense, although glucose could readily enter at any level of the cell, insulin receptors (IR) have a very specific pattern of expression, contributing to local signaling pathways. In peripheral nerves, IR are expressed in high densities in paranodal loops and small size primary sensory neurons [419, 420]

f) Time course

It is also possible, that activation of MAPK may be more transient. We only measured kinase activation at 8 weeks post DM induction and we could have missed the window of MAPK kinase activation if at all present. A time course study of activation of all of the kinases studied in this thesis would be needed to properly address this issue. Many reports also show kinase activation only after 12 weeks of DM induction [417, 418], thus our kinase studies would need to be extended to 12 weeks of DM or longer to make any type of comparison.

2. Kinase activation in DM nerves and fast axonal transport

It was previously shown that activation of GSK3 beta leads to decreased anterograde axonal transport, increased phosphorylation of kinesin light chain and detachment of kinesin from vesicles [158]. This result is in accordance with the increased GSK3 beta activation found in DM sciatic and vagus nerves and is consistent with several reports showing deficits in anterograde transport in DM nerves [126, 171, 369]. Further studies will focus on evaluating kinesin vesicles binding in DM sciatic and vagus nerve to link GSK3 beta activation to changes in molecular motor function.

It was also previously reported from Brady lab that JNK was able to phosphorylate kinesin in vitro and to regulate FAT regulation in a isoform specific-manner, where JNK1 had no effect on FAT, JNK2 reduced the anterograde direction and JNK3 reduced both anterograde and retrograde transport [166]. Moreover, activation of JNK produces reduced kinesin binding to microtubules [165]. The present study extended the analysis of the effect of disease-modifying kinase signaling pathways and showed isoform specific effects of active p38 on FAT. Specifically, p38

alpha reduced anterograde transport, whereas p38 beta reduced both anterograde and retrograde transport. In the present study there was a decreased activation for both JNK and p38 in DM nerves. However, as shown in the case of JNK, this effect may be isoform specific and a more detailed study on specific JNK and p38 isoforms would need to be perform in future studies to completely elucidate the pattern of kinase activation relevant to axonal transport. Nonetheless, changes in kinesin binding to microtubules was observed in DM nerves, in particular to vagus nerves, suggesting that kinases other than JNK and p38, may be affecting this binding.

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V. DISCUSSION AND FUTURE DIRECTIONS

1. Co-presentation of different types of neuropathies: role in prediction and neuronal vulnerability

Co-presentation or sequential presentation of different types of neuropathies with similar degeneration processes may be useful in both the clinical and laboratory setting. Clinically, presence of one type of neuropathy may serve to predict other undiagnosed or subclinical neuropathies and aid in prevention and better management of complications. Experimentally, animal models that resemble the pattern of appearance of different types of neuropathy may aid in the identification of true underlying pathology shared by different types of diabetic nerves. Epidemiological studies on DM patients show a clear association of cardiovascular autonomic neuropathy (CAN) with sensory neuropathy , however, this association is not frequently measured or found in animal models of DN, especially in relationship with insensate neuropathy. This work represents the first report showing association of insensate neuropathy with CAN in animal models, as exemplified by decreased heart rate, decreased myocardial tissue NE and increased urinary NE levels (TABLE XIII).

TABLE XIII

CO-PRESENTATION OF MEASUREMENTS

	4/5 weeks	8 weeks	12 weeks
↓Tactile sensitivity	+	++	N/M
Thermal hypoalgesia	++	+++	N/M
Bradycardia	+	+++	+++
Diastolic & systolic dysfunction	++	+++	+++
Cardiac dilation	++	+++	+++
↓Cardiac NE/↑ Urine NE	N/M	+++	N/M
↑GSK3 activation	N/M	+++	N/M
↓MAPK activation	N/M	+	N/M
↓Kinesin bound-MT	N/M	+++	N/M

+= mild ++= moderate +++= significant

N/M: not measured

NE: norepinephrine

MT: microtubules

Moreover, the present study showed altered cardiac sensory innervation by measuring common cardiac sensory markers in diabetic heart tissue, suggesting that early signs of cardiac afferent dysfunction may be already present at times of sensory deficits. These studies suggest that somatic and visceral sensory fibers may be similarly vulnerable to degeneration and may share a common pathogenic mechanism. In the present study, alterations of some echo parameters are already present before evident signs of sensory dysfunction and continue to be present as sensory neuropathy develops. Thus, indicating that autonomic fibers may be more vulnerable that sensory ones and that presence of CAN could also serve as a predictor of insensate neuropathy. A detail analysis of different nerve fibers in future studies would help elucidate the pattern and time course of degeneration in relationship to clinical signs.

2. Mechanism of fast axonal transport deficits in DN

Fast axonal transport deficits have been described for both vagus and sciatic nerves of animal models of diabetes but their relative contribution to the pathogenesis of diabetic neuropathies could not be established due to lack of a mechanistic pathway. The present study shows for the first time a molecular mechanism that could explain FAT deficits observed in diabetic neuropathies, via differential kinase activation and reduction of kinesin binding to microtubules occurring at the same time of signs of insensate neuropathy (Figure 31).

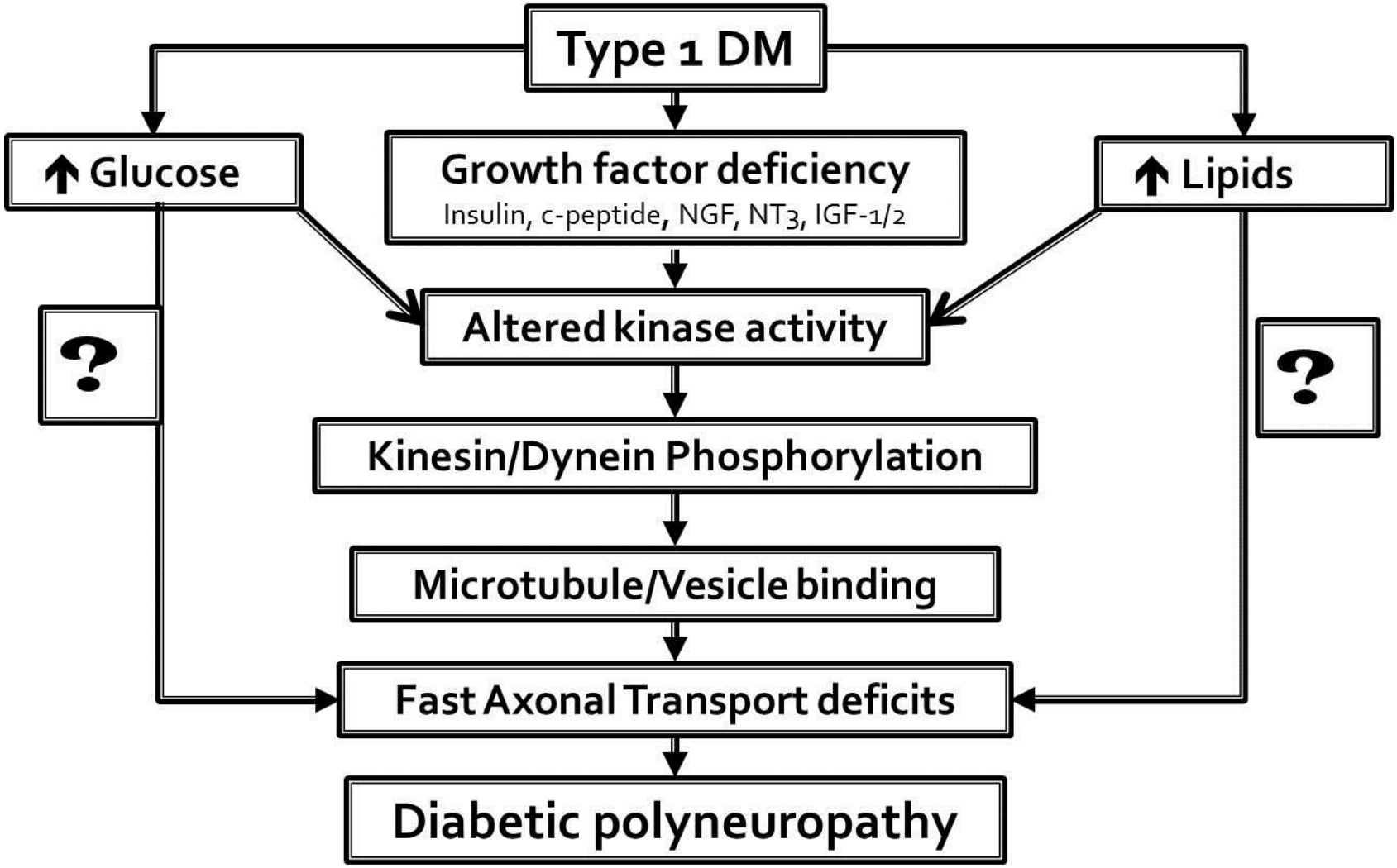


Figure 31. Proposed pathogenic mechanism of FAT deficits in DPN

Reduction of microtubule binding was associated with a very specific pattern of kinase activation/deactivation derived from insulin signaling deficiency. These experiments suggest that changes in kinesin binding to MT could be derived directly from its phosphorylation state. Moreover, phosphorylation of kinesin light chain and its interaction with the heavy chain could also be affecting the way the heavy chain interacts with the microtubules [421]. Many of the already published work has focused on activation of kinases that lead to increased kinesin phosphorylation at specific sites and disruption of FAT. However, interaction of molecular motors with MT could be dependent on the differential state of phosphorylation of different motifs specific for certain kinases, similar to what happens with tau protein [422].

Furthermore, phosphorylation of kinesin can also affect its binding to cargo vesicles. It was previously published that both light and heavy chains can be phosphorylated in different degrees by kinase A, affecting their binding to vesicles and acting as a normal point of regulation for cargo delivery [340]. Also, other patterns of kinesin phosphorylation have been implicated in kinesin association to vesicles and neurite outgrowth [423]. In addition, kinesin dephosphorylation has been shown to be necessary for transport of insulin in pancreatic beta cells, suggesting a role of phosphatases in regulation of molecular motor functions in addition to kinases [328]. Another function of kinesin, namely ATPase activity necessary for kinesin processing, can also be regulated by phosphorylation [424].

It has been shown that different kinesin isoforms are responsible for transporting specific cargoes [332], suggesting that phospho-dependent regulation of molecular motor's function could also be isoform specific. In addition, selection of cargo could also

be dependent on associated signaling proteins [425]. To complement the findings of the present study, future experiments would need to address the above-mentioned issues.

Retrograde transport is also affected in diabetic nerves [173, 175, 371] and similar studies as the ones proposed for kinesin, could also be performed on dynein, the retrograde motor. Furthermore, future mass spec analysis intended to identify specific phosphorylation sites on molecular motors and help identify novel sites and novel kinases important for the regulation of FAT in health and disease. In this sense, many other kinases are also involve in diabetes, which could also affect some aspect of the regulation of FAT, such as Src [426], PKCs [427] and AMPK [428], many of which have been also implicated in DN pathogenesis [429, 430].

Finally, other types of modifications frequently occurring in diabetes, such as glycation [431] could also affect molecular motors and tubulin [361], with the potential of changing crucial protein-protein interactions that can lead to dysregulation of FAT in DN. In the present study, the protocol used for microtubule binding is independent of glycation of tubulin, since fresh previously assembled MT from pig brain were used for the binding studies. To further assess the role of glycation of tubulin in binding to motors, future experiments would need to use tubulin from DM animals. Although glycation is frequent in a hyperglycemic state, it is largely irreversible, thus not subject to manipulation. On the contrary, the activity of kinases and phosphatases can be manipulated by inhibitors or activators, serving as a possible target for therapy.

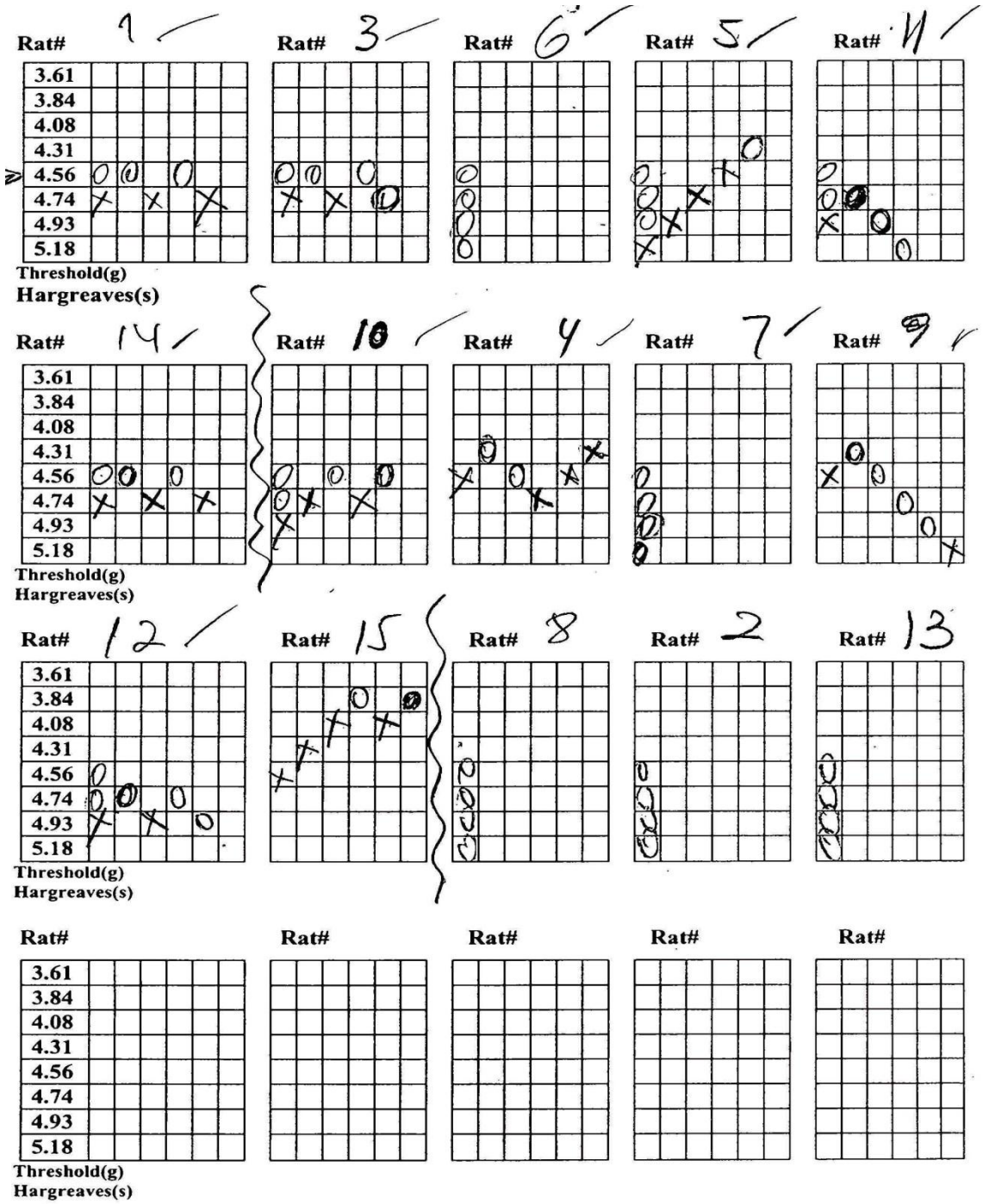
3. The future of DN therapies

Based on the complex heterogeneity of the pathogenesis of diabetic neuropathy, the future of DN therapies may likely be multifactorial. Inclusion of agents that cover the

glucose toxicity pathway, such as lipoic acid and aldose reductase inhibitors, are obviously needed to preserve normal neuronal function. Another important aspect would be to include growth factor replacement (by different means as the ones already tried) that could contribute to neuronal regeneration and maintenance of proper connections, especially for insulin independent patients that usually do not implement insulin as part of their regular treatment. Finally, the use of a specific kinase/phosphatase therapy could aid in the normal transport of not only neurotrophic factors but would also ensure proper neuronal transmission, connectivity, and transport of signaling molecules and overall neuronal health. Many GSK3 beta inhibitors are already being evaluated to treat other neurodegenerative diseases [432-434] and insulin resistance [134] and may represent a useful drug therapy to treat DN as well.

APPENDICES

Appendix A. Example of a Von Frey reading



Appendix B. Values of k based on response patterns

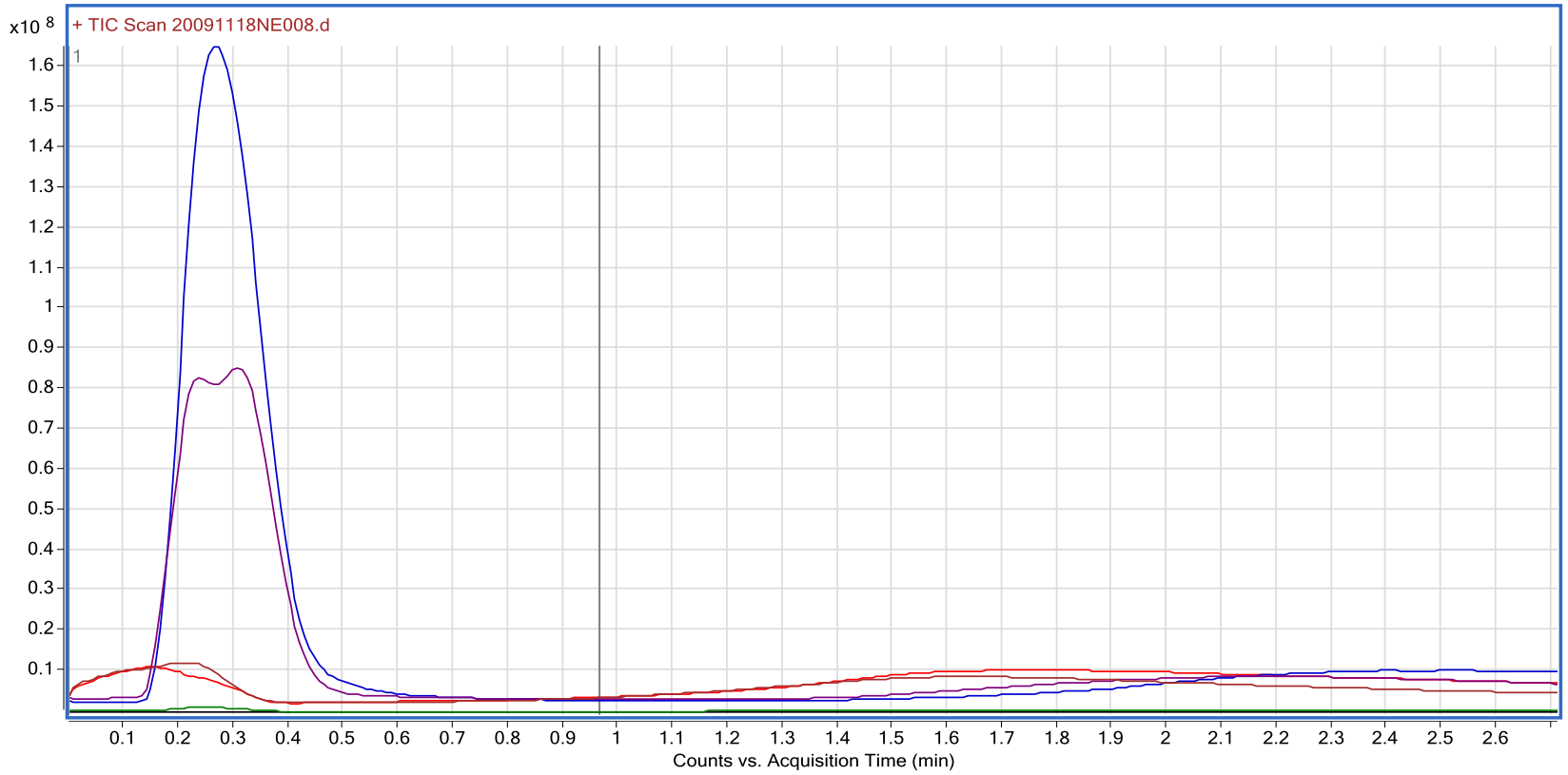
N	Second part of series	k for test series whose first part is: O	OO	OOO	OOOO		Standard error of LD_{50}
2	X	−0.500	−0.388	−0.378	−0.377	O	0.88 σ
3	XO	0.842	0.890	0.894	0.894	OX	0.76 σ
	XX	−0.178	0.000	0.026	0.028	OO	
4	XOO	0.299	0.314	0.315	0.315	OXX	0.67 σ
	XOX	−0.500	−0.439	−0.432	−0.432	OXO	
	XXO	1.000	1.122	1.139	1.140	OOX	
	XXX	0.194	0.449	0.500	0.506	OOO	
5	XOOO	−0.157	−0.154	−0.154	−0.154	OXXX	0.61 σ
	XOOX	−0.878	−0.861	−0.860	−0.860	OXXO	
	XOXO	0.701	0.737	0.741	0.741	OXOX	
	XOXX	0.084	0.169	0.181	0.182	OXOO	
	XXOO	0.305	0.372	0.380	0.381	OOXX	
	XXOX	−0.305	−0.169	−0.144	−0.142	OOXO	
	XXXO	1.288	1.500	1.544	1.549	OOOX	
	XXXX	0.555	0.897	0.985	1.000^{+1}	OOOO	
6	XOOOO	−0.547	−0.547	−0.547	−0.547	OXXXX	0.56 σ
	XOOOX	−1.250	−1.247	−1.246	−1.246	OXXXO	
	XOOXO	0.372	0.380	0.381	0.381	OXXOX	
	XOOXX	−0.169	−0.144	−0.142	−0.142	OXXOO	
	XOXOO	0.022	0.039	0.040	0.040	OXOXX	
	XOXOX	−0.500	−0.458	−0.453	−0.453	OXOXO	
	XOXXO	1.169	1.237	1.247	1.248	OXOOX	
	XOXXX	0.611	0.732	0.756	0.758	OXOOO	
	XXOOO	−0.296	−0.266	−0.263	−0.263	OOXXX	
	XXOOX	−0.831	−0.763	−0.753	−0.752	OOXXO	
	XXOXO	0.831	0.935	0.952	0.954	OOXOX	
	XXOXX	0.296	0.463	0.500	0.504^{+1}	OOXOO	
	XXXOO	0.500	0.648	0.678	0.681	OOOXX	
	XXXOX	−0.043	0.187	0.244	0.252^{+1}	OOOXO	
	XXXXO	1.603	1.917	2.000	2.014^{+1}	OOOOX	
	XXXXX	0.893	1.329	1.465	1.496^{+1}	OOOOO	
		X	XX	XXX	XXXX		Second part of series
		−k for series whose first part is					

*Values of k for estimating LD_{50} from up-and-down sequence of trials of nominal length N. The estimate of LD_{50} is $x_f + kd$ where x_f is the final test level and d is the interval between dose levels. If the table is entered from the foot, the sign of k is to be reversed.

Appendix C. Typical chromatogram obtained from a NE standard

NE: norepinephrine; d-NE: deuterium norepinephrine

Appendix D. Chromatogram comparing homogenization buffer with and without sodium metabisulfite as antioxidant



Purple trace is 5mM NE with 0.1% $Na_2(SO3)$--20091118NE007
Blue trace is 5mM NE without 0.1% $Na_2(SO3)$---20091118NE006
Red trace is a water blank---20091118NE005

Appendix E. Pilot echo study

ECHO PARAMETERS	CONTROL	STZ DM
HR (beats/min)	342	256
LVDD (cm)	0.724	0.870
LVSD (cm)	0.389	0.507
IVSD	0.087	0.095
IVSS	0.190	0.172
LVPWD	0.095	0.096

HR: Heart Rate; LVDD: diameter in Dyastole; LVSD: Left Ventricular Diameter in systole; IVSD: Interventricular Septum in diastole, IVSS: Interventricular septum in Systole; LVPWD: Left Ventricular Posterior Wall Diameter

Data is expressed as mean ± SEM n=1

CITED LITERATURE

1. Saltiel A.R. and Kahn C.R. Insulin signalling and the regulation of glucose and lipid metabolism. Nature, 2001. 414(6865): p. 799-806.

2. Neubauer N. and Kulkarni R.N. Molecular approaches to study control of glucose homeostasis. ILAR journal / National Research Council, Institute of Laboratory Animal Resources, 2006. 47(3): p. 199-211.

3. Morris D.L. and Rui L. Recent advances in understanding leptin signaling and leptin resistance. American journal of physiology. Endocrinology and metabolism, 2009. 297(6): p. E1247-1259.

4. Kadowaki T., Yamauchi T., and Kubota N. The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. FEBS letters, 2008. 582(1): p. 74-80.

5. German J., Kim F., Schwartz G.J., Havel P.J., Rhodes C.J., Schwartz M.W., and Morton G.J. Hypothalamic leptin signaling regulates hepatic insulin sensitivity via a neurocircuit involving the vagus nerve. Endocrinology, 2009. 150(10): p. 4502-4511.

6. Nonogaki K. New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia, 2000. 43(5): p. 533-549.

7. Kulkarni R.N., Wang Z.L., Wang R.M., Hurley J.D., Smith D.M., Ghatei M.A., Withers D.J., Gardiner J.V., Bailey C.J., and Bloom S.R. Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. The Journal of clinical investigation, 1997. 100(11): p. 2729-2736.

8. Cho N. and Momose Y. Peroxisome proliferator-activated receptor gamma agonists as insulin sensitizers: from the discovery to recent progress. Current topics in medicinal chemistry, 2008. 8(17): p. 1483-1507.

9. Alberti K.G. and Zimmet P.Z. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabetic medicine : a journal of the British Diabetic Association, 1998. 15(7): p. 539-553.

10. WHO Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia. Report of a WHO/IDF Consultation. 2006. 1-46.

CITED LITERATURE (continued)

11. Nathan D.M., Turgeon H., and Regan S. Relationship between glycated haemoglobin levels and mean glucose levels over time. Diabetologia, 2007. 50(11): p. 2239-2244.

12. Association, Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus. Abbreviated Report of a WHO Consultation, Journal, 2011.

13. Goldstein D.E., Little R.R., Lorenz R.A., Malone J.I., Nathan D., Peterson C.M., and Sacks D.B. Tests of glycemia in diabetes. Diabetes care, 2004. 27(7): p. 1761-1773.

14. Sacks D.B., Arnold M., Bakris G.L., Bruns D.E., Horvath A.R., Kirkman M.S., Lernmark A., Metzger B.E., and Nathan D.M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Diabetes care, 2011. 34(6): p. e61-99.

15. Association A.D. Diagnosis and classification of diabetes mellitus. Diabetes care, 2011. 34 Suppl 1: p. S62-69.

16. Smith A.G., Russell J., Feldman E.L., Goldstein J., Peltier A., Smith S., Hamwi J., Pollari D., Bixby B., Howard J., and Singleton J.R. Lifestyle intervention for pre-diabetic neuropathy. Diabetes care, 2006. 29(6): p. 1294-1299.

17. Reisin E. and Alpert M.A. Definition of the metabolic syndrome: current proposals and controversies. The American journal of the medical sciences, 2005. 330(6): p. 269-272.

18. Simmons R.K., Alberti K.G., Gale E.A., Colagiuri S., Tuomilehto J., Qiao Q., Ramachandran A., Tajima N., Brajkovich Mirchov I., Ben-Nakhi A., Reaven G., Hama Sambo B., Mendis S., and Roglic G. The metabolic syndrome: useful concept or clinical tool? Report of a WHO Expert Consultation. Diabetologia, 2010. 53(4): p. 600-605.

19. Wildman R.P., McGinn A.P., Kim M., Muntner P., Wang D., Cohen H.W., Ogorodnikova A.D., Reynolds K., and Fonseca V. Empirical derivation to improve the definition of the metabolic syndrome in the evaluation of cardiovascular disease risk. Diabetes care, 2011. 34(3): p. 746-748.

CITED LITERATURE (continued)

20. Aronson S.M. A physician's lexicon: the many words of diabetes mellitus. Medicine and health, Rhode Island, 2005. 88(11): p. 409.

21. Fishbein H. and Palumbo P., 13:Acute metabolic complications in diabetes, In: Diabetes in America, 2nd, 283-292. 1995, National Diabetes Data Group, National Intitue of Health, National Institute of Diabetes and Digestive and Kidney Diseases: Bethesda, MD, NIH Publication No. 95-1468.

22. Portuese E. and Orchard T., 10:Mortality in Insulin-Dependent Diabetes, In: Diabetes in America, second, 1995, National Diabetes Data group, National institues of Health, National Institute of Diabetes and Digestive and Kidney Disease: Bethesda, MD, NIH Publication No 95-1468.

23. Geiss L.S., Herman W.H., and Smith P.J., 11:Mortality in Non-Insulin-Dependent Diabetes, In: Diabetes in America, second, 1995, National Diabetes Data group, National institues of Health, National Institute of Diabetes and Digestive and Kidney Disease: Bethesda, MD,

24. Melendez-Ramirez L.Y., Richards R.J., and Cefalu W.T. Complications of type 1 diabetes. Endocrinology and metabolism clinics of North America, 2010. 39(3): p. 625-640.

25. Jax T.W. Metabolic memory: a vascular perspective. Cardiovascular diabetology, 2010. 9: p. 51.

26. Balakumar P., Arora M.K., Reddy J., and Anand-Srivastava M.B. Pathophysiology of diabetic nephropathy: involvement of multifaceted signalling mechanism. Journal of cardiovascular pharmacology, 2009. 54(2): p. 129-138.

27. Sima A.A. The heterogeneity of diabetic neuropathy. Frontiers in bioscience : a journal and virtual library, 2008. 13: p. 4809-4816.

28. Frank R.N. Diabetic retinopathy. The New England journal of medicine, 2004. 350(1): p. 48-58.

29. Fang Z.Y., Prins J.B., and Marwick T.H. Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. Endocrine reviews, 2004. 25(4): p. 543-567.

CITED LITERATURE (continued)

30. Jude E.B., Eleftheriadou I., and Tentolouris N. Peripheral arterial disease in diabetes--a review. Diabetic medicine : a journal of the British Diabetic Association, 2010. 27(1): p. 4-14.

31. Bejot Y. and Giroud M. Stroke in diabetic patients. Diabetes & metabolism, 2010. 36 Suppl 3: p. S84-87.

32. Sima A.A. Encephalopathies: the emerging diabetic complications. Acta diabetologica, 2010. 47(4): p. 279-293.

33. Houlden H., Blake J., and Reilly M.M. Hereditary sensory neuropathies. Current opinion in neurology, 2004. 17(5): p. 569-577.

34. Keller M.P. and Chance P.F. Inherited peripheral neuropathy. Seminars in neurology, 1999. 19(4): p. 353-362.

35. Reilly M.M. and Shy M.E. Diagnosis and new treatments in genetic neuropathies. Journal of neurology, neurosurgery, and psychiatry, 2009. 80(12): p. 1304-1314.

36. Chalk C.H. Acquired peripheral neuropathy. Neurologic clinics, 1997. 15(3): p. 501-528.

37. Weimer L.H. and Sachdev N. Update on medication-induced peripheral neuropathy. Current neurology and neuroscience reports, 2009. 9(1): p. 69-75.

38. Peltier A.C. and Russell J.W. Advances in understanding drug-induced neuropathies. Drug safety : an international journal of medical toxicology and drug experience, 2006. 29(1): p. 23-30.

39. Umapathi T. and Chaudhry V. Toxic neuropathy. Current opinion in neurology, 2005. 18(5): p. 574-580.

40. Gordon Smith A. and Robinson Singleton J. Idiopathic neuropathy, prediabetes and the metabolic syndrome. Journal of the neurological sciences, 2006. 242(1-2): p. 9-14.

CITED LITERATURE (continued)

41. Smith A.G. and Singleton J.R. Impaired glucose tolerance and neuropathy. The neurologist, 2008. 14(1): p. 23-29.

42. Singleton J.R. and Smith A.G. Neuropathy associated with prediabetes: what is new in 2007? Current diabetes reports, 2007. 7(6): p. 420-424.

43. Gordois A., Scuffham P., Shearer A., and Oglesby A. The health care costs of diabetic nephropathy in the United States and the United Kingdom. Journal of diabetes and its complications, 2004. 18(1): p. 18-26.

44. Sima A.A. Pathological mechanisms involved in diabetic neuropathy: can we slow the process? Current opinion in investigational drugs, 2006. 7(4): p. 324-337.

45. Boulton A.J. Guidelines for diagnosis and outpatient management of diabetic peripheral neuropathy. European Association for the Study of Diabetes, Neurodiab. Diabetes & metabolism, 1998. 24 Suppl 3: p. 55-65.

46. Freeman R. Not all neuropathy in diabetes is of diabetic etiology: differential diagnosis of diabetic neuropathy. Current diabetes reports, 2009. 9(6): p. 423-431.

47. Bell D.S. Metformin-induced vitamin B12 deficiency presenting as a peripheral neuropathy. Southern medical journal, 2010. 103(3): p. 265-267.

48. Boulton A.J. and Ward J.D. Diabetic neuropathies and pain. Clinics in endocrinology and metabolism, 1986. 15(4): p. 917-931.

49. Boulton A.J. and Malik R.A. Diabetic neuropathy. The Medical clinics of North America, 1998. 82(4): p. 909-929.

50. Thomas P.K. Classification, differential diagnosis, and staging of diabetic peripheral neuropathy. Diabetes, 1997. 46 Suppl 2: p. S54-57.

51. Vinik A., Ullal J., Parson H.K., and Casellini C.M. Diabetic neuropathies: clinical manifestations and current treatment options. Nature clinical practice. Endocrinology & metabolism, 2006. 2(5): p. 269-281.

CITED LITERATURE (continued)

52. England J.D. and Asbury A.K. Peripheral neuropathy. Lancet, 2004. 363(9427): p. 2151-2161.

53. Boulton A.J., Malik R.A., Arezzo J.C., and Sosenko J.M. Diabetic somatic neuropathies. Diabetes care, 2004. 27(6): p. 1458-1486.

54. Sima A.A. and Kamiya H. Diabetic neuropathy differs in type 1 and type 2 diabetes. Annals of the New York Academy of Sciences, 2006. 1084: p. 235-249.

55. Said G., Slama G., and Selva J. Progressive centripetal degeneration of axons in small fibre diabetic polyneuropathy. Brain : a journal of neurology, 1983. 106 (Pt 4): p. 791-807.

56. Christen W.G., Manson J.E., Bubes V., and Glynn R.J. Risk factors for progression of distal symmetric polyneuropathy in type 1 diabetes mellitus. Sorbinil Retinopathy Trial Research Group. American journal of epidemiology, 1999. 150(11): p. 1142-1151.

57. Kilpatrick E.S., Rigby A.S., and Atkin S.L. The Diabetes Control and Complications Trial: the gift that keeps giving. Nature reviews. Endocrinology, 2009. 5(10): p. 537-545.

58. DCCT. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. The New England journal of medicine, 1993. 329(14): p. 977-986.

59. Tesfaye S., Chaturvedi N., Eaton S.E., Ward J.D., Manes C., Ionescu-Tirgoviste C., Witte D.R., and Fuller J.H. Vascular risk factors and diabetic neuropathy. The New England journal of medicine, 2005. 352(4): p. 341-350.

60. Wiggin T.D., Sullivan K.A., Pop-Busui R., Amato A., Sima A.A., and Feldman E.L. Elevated triglycerides correlate with progression of diabetic neuropathy. Diabetes, 2009. 58(7): p. 1634-1640.

61. Kempler P., Tesfaye S., Chaturvedi N., Stevens L.K., Webb D.J., Eaton S., Kerenyi Z., Tamas G., Ward J.D., and Fuller J.H. Autonomic neuropathy is associated with increased cardiovascular risk factors: the EURODIAB IDDM Complications Study.

CITED LITERATURE (continued)

Diabetic medicine : a journal of the British Diabetic Association, 2002. 19(11): p. 900-909.

62. Dobretsov M., Romanovsky D., and Stimers J.R. Early diabetic neuropathy: triggers and mechanisms. World journal of gastroenterology : WJG, 2007. 13(2): p. 175-191.

63. Sima A.A. Diabetic neuropathy in type 1 and type 2 diabetes and the effects of C-peptide. Journal of the neurological sciences, 2004. 220(1-2): p. 133-136.

64. Elliott J., Tesfaye S., Chaturvedi N., Gandhi R.A., Stevens L.K., Emery C., and Fuller J.H. Large-fiber dysfunction in diabetic peripheral neuropathy is predicted by cardiovascular risk factors. Diabetes care, 2009. 32(10): p. 1896-1900.

65. Karvestedt L., Martensson E., Grill V., Elofsson S., von Wendt G., Hamsten A., and Brismar K. Peripheral sensory neuropathy associates with micro- or macroangiopathy: results from a population-based study of type 2 diabetic patients in Sweden. Diabetes care, 2009. 32(2): p. 317-322.

66. Messmer E.M., Schmid-Tannwald C., Zapp D., and Kampik A. In vivo confocal microscopy of corneal small fiber damage in diabetes mellitus. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 2010. 248(9): p. 1307-1312.

67. Kallinikos P., Berhanu M., O'Donnell C., Boulton A.J., Efron N., and Malik R.A. Corneal nerve tortuosity in diabetic patients with neuropathy. Investigative ophthalmology & visual science, 2004. 45(2): p. 418-422.

68. Quattrini C., Tavakoli M., Jeziorska M., Kallinikos P., Tesfaye S., Finnigan J., Marshall A., Boulton A.J., Efron N., and Malik R.A. Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes, 2007. 56(8): p. 2148-2154.

69. Ziegler D., Gries F.A., Muhlen H., Rathmann W., Spuler M., and Lessmann F. Prevalence and clinical correlates of cardiovascular autonomic and peripheral diabetic neuropathy in patients attending diabetes centers. The Diacan Multicenter Study Group. Diabete & metabolisme, 1993. 19(1 Pt 2): p. 143-151.

CITED LITERATURE (continued)

70. Pop-Busui R. Cardiac autonomic neuropathy in diabetes: a clinical perspective. Diabetes care, 2010. 33(2): p. 434-441.

71. Spallone V., Morganti R., D'Amato C., Cacciotti L., Fedele T., Maiello M.R., and Marfia G. Clinical correlates of painful diabetic neuropathy and relationship of neuropathic pain with sensorimotor and autonomic nerve function. European journal of pain, 2011. 15(2): p. 153-160.

72. Pop-Busui R., Herman W.H., Feldman E.L., Low P.A., Martin C.L., Cleary P.A., Waberski B.H., Lachin J.M., and Albers J.W. DCCT and EDIC studies in type 1 diabetes: lessons for diabetic neuropathy regarding metabolic memory and natural history. Current diabetes reports, 2010. 10(4): p. 276-282.

73. Rolim L.C., Sa J.R., Chacra A.R., and Dib S.A. Diabetic cardiovascular autonomic neuropathy: risk factors, clinical impact and early diagnosis. Arquivos brasileiros de cardiologia, 2008. 90(4): p. e24-31.

74. Pop-Busui R., Kirkwood I., Schmid H., Marinescu V., Schroeder J., Larkin D., Yamada E., Raffel D.M., and Stevens M.J. Sympathetic dysfunction in type 1 diabetes: association with impaired myocardial blood flow reserve and diastolic dysfunction. Journal of the American College of Cardiology, 2004. 44(12): p. 2368-2374.

75. Flotats A. and Carrio I. Is cardiac autonomic neuropathy the basis of nonischemic diabetic cardiomyopathy? JACC. Cardiovascular imaging, 2010. 3(12): p. 1216-1218.

76. Scholte A.J., Schuijf J.D., Delgado V., Kok J.A., Bus M.T., Maan A.C., Stokkel M.P., Kharagitsingh A.V., Dibbets-Schneider P., van der Wall E.E., and Bax J.J. Cardiac autonomic neuropathy in patients with diabetes and no symptoms of coronary artery disease: comparison of 123I-metaiodobenzylguanidine myocardial scintigraphy and heart rate variability. European journal of nuclear medicine and molecular imaging, 2010. 37(9): p. 1698-1705.

77. Moon S.S., Choi Y.K., Seo H.A., Jeon J.H., Lee J.E., Jeong J.Y., Lee J.Y., Lee D.H., Park W.J., Kim J.G., Kim B.W., and Lee I.K. Relationship between cardiovascular autonomic neuropathy and coronary artery calcification in patients with type 2 diabetes. Endocrine journal, 2010. 57(5): p. 445-454.

78. Poanta L., Porojan M., and Dumitrascu D.L. Heart rate variability and diastolic dysfunction in patients with type 2 diabetes mellitus. Acta diabetologica, 2011.

CITED LITERATURE (continued)

79. Tesfaye S., Boulton A.J., Dyck P.J., Freeman R., Horowitz M., Kempler P., Lauria G., Malik R.A., Spallone V., Vinik A., Bernardi L., and Valensi P. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes care, 2010. 33(10): p. 2285-2293.

80. Boulton A.J., Gries F.A., and Jervell J.A. Guidelines for the diagnosis and outpatient management of diabetic peripheral neuropathy. Diabetic medicine : a journal of the British Diabetic Association, 1998. 15(6): p. 508-514.

81. Van Acker K., Bouhassira D., De Bacquer D., Weiss S., Matthys K., Raemen H., Mathieu C., and Colin I.M. Prevalence and impact on quality of life of peripheral neuropathy with or without neuropathic pain in type 1 and type 2 diabetic patients attending hospital outpatients clinics. Diabetes & metabolism, 2009. 35(3): p. 206-213.

82. Ribu L., Hanestad B.R., Moum T., Birkeland K., and Rustoen T. A comparison of the health-related quality of life in patients with diabetic foot ulcers, with a diabetes group and a nondiabetes group from the general population. Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation, 2007. 16(2): p. 179-189.

83. Quattrini C. and Tesfaye S. Understanding the impact of painful diabetic neuropathy. Diabetes/metabolism research and reviews, 2003. 19 Suppl 1: p. S2-8.

84. Fosse S., Hartemann-Heurtier A., Jacqueminet S., Ha Van G., Grimaldi A., and Fagot-Campagna A. Incidence and characteristics of lower limb amputations in people with diabetes. Diabetic medicine : a journal of the British Diabetic Association, 2009. 26(4): p. 391-396.

85. van Baal J., Hubbard R., Game F., and Jeffcoate W. Mortality associated with acute Charcot foot and neuropathic foot ulceration. Diabetes care, 2010. 33(5): p. 1086-1089.

86. Boyko E.J., Ahroni J.H., Smith D.G., and Davignon D. Increased mortality associated with diabetic foot ulcer. Diabetic medicine : a journal of the British Diabetic Association, 1996. 13(11): p. 967-972.

87. Pop-Busui R., Evans G.W., Gerstein H.C., Fonseca V., Fleg J.L., Hoogwerf B.J., Genuth S., Grimm R.H., Corson M.A., and Prineas R. Effects of cardiac autonomic

CITED LITERATURE (continued)

dysfunction on mortality risk in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial. Diabetes care, 2010. 33(7): p. 1578-1584.

88. Maser R.E., Mitchell B.D., Vinik A.I., and Freeman R. The association between cardiovascular autonomic neuropathy and mortality in individuals with diabetes: a meta-analysis. Diabetes care, 2003. 26(6): p. 1895-1901.

89. Chang E.B., Bergenstal R.M., and Field M. Diarrhea in streptozocin-treated rats. Loss of adrenergic regulation of intestinal fluid and electrolyte transport. The Journal of clinical investigation, 1985. 75(5): p. 1666-1670.

90. Tracy J.A. and Dyck P.J. The spectrum of diabetic neuropathies. Physical medicine and rehabilitation clinics of North America, 2008. 19(1): p. 1-26, v.

91. Zochodne D.W., Ramji N., and Toth C. Neuronal targeting in diabetes mellitus: a story of sensory neurons and motor neurons. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry, 2008. 14(4): p. 311-318.

92. Grandinetti A., Chow D.C., Sletten D.M., Oyama J.K., Theriault A.G., Schatz I.J., and Low P.A. Impaired glucose tolerance is associated with postganglionic sudomotor impairment. Clinical autonomic research : official journal of the Clinical Autonomic Research Society, 2007. 17(4): p. 231-233.

93. Papanas N., Vinik A.I., and Ziegler D. Neuropathy in prediabetes: does the clock start ticking early? Nature reviews. Endocrinology, 2011.

94. Dyck P.J. and Giannini C. Pathologic alterations in the diabetic neuropathies of humans: a review. Journal of neuropathology and experimental neurology, 1996. 55(12): p. 1181-1193.

95. Toth C., Brussee V., Cheng C., and Zochodne D.W. Diabetes mellitus and the sensory neuron. Journal of neuropathology and experimental neurology, 2004. 63(6): p. 561-573.

96. Schmidt R.E., Plurad S.B., and Modert C.W. Experimental diabetic autonomic neuropathy characterization in streptozotocin-diabetic Sprague-Dawley rats. Laboratory investigation; a journal of technical methods and pathology, 1983. 49(5): p. 538-552.

CITED LITERATURE (continued)

97. Ramji N., Toth C., Kennedy J., and Zochodne D.W. Does diabetes mellitus target motor neurons? Neurobiology of disease, 2007. 26(2): p. 301-311.

98. Schmidt R.E. Neuropathology and pathogenesis of diabetic autonomic neuropathy. International review of neurobiology, 2002. 50: p. 257-292.

99. Sugimoto K., Murakawa Y., and Sima A.A. Diabetic neuropathy--a continuing enigma. Diabetes/metabolism research and reviews, 2000. 16(6): p. 408-433.

100. Said G. Diabetic neuropathy--a review. Nature clinical practice. Neurology, 2007. 3(6): p. 331-340.

101. Sinnreich M., Taylor B.V., and Dyck P.J. Diabetic neuropathies. Classification, clinical features, and pathophysiological basis. The neurologist, 2005. 11(2): p. 63-79.

102. Dyck P.J., Lais A., Karnes J.L., O'Brien P., and Rizza R. Fiber loss is primary and multifocal in sural nerves in diabetic polyneuropathy. Annals of neurology, 1986. 19(5): p. 425-439.

103. Vinik A.I. Neuropathy: new concepts in evaluation and treatment. Southern medical journal, 2002. 95(1): p. 21-23.

104. Leinninger G.M., Vincent A.M., and Feldman E.L. The role of growth factors in diabetic peripheral neuropathy. Journal of the peripheral nervous system : JPNS, 2004. 9(1): p. 26-53.

105. Vincent A.M., Hinder L.M., Pop-Busui R., and Feldman E.L. Hyperlipidemia: a new therapeutic target for diabetic neuropathy. Journal of the peripheral nervous system : JPNS, 2009. 14(4): p. 257-267.

106. Chung S.S.M. Contribution of Polyol Pathway to Diabetes-Induced Oxidative Stress. Journal of the American Society of Nephrology, 2003. 14(90003): p. 233S-236.

107. Bonnefont-Rousselot D. Glucose and reactive oxygen species. Current opinion in clinical nutrition and metabolic care, 2002. 5(5): p. 561-568.

108. Vincent A.M., Russell J.W., Low P., and Feldman E.L. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocrine reviews, 2004. 25(4): p. 612-628.

109. Pop-Busui R., Sima A., and Stevens M. Diabetic neuropathy and oxidative stress. Diabetes/metabolism research and reviews, 2006. 22(4): p. 257-273.

110. Vlassara H. The AGE-receptor in the pathogenesis of diabetic complications. Diabetes/metabolism research and reviews, 2001. 17(6): p. 436-443.

111. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature, 2001. 414(6865): p. 813-820.

112. Yorek M.A. The role of oxidative stress in diabetic vascular and neural disease. Free radical research, 2003. 37(5): p. 471-480.

113. Teunissen L.L., Notermans N.C., and Wokke J.H. Relationship between ischemia and neuropathy. European neurology, 2000. 44(1): p. 1-7.

114. Fernyhough P., Roy Chowdhury S.K., and Schmidt R.E. Mitochondrial stress and the pathogenesis of diabetic neuropathy. Expert review of endocrinology & metabolism, 2010. 5(1): p. 39-49.

115. Kaul K., Hodgkinson A., Tarr J.M., Kohner E.M., and Chibber R. Is inflammation a common retinal-renal-nerve pathogenic link in diabetes? Current diabetes reviews, 2010. 6(5): p. 294-303.

116. Price S.A., Zeef L.A., Wardleworth L., Hayes A., and Tomlinson D.R. Identification of changes in gene expression in dorsal root ganglia in diabetic neuropathy: correlation with functional deficits. Journal of neuropathology and experimental neurology, 2006. 65(7): p. 722-732.

117. Yasuda H., Terada M., Maeda K., Kogawa S., Sanada M., Haneda M., Kashiwagi A., and Kikkawa R. Diabetic neuropathy and nerve regeneration. Progress in neurobiology, 2003. 69(4): p. 229-285.

118. Tomlinson D.R. Mitogen-activated protein kinases as glucose transducers for diabetic complications. Diabetologia, 1999. 42(11): p. 1271-1281.

119. Geraldes P. and King G.L. Activation of protein kinase C isoforms and its impact on diabetic complications. Circulation research, 2010. 106(8): p. 1319-1331.

CITED LITERATURE (continued)

120. Siegelaar S.E., Holleman F., Hoekstra J.B., and DeVries J.H. Glucose variability; does it matter? Endocrine reviews, 2010. 31(2): p. 171-182.

121. Zherebitskaya E., Akude E., Smith D.R., and Fernyhough P. Development of selective axonopathy in adult sensory neurons isolated from diabetic rats: role of glucose-induced oxidative stress. Diabetes, 2009. 58(6): p. 1356-1364.

122. Chiu S.L. and Cline H.T. Insulin receptor signaling in the development of neuronal structure and function. Neural development, 2010. 5: p. 7.

123. Luppi P., Cifarelli V., and Wahren J. C-peptide and long-term complications of diabetes. Pediatric diabetes, 2010.

124. Chiarelli F., Santilli F., and Mohn A. Role of growth factors in the development of diabetic complications. Hormone research, 2000. 53(2): p. 53-67.

125. Calcutt N.A., Jolivalt C.G., and Fernyhough P. Growth factors as therapeutics for diabetic neuropathy. Current drug targets, 2008. 9(1): p. 47-59.

126. Fernyhough P., Diemel L.T., and Tomlinson D.R. Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin-diabetic rats. Diabetologia, 1998. 41(3): p. 300-306.

127. Pierson C.R., Zhang W., Murakawa Y., and Sima A.A. Insulin deficiency rather than hyperglycemia accounts for impaired neurotrophic responses and nerve fiber regeneration in type 1 diabetic neuropathy. Journal of neuropathology and experimental neurology, 2003. 62(3): p. 260-271.

128. Sima A.A. New insights into the metabolic and molecular basis for diabetic neuropathy. Cellular and molecular life sciences : CMLS, 2003. 60(11): p. 2445-2464.

129. van der Heide L.P., Ramakers G.M., and Smidt M.P. Insulin signaling in the central nervous system: learning to survive. Progress in neurobiology, 2006. 79(4): p. 205-221.

130. Lee S.S., Han H.S., and Kim H. A 5-yr follow-up nerve conduction study for the detection of subclinical diabetic neuropathy in children with newly diagnosed insulin-dependent diabetes mellitus. Pediatric diabetes, 2010. 11(8): p. 521-528.

CITED LITERATURE (continued)

131. Sun X.J. and Liu F. Phosphorylation of IRS proteins Yin-Yang regulation of insulin signaling. Vitamins and hormones, 2009. 80: p. 351-387.

132. Thirone A.C., Huang C., and Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends in endocrinology and metabolism: TEM, 2006. 17(2): p. 72-78.

133. Muniyappa R., Montagnani M., Koh K.K., and Quon M.J. Cardiovascular actions of insulin. Endocrine reviews, 2007. 28(5): p. 463-491.

134. Wada A. GSK-3 inhibitors and insulin receptor signaling in health, disease, and therapeutics. Frontiers in bioscience : a journal and virtual library, 2009. 14: p. 1558-1570.

135. Nemoto T., Yanagita T., Kanai T., and Wada A. Drug development targeting the glycogen synthase kinase-3beta (GSK-3beta)-mediated signal transduction pathway: the role of GSK-3beta in the maintenance of steady-state levels of insulin receptor signaling molecules and Na(v)1.7 sodium channel in adrenal chromaffin cells. Journal of pharmacological sciences, 2009. 109(2): p. 157-161.

136. Wada A. Lithium and neuropsychiatric therapeutics: neuroplasticity via glycogen synthase kinase-3beta, beta-catenin, and neurotrophin cascades. Journal of pharmacological sciences, 2009. 110(1): p. 14-28.

137. Chu Q., Moreland R., Yew N.S., Foley J., Ziegler R., and Scheule R.K. Systemic Insulin-like growth factor-1 reverses hypoalgesia and improves mobility in a mouse model of diabetic peripheral neuropathy. Molecular therapy : the journal of the American Society of Gene Therapy, 2008. 16(8): p. 1400-1408.

138. Francis G.J., Martinez J.A., Liu W.Q., Zochodne D.W., Hanson L.R., Frey W.H., 2nd, and Toth C. Motor end plate innervation loss in diabetes and the role of insulin. Journal of neuropathology and experimental neurology, 2011. 70(5): p. 323-339.

139. Sampson S.R. and Cooper D.R. Specific protein kinase C isoforms as transducers and modulators of insulin signaling. Molecular genetics and metabolism, 2006. 89(1-2): p. 32-47.

CITED LITERATURE (continued)

140. Ziegler D. and Luft D. Clinical trials for drugs against diabetic neuropathy: can we combine scientific needs with clinical practicalities? International review of neurobiology, 2002. 50: p. 431-463.

141. Foster T.S. Efficacy and safety of alpha-lipoic acid supplementation in the treatment of symptomatic diabetic neuropathy. The Diabetes educator, 2007. 33(1): p. 111-117.

142. Hotta N., Akanuma Y., Kawamori R., Matsuoka K., Oka Y., Shichiri M., Toyota T., Nakashima M., Yoshimura I., Sakamoto N., and Shigeta Y. Long-term clinical effects of epalrestat, an aldose reductase inhibitor, on diabetic peripheral neuropathy: the 3-year, multicenter, comparative Aldose Reductase Inhibitor-Diabetes Complications Trial. Diabetes care, 2006. 29(7): p. 1538-1544.

143. Ekberg K. and Johansson B.L. Effect of C-peptide on diabetic neuropathy in patients with type 1 diabetes. Experimental diabetes research, 2008. 2008: p. 457912.

144. Tahrani A.A., Askwith T., and Stevens M.J. Emerging drugs for diabetic neuropathy. Expert opinion on emerging drugs, 2010. 15(4): p. 661-683.

145. Tam J., Diamond J., and Maysinger D. Dual-action peptides: a new strategy in the treatment of diabetes-associated neuropathy. Drug discovery today, 2006. 11(5-6): p. 254-260.

146. Calcutt N.A. Future treatments for diabetic neuropathy: clues from experimental neuropathy. Current diabetes reports, 2002. 2(6): p. 482-488.

147. Apfel S. Diabetic neuropathy models: Are they relevant? Drug Discovery Today: Disease Models, 2006. 3(4): p. 397-402.

148. Obrosova I.G. Diabetic painful and insensate neuropathy: pathogenesis and potential treatments. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 2009. 6(4): p. 638-647.

149. Gordon D.B. and Love G. Pharmacologic management of neuropathic pain. Pain management nursing : official journal of the American Society of Pain Management Nurses, 2004. 5(4 Suppl 1): p. 19-33.

CITED LITERATURE (continued)

150. Coleman M. Axon degeneration mechanisms: commonality amid diversity. Nature reviews. Neuroscience, 2005. 6(11): p. 889-898.

151. Ino-Ue M., Zhang L., Naka H., Kuriyama H., and Yamamoto M. Polyol metabolism of retrograde axonal transport in diabetic rat large optic nerve fiber. Investigative ophthalmology & visual science, 2000. 41(13): p. 4055-4058.

152. Kuruvilla R., Ye H., and Ginty D.D. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron, 2000. 27(3): p. 499-512.

153. Hirokawa N. and Noda Y. Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. Physiological reviews, 2008. 88(3): p. 1089-1118.

154. Pfister K.K., Shah P.R., Hummerich H., Russ A., Cotton J., Annuar A.A., King S.M., and Fisher E.M. Genetic analysis of the cytoplasmic dynein subunit families. PLoS genetics, 2006. 2(1): p. e1.

155. Schlager M.A. and Hoogenraad C.C. Basic mechanisms for recognition and transport of synaptic cargos. Molecular brain, 2009. 2: p. 25.

156. Hirokawa N., Niwa S., and Tanaka Y. Molecular Motors in Neurons: Transport Mechanisms and Roles in Brain Function, Development, and Disease. Neuron, 2010. 68(4): p. 610-638.

157. Mallik R. and Gross S.P. Molecular motors: strategies to get along. Current biology : CB, 2004. 14(22): p. R971-982.

158. Morfini G., Szebenyi G., Elluru R., Ratner N., and Brady S.T. Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. The EMBO journal, 2002. 21(3): p. 281-293.

159. Morfini G., Szebenyi G., Brown H., Pant H.C., Pigino G., DeBoer S., Beffert U., and Brady S.T. A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons. The EMBO journal, 2004. 23(11): p. 2235-2245.

CITED LITERATURE (continued)

160. Akhmanova A. and Hammer J.A., 3rd. Linking molecular motors to membrane cargo. Current opinion in cell biology, 2010. 22(4): p. 479-487.

161. Roy S., Zhang B., Lee V.M., and Trojanowski J.Q. Axonal transport defects: a common theme in neurodegenerative diseases. Acta neuropathologica, 2005. 109(1): p. 5-13.

162. Morfini G.A., Burns M., Binder L.I., Kanaan N.M., LaPointe N., Bosco D.A., Brown R.H., Jr., Brown H., Tiwari A., Hayward L., Edgar J., Nave K.A., Garberrn J., Atagi Y., Song Y., Pigino G., and Brady S.T. Axonal transport defects in neurodegenerative diseases. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2009. 29(41): p. 12776-12786.

163. Duncan J.E. and Goldstein L.S. The genetics of axonal transport and axonal transport disorders. PLoS genetics, 2006. 2(9): p. e124.

164. Fichera M., Lo Giudice M., Falco M., Sturnio M., Amata S., Calabrese O., Bigoni S., Calzolari E., and Neri M. Evidence of kinesin heavy chain (KIF5A) involvement in pure hereditary spastic paraplegia. Neurology, 2004. 63(6): p. 1108-1110.

165. Morfini G., Pigino G., Szebenyi G., You Y., Pollema S., and Brady S.T. JNK mediates pathogenic effects of polyglutamine-expanded androgen receptor on fast axonal transport. Nature neuroscience, 2006. 9(7): p. 907-916.

166. Morfini G.A., You Y.M., Pollema S.L., Kaminska A., Liu K., Yoshioka K., Bjorkblom B., Coffey E.T., Bagnato C., Han D., Huang C.F., Banker G., Pigino G., and Brady S.T. Pathogenic huntingtin inhibits fast axonal transport by activating JNK3 and phosphorylating kinesin. Nature neuroscience, 2009. 12(7): p. 864-871.

167. Pigino G., Morfini G., Atagi Y., Deshpande A., Yu C., Jungbauer L., LaDu M., Busciglio J., and Brady S. Disruption of fast axonal transport is a pathogenic mechanism for intraneuronal amyloid beta. Proceedings of the National Academy of Sciences of the United States of America, 2009. 106(14): p. 5907-5912.

168. Morfini G., Pigino G., Opalach K., Serulle Y., Moreira J.E., Sugimori M., Llinas R.R., and Brady S.T. 1-Methyl-4-phenylpyridinium affects fast axonal transport by activation of caspase and protein kinase C. Proceedings of the National Academy of Sciences of the United States of America, 2007. 104(7): p. 2442-2447.

CITED LITERATURE (continued)

169. Serulle Y., Morfini G., Pigino G., Moreira J.E., Sugimori M., Brady S.T., and Llinas R.R. 1-Methyl-4-phenylpyridinium induces synaptic dysfunction through a pathway involving caspase and PKCdelta enzymatic activities. Proceedings of the National Academy of Sciences of the United States of America, 2007. 104(7): p. 2437-2441.

170. Jakobsen J. and Sidenius P. Decreased axonal transport of structural proteins in streptozotocin diabetic rats. The Journal of clinical investigation, 1980. 66(2): p. 292-297.

171. Mayer J.H. and Tomlinson D.R. Axonal transport of cholinergic transmitter enzymes in vagus and sciatic nerves of rats with acute experimental diabetes mellitus; correlation with motor nerve conduction velocity and effects of insulin. Neuroscience, 1983. 9(4): p. 951-957.

172. Lee P.G., Cai F., and Helke C.J. Streptozotocin-induced diabetes reduces retrograde axonal transport in the afferent and efferent vagus nerve. Brain research, 2002. 941(1-2): p. 127-136.

173. Lee P.G., Hohman T.C., Cai F., Regalia J., and Helke C.J. Streptozotocin-induced diabetes causes metabolic changes and alterations in neurotrophin content and retrograde transport in the cervical vagus nerve. Experimental neurology, 2001. 170(1): p. 149-161.

174. Mizisin A.P., DiStefano P.S., Liu X., Garrett D.N., and Tonra J.R. Decreased accumulation of endogenous brain-derived neurotrophic factor against constricting sciatic nerve ligatures in streptozotocin-diabetic and galactose-fed rats. Neuroscience letters, 1999. 263(2-3): p. 149-152.

175. Hellweg R., Raivich G., Hartung H.D., Hock C., and Kreutzberg G.W. Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy. Experimental neurology, 1994. 130(1): p. 24-30.

176. Wild S., Roglic G., Green A., Sicree R., and King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes care, 2004. 27(5): p. 1047-1053.

CITED LITERATURE (continued)

177. Shaw J.E., Sicree R.A., and Zimmet P.Z. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes research and clinical practice, 2010. 87(1): p. 4-14.

178. Srinivasan K. and Ramarao P. Animal models in type 2 diabetes research: an overview. The Indian journal of medical research, 2007. 125(3): p. 451-472.

179. Rees D.A. and Alcolado J.C. Animal models of diabetes mellitus. Diabetic medicine : a journal of the British Diabetic Association, 2005. 22(4): p. 359-370.

180. Van Belle T.L., Taylor P., and von Herrath M.G. Mouse Models for Type 1 Diabetes. Drug discovery today. Disease models, 2009. 6(2): p. 41-45.

181. Chatzigeorgiou A., Halapas A., Kalafatakis K., and Kamper E. The use of animal models in the study of diabetes mellitus. In vivo, 2009. 23(2): p. 245-258.

182. Nakhooda A.F., Like A.A., Chappel C.I., Murray F.T., and Marliss E.B. The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes, 1977. 26(2): p. 100-112.

183. Marliss E.B., Nakhooda A.F., Poussier P., and Sima A.A. The diabetic syndrome of the 'BB' Wistar rat: possible relevance to type 1 (insulin-dependent) diabetes in man. Diabetologia, 1982. 22(4): p. 225-232.

184. Rakieten N., Rakieten M.L., and Nadkarni M.R. Studies on the diabetogenic action of streptozotocin (NSC-37917). Cancer chemotherapy reports. Part 1, 1963. 29: p. 91-98.

185. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiological research / Academia Scientiarum Bohemoslovaca, 2001. 50(6): p. 537-546.

186. Bolzan A.D. and Bianchi M.S. Genotoxicity of streptozotocin. Mutation research, 2002. 512(2-3): p. 121-134.

187. Pessin J.E. and Bell G.I. Mammalian facilitative glucose transporter family: structure and molecular regulation. Annual review of physiology, 1992. 54: p. 911-930.

CITED LITERATURE (continued)

188. Elsner M., Tiedge M., and Lenzen S. Mechanism underlying resistance of human pancreatic beta cells against toxicity of streptozotocin and alloxan. Diabetologia, 2003. 46(12): p. 1713-1714.

189. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia, 2008. 51(2): p. 216-226.

190. Mythili M.D., Vyas R., Akila G., and Gunasekaran S. Effect of streptozotocin on the ultrastructure of rat pancreatic islets. Microscopy research and technique, 2004. 63(5): p. 274-281.

191. http://www.criver.com/en-US/TrainEducation/RMS/Species/Pages/home.aspx.

192. Lee J.Y., Kim M.J., Moon C.K., and Chung J.H. Degradation products of streptozotocin do not induce hyperglycemia in rats. Biochemical Pharmacology, 1993. 46(11): p. 2111-2113.

193. http://www.brl.uic.edu/ANIMAL_USERS_MANUAL/Rat/rats.htm.

194. Hummel K.P., Dickie M.M., and Coleman D.L. Diabetes, a new mutation in the mouse. Science, 1966. 153(740): p. 1127-1128.

195. Sima A.A., Zhang W., Xu G., Sugimoto K., Guberski D., and Yorek M.A. A comparison of diabetic polyneuropathy in type II diabetic BBZDR/Wor rats and in type I diabetic BB/Wor rats. Diabetologia, 2000. 43(6): p. 786-793.

196. Sima A.A. and Sugimoto K. Experimental diabetic neuropathy: an update. Diabetologia, 1999. 42(7): p. 773-788.

197. Moore S.A., Peterson R.G., Felten D.L., and O'Connor B.L. A quantitative comparison of motor and sensory conduction velocities in short- and long-term streptozotocin- and alloxan-diabetic rats. Journal of the neurological sciences, 1980. 48(1): p. 133-152.

198. Schmidt R.E., Dorsey D.A., Beaudet L.N., Parvin C.A., Zhang W., and Sima A.A. Experimental rat models of types 1 and 2 diabetes differ in sympathetic neuroaxonal dystrophy. Journal of neuropathology and experimental neurology, 2004. 63(5): p. 450-460.

CITED LITERATURE (continued)

199. Sullivan K.A., Hayes J.M., Wiggin T.D., Backus C., Su Oh S., Lentz S.I., Brosius F., 3rd, and Feldman E.L. Mouse models of diabetic neuropathy. Neurobiology of disease, 2007. 28(3): p. 276-285.

200. Robertson D.M. and Sima A.A. Diabetic neuropathy in the mutant mouse [C57BL/ks(db/db)]: a morphometric study. Diabetes, 1980. 29(1): p. 60-67.

201. Shaikh A.S. and Somani R.S. Animal models and biomarkers of neuropathy in diabetic rodents. Indian journal of pharmacology, 2010. 42(3): p. 129-134.

202. Sullivan K.A., Lentz S.I., Roberts J.L., Jr., and Feldman E.L. Criteria for creating and assessing mouse models of diabetic neuropathy. Current drug targets, 2008. 9(1): p. 3-13.

203. Jakobsen J. and Sidenius P. Nerve morphology in experimental diabetes. Clinical physiology, 1985. 5 Suppl 5: p. 9-13.

204. Schmidt R.E., Dorsey D.A., Beaudet L.N., Frederick K.E., Parvin C.A., Plurad S.B., and Levisetti M.G. Non-obese diabetic mice rapidly develop dramatic sympathetic neuritic dystrophy: a new experimental model of diabetic autonomic neuropathy. The American journal of pathology, 2003. 163(5): p. 2077-2091.

205. Caccia M.R., Osio M., Dezuanni E., Bevilacqua M., Bertora P.L., Salvaggio A., Mangoni A., and Norbiato G. Nerve conduction velocity distribution in normal subjects and in diabetic patients without clinical neuropathy. I. Motor nerve. Electromyography and clinical neurophysiology, 1992. 32(7-8): p. 403-409.

206. Caccia M.R., Osio M., Dezuanni E., Bevilacqua M., Bertora P.L., Salvaggio A., Mangoni A., and Norbiato G. Nerve conduction velocity distribution in normal subjects and in diabetic patients without clinical signs of neuropathy. II. Sensory nerve. Electromyography and clinical neurophysiology, 1992. 32(7-8): p. 411-416.

207. Pehlivan F., Dalkilic N., and Kiziltan E. Does the conduction velocity distribution change along the nerve? Medical engineering & physics, 2004. 26(5): p. 395-401.

208. Tuncer S., Dalkilic N., Esen H.H., and Avunduk M.C. An early diagnostic tool for diabetic neuropathy: conduction velocity distribution. Muscle & nerve, 2011. 43(2): p. 237-244.

CITED LITERATURE (continued)

209. Beiswenger K.K., Calcutt N.A., and Mizisin A.P. Epidermal nerve fiber quantification in the assessment of diabetic neuropathy. Acta histochemica, 2008. 110(5): p. 351-362.

210. Christianson J.A., Riekhof J.T., and Wright D.E. Restorative effects of neurotrophin treatment on diabetes-induced cutaneous axon loss in mice. Experimental neurology, 2003. 179(2): p. 188-199.

211. Christianson J.A., Ryals J.M., Johnson M.S., Dobrowsky R.T., and Wright D.E. Neurotrophic modulation of myelinated cutaneous innervation and mechanical sensory loss in diabetic mice. Neuroscience, 2007. 145(1): p. 303-313.

212. Polydefkis M., Hauer P., Griffin J.W., and McArthur J.C. Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. Diabetes technology & therapeutics, 2001. 3(1): p. 23-28.

213. Lauria G., Hsieh S.T., Johansson O., Kennedy W.R., Leger J.M., Mellgren S.I., Nolano M., Merkies I.S., Polydefkis M., Smith A.G., Sommer C., and Valls-Sole J. European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. European journal of neurology : the official journal of the European Federation of Neurological Societies, 2010. 17(7): p. 903-912, e944-909.

214. Mabe A.M. and Hoover D.B. Remodeling of cardiac cholinergic innervation and control of heart rate in mice with streptozotocin-induced diabetes. Autonomic neuroscience : basic & clinical, 2011.

215. Ieda M., Kanazawa H., Ieda Y., Kimura K., Matsumura K., Tomita Y., Yagi T., Onizuka T., Shimoji K., Ogawa S., Makino S., Sano M., and Fukuda K. Nerve growth factor is critical for cardiac sensory innervation and rescues neuropathy in diabetic hearts. Circulation, 2006. 114(22): p. 2351-2363.

216. Spangeus A., Suhr O., and El-Salhy M. Diabetic state affects the innervation of gut in an animal model of human type 1 diabetes. Histology and histopathology, 2000. 15(3): p. 739-744.

CITED LITERATURE (continued)

217. Johnson M.S., Ryals J.M., and Wright D.E. Early loss of peptidergic intraepidermal nerve fibers in an STZ-induced mouse model of insensate diabetic neuropathy. Pain, 2008. 140(1): p. 35-47.

218. Chen Y.S., Chung S.S., and Chung S.K. Noninvasive monitoring of diabetes-induced cutaneous nerve fiber loss and hypoalgesia in thy1-YFP transgenic mice. Diabetes, 2005. 54(11): p. 3112-3118.

219. Beiswenger K.K., Calcutt N.A., and Mizisin A.P. Dissociation of thermal hypoalgesia and epidermal denervation in streptozotocin-diabetic mice. Neuroscience letters, 2008. 442(3): p. 267-272.

220. Hargreaves K., Dubner R., Brown F., Flores C., and Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain, 1988. 32(1): p. 77-88.

221. Chaplan S.R., Bach F.W., Pogrel J.W., Chung J.M., and Yaksh T.L. Quantitative assessment of tactile allodynia in the rat paw. Journal of neuroscience methods, 1994. 53(1): p. 55-63.

222. Dixon W.J. Efficient analysis of experimental observations. Annual review of pharmacology and toxicology, 1980. 20: p. 441-462.

223. Dixon W.J. Staircase bioassay: the up-and-down method. Neuroscience and biobehavioral reviews, 1991. 15(1): p. 47-50.

224. Sakakibara Y., Mitha A.P., Ayoub I.A., Ogilvy C.S., and Maynard K.I. Delayed treatment with nicotinamide (vitamin B3) reduces the infarct volume following focal cerebral ischemia in spontaneously hypertensive rats, diabetic and non-diabetic Fischer 344 rats. Brain research, 2002. 931(1): p. 68-73.

225. Rehman J., Chenven E., Brink P., Peterson B., Walcott B., Wen Y.P., Melman A., and Christ G. Diminished neurogenic but not pharmacological erections in the 2- to 3-month experimentally diabetic F-344 rat. The American journal of physiology, 1997. 272(4 Pt 2): p. H1960-1971.

CITED LITERATURE (continued)

226. Mooradian A.D., Haas M.J., Batejko O., Hovsepyan M., and Feman S.S. Statins ameliorate endothelial barrier permeability changes in the cerebral tissue of streptozotocin-induced diabetic rats. Diabetes, 2005. 54(10): p. 2977-2982.

227. Christ G.J., Day N., Santizo C., Sato Y., Zhao W., Sclafani T., Bakal R., Salman M., Davies K., and Melman A. Intracorporal injection of hSlo cDNA restores erectile capacity in STZ-diabetic F-344 rats in vivo. American journal of physiology. Heart and circulatory physiology, 2004. 287(4): p. H1544-1553.

228. Harris G.D., Lohr J.W., Fiordalisi I., and Acara M. Brain osmoregulation during extreme and moderate dehydration in a rat model of severe DKA. Life sciences, 1993. 53(3): p. 185-191.

229. Khan A.J., Fligiel S.E., Liu L., Jaszewski R., Chandok A., and Majumdar A.P. Induction of EGFR tyrosine kinase in the gastric mucosa of diabetic rats. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine, 1999. 221(2): p. 105-110.

230. Warren B.K., Mozaffari M.S., and Patel C. Effects of inorganic fluoride on renal excretion of fluid and sodium in diabetic rats. Methods and findings in experimental and clinical pharmacology, 1998. 20(9): p. 761-769.

231. Ikebukuro K., Adachi Y., Yamada Y., Fujimoto S., Seino Y., Oyaizu H., Hioki K., and Ikehara S. Treatment of streptozotocin-induced diabetes mellitus by transplantation of islet cells plus bone marrow cells via portal vein in rats. Transplantation, 2002. 73(4): p. 512-518.

232. Aughsteen A.A. and Mohammed F.I. Insulin enhances amylase and lipase activity in the pancreas of streptozotocin-diabetic rats. An in vivo study. Saudi medical journal, 2002. 23(7): p. 838-844.

233. Haughton C.L., Dillehay D.L., and Phillips L.S. Insulin replacement therapy for the rat model of streptozotocin-induced diabetes mellitus. Laboratory animal science, 1999. 49(6): p. 639-644.

234. Sugimoto K., Rashid I.B., Shoji M., Suda T., and Yasujima M. Early changes in insulin receptor signaling and pain sensation in streptozotocin-induced diabetic neuropathy in rats. The journal of pain : official journal of the American Pain Society, 2008. 9(3): p. 237-245.

CITED LITERATURE (continued)

235. Obrosova I.G., Xu W., Lyzogubov V.V., Ilnytska O., Mashtalir N., Vareniuk I., Pavlov I.A., Zhang J., Slusher B., and Drel V.R. PARP inhibition or gene deficiency counteracts intraepidermal nerve fiber loss and neuropathic pain in advanced diabetic neuropathy. Free radical biology & medicine, 2008. 44(6): p. 972-981.

236. Calcutt N.A., Freshwater J.D., and Mizisin A.P. Prevention of sensory disorders in diabetic Sprague-Dawley rats by aldose reductase inhibition or treatment with ciliary neurotrophic factor. Diabetologia, 2004. 47(4): p. 718-724.

237. Anseloni V.C., Ennis M., and Lidow M.S. Optimization of the mechanical nociceptive threshold testing with the Randall-Selitto assay. Journal of neuroscience methods, 2003. 131(1-2): p. 93-97.

238. Christianson J.A., Ryals J.M., McCarson K.E., and Wright D.E. Beneficial actions of neurotrophin treatment on diabetes-induced hypoalgesia in mice. The journal of pain : official journal of the American Pain Society, 2003. 4(9): p. 493-504.

239. Navarro X. and Udina E. Chapter 6: Methods and protocols in peripheral nerve regeneration experimental research: part III-electrophysiological evaluation. International review of neurobiology, 2009. 87: p. 105-126.

240. Toth C., Brussee V., and Zochodne D.W. Remote neurotrophic support of epidermal nerve fibres in experimental diabetes. Diabetologia, 2006. 49(5): p. 1081-1088.

241. Lauria G., Lombardi R., Borgna M., Penza P., Bianchi R., Savino C., Canta A., Nicolini G., Marmiroli P., and Cavaletti G. Intraepidermal nerve fiber density in rat foot pad: neuropathologic-neurophysiologic correlation. Journal of the peripheral nervous system : JPNS, 2005. 10(2): p. 202-208.

242. Shir Y., Zeltser R., Vatine J.J., Carmi G., Belfer I., Zangen A., Overstreet D., Raber P., and Seltzer Z. Correlation of intact sensibility and neuropathic pain-related behaviors in eight inbred and outbred rat strains and selection lines. Pain, 2001. 90(1-2): p. 75-82.

243. Fecho K. and Valtschanoff J.G. Acute inflammatory and neuropathic pain in Lewis and Fischer rats. Journal of neuroendocrinology, 2006. 18(7): p. 504-513.

CITED LITERATURE (continued)

244. Kosten T.A. and Ambrosio E. HPA axis function and drug addictive behaviors: insights from studies with Lewis and Fischer 344 inbred rats. Psychoneuroendocrinology, 2002. 27(1-2): p. 35-69.

245. Nolte J. The human brain: An introduction to its functional anatomy. 2002.

246. Furness J.B. The organisation of the autonomic nervous system: peripheral connections. Autonomic neuroscience : basic & clinical, 2006. 130(1-2): p. 1-5.

247. Furness J.B. Novel gut afferents: Intrinsic afferent neurons and intestinofugal neurons. Autonomic neuroscience : basic & clinical, 2006. 125(1-2): p. 81-85.

248. Lomax A.E., Sharkey K.A., and Furness J.B. The participation of the sympathetic innervation of the gastrointestinal tract in disease states. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society, 2010. 22(1): p. 7-18.

249. Zhao J., Frokjaer J.B., Drewes A.M., and Ejskjaer N. Upper gastrointestinal sensory-motor dysfunction in diabetes mellitus. World journal of gastroenterology : WJG, 2006. 12(18): p. 2846-2857.

250. Armour J.A. Potential clinical relevance of the 'little brain' on the mammalian heart. Experimental Physiology, 2008. 93(2): p. 165-176.

251. Moody T.W., Ito T., Osefo N., and Jensen R.T. VIP and PACAP: recent insights into their functions/roles in physiology and disease from molecular and genetic studies. Current opinion in endocrinology, diabetes, and obesity, 2011. 18(1): p. 61-67.

252. van Rossum D., Hanisch U.K., and Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neuroscience and biobehavioral reviews, 1997. 21(5): p. 649-678.

253. Snyder S.H. Nitric oxide: first in a new class of neurotransmitters. Science, 1992. 257(5069): p. 494-496.

254. Freeman R. Autonomic peripheral neuropathy. Neurologic clinics, 2007. 25(1): p. 277-301.

CITED LITERATURE (continued)

255. Goldstein D.S., Robertson D., Esler M., Straus S.E., and Eisenhofer G. Dysautonomias: clinical disorders of the autonomic nervous system. Annals of internal medicine, 2002. 137(9): p. 753-763.

256. Jänig W., The integrative action of the autonomic nervous system: neurobiology of homeostasis2006: Cambridge University Press.

257. Vinik A.I., Freeman R., and Erbas T. Diabetic autonomic neuropathy. Seminars in neurology, 2003. 23(4): p. 365-372.

258. Astrup A.S., Tarnow L., Rossing P., Hansen B.V., Hilsted J., and Parving H.H. Cardiac autonomic neuropathy predicts cardiovascular morbidity and mortality in type 1 diabetic patients with diabetic nephropathy. Diabetes care, 2006. 29(2): p. 334-339.

259. Vinik A.I. and Ziegler D. Diabetic cardiovascular autonomic neuropathy. Circulation, 2007. 115(3): p. 387-397.

260. Camici P.G. and Pagani M. Cardiac nociception. Circulation, 2006. 114(22): p. 2309-2312.

261. Ieda M. and Fukuda K. Cardiac innervation and sudden cardiac death. Current cardiology reviews, 2009. 5(4): p. 289-295.

262. Voulgari C., Papadogiannis D., and Tentolouris N. Diabetic cardiomyopathy: from the pathophysiology of the cardiac myocytes to current diagnosis and management strategies. Vascular health and risk management, 2010. 6: p. 883-903.

263. Taskiran M., Rasmussen V., Rasmussen B., Fritz-Hansen T., Larsson H.B., Jensen G.B., and Hilsted J. Left ventricular dysfunction in normotensive Type 1 diabetic patients: the impact of autonomic neuropathy. Diabetic medicine : a journal of the British Diabetic Association, 2004. 21(6): p. 524-530.

264. Debono M. and Cachia E. The impact of Cardiovascular Autonomic Neuropathy in diabetes: is it associated with left ventricular dysfunction? Autonomic neuroscience : basic & clinical, 2007. 132(1-2): p. 1-7.

265. Weimer L.H. Autonomic testing: common techniques and clinical applications. The neurologist, 2010. 16(4): p. 215-222.

CITED LITERATURE (continued)

266. Scott L.A. and Kench P.L. Cardiac autonomic neuropathy in the diabetic patient: does 123I-MIBG imaging have a role to play in early diagnosis? Journal of nuclear medicine technology, 2004. 32(2): p. 66-71.

267. Patel A.D. and Iskandrian A.E. MIBG imaging. Journal of nuclear cardiology : official publication of the American Society of Nuclear Cardiology, 2002. 9(1): p. 75-94.

268. Allman K.C., Stevens M.J., Wieland D.M., Hutchins G.D., Wolfe E.R., Jr., Greene D.A., and Schwaiger M. Noninvasive assessment of cardiac diabetic neuropathy by carbon-11 hydroxyephedrine and positron emission tomography. Journal of the American College of Cardiology, 1993. 22(5): p. 1425-1432.

269. Kiyono Y., Iida Y., Kawashima H., Tamaki N., Nishimura H., and Saji H. Regional alterations of myocardial norepinephrine transporter density in streptozotocin-induced diabetic rats: implications for heterogeneous cardiac accumulation of MIBG in diabetes. European journal of nuclear medicine, 2001. 28(7): p. 894-899.

270. Ryden L., Standl E., Bartnik M., Van den Berghe G., Betteridge J., de Boer M.J., Cosentino F., Jonsson B., Laakso M., Malmberg K., Priori S., Ostergren J., Tuomilehto J., Thrainsdottir I., Vanhorebeek I., Stramba-Badiale M., Lindgren P., Qiao Q., Priori S.G., Blanc J.J., Budaj A., Camm J., Dean V., Deckers J., Dickstein K., Lekakis J., McGregor K., Metra M., Morais J., Osterspey A., Tamargo J., Zamorano J.L., Deckers J.W., Bertrand M., Charbonnel B., Erdmann E., Ferrannini E., Flyvbjerg A., Gohlke H., Juanatey J.R., Graham I., Monteiro P.F., Parhofer K., Pyorala K., Raz I., Schernthaner G., Volpe M., and Wood D. Guidelines on diabetes, pre-diabetes, and cardiovascular diseases: executive summary. The Task Force on Diabetes and Cardiovascular Diseases of the European Society of Cardiology (ESC) and of the European Association for the Study of Diabetes (EASD). European heart journal, 2007. 28(1): p. 88-136.

271. Boudina S. and Abel E.D. Diabetic cardiomyopathy, causes and effects. Reviews in endocrine & metabolic disorders, 2010. 11(1): p. 31-39.

272. Cosson S. and Kevorkian J.P. Left ventricular diastolic dysfunction: an early sign of diabetic cardiomyopathy? Diabetes & metabolism, 2003. 29(5): p. 455-466.

273. Raev D.C. Which left ventricular function is impaired earlier in the evolution of diabetic cardiomyopathy? An echocardiographic study of young type I diabetic patients. Diabetes care, 1994. 17(7): p. 633-639.

CITED LITERATURE (continued)

274. Sacre J.W., Franjic B., Jellis C.L., Jenkins C., Coombes J.S., and Marwick T.H. Association of cardiac autonomic neuropathy with subclinical myocardial dysfunction in type 2 diabetes. JACC. Cardiovascular imaging, 2010. 3(12): p. 1207-1215.

275. Felicio J.S., Santos F.M., de Souza A.C., Felicio K.M., Ribeiro A.B., and Zanella M.T. Autonomic neuropathy tests correlate with left ventricular mass and cardiac diastolic function in normotensive patients with type 2 diabetes mellitus and without left ventricular hypertrophy. Experimental and clinical cardiology, 2010. 15(1): p. e5-9.

276. Karamitsos T.D., Karvounis H.I., Didangelos T., Parcharidis G.E., and Karamitsos D.T. Impact of autonomic neuropathy on left ventricular function in normotensive type 1 diabetic patients: a tissue Doppler echocardiographic study. Diabetes care, 2008. 31(2): p. 325-327.

277. Didangelos T.P., Arsos G.A., Karamitsos D.T., Athyros V.G., and Karatzas N.D. Left ventricular systolic and diastolic function in normotensive type 1 diabetic patients with or without autonomic neuropathy: a radionuclide ventriculography study. Diabetes care, 2003. 26(7): p. 1955-1960.

278. Raev D.C. Left ventricular function and specific diabetic complications in other target organs in young insulin-dependent diabetics: an echocardiographic study. Heart and vessels, 1994. 9(3): p. 121-128.

279. Lacigova S., Bartunek L., Cechurova D., Visek J., Gruberova J., Krcma M., Jankovec Z., Rusavy Z., and Zourek M. Influence of cardiovascular autonomic neuropathy on atherogenesis and heart function in patients with type 1 diabetes. Diabetes research and clinical practice, 2009. 83(1): p. 26-31.

280. Stevens M.J., Raffel D.M., Allman K.C., Dayanikli F., Ficaro E., Sandford T., Wieland D.M., Pfeifer M.A., and Schwaiger M. Cardiac sympathetic dysinnervation in diabetes: implications for enhanced cardiovascular risk. Circulation, 1998. 98(10): p. 961-968.

281. Schnell O., Hammer K., Muhr-Becker D., Ziegler A., Weiss M., Tatsch K., and Standl E. Cardiac sympathetic dysinnervation in Type 2 diabetes mellitus with and without ECG-based cardiac autonomic neuropathy. Journal of diabetes and its complications, 2002. 16(3): p. 220-227.

CITED LITERATURE (continued)

282. Lluch I., Hernandez A., Real J.T., Morillas C., Tenes S., Sanchez C., and Ascaso J.F. Cardiovascular autonomic neuropathy in type 1 diabetic patients with and without peripheral neuropathy. Diabetes research and clinical practice, 1998. 42(1): p. 35-40.

283. Gandhi R.A., Marques J.L., Selvarajah D., Emery C.J., and Tesfaye S. Painful diabetic neuropathy is associated with greater autonomic dysfunction than painless diabetic neuropathy. Diabetes care, 2010. 33(7): p. 1585-1590.

284. Tack C.J., van Gurp P.J., Holmes C., and Goldstein D.S. Local sympathetic denervation in painful diabetic neuropathy. Diabetes, 2002. 51(12): p. 3545-3553.

285. Lanting P., Faes T.J., Ijff G.A., Bertelsmann F.W., Heimans J.J., and van der Veen E.A. Autonomic and somatic peripheral nerve function and the correlation with neuropathic pain in diabetic patients. Journal of the neurological sciences, 1989. 94(1-3): p. 307-317.

286. Young R.J., Zhou Y.Q., Rodriguez E., Prescott R.J., Ewing D.J., and Clarke B.F. Variable relationship between peripheral somatic and autonomic neuropathy in patients with different syndromes of diabetic polyneuropathy. Diabetes, 1986. 35(2): p. 192-197.

287. Wichi R., Malfitano C., Rosa K., De Souza S.B., Salemi V., Mostarda C., De Angelis K., and Irigoyen M.C. Noninvasive and invasive evaluation of cardiac dysfunction in experimental diabetes in rodents. Cardiovascular diabetology, 2007. 6: p. 14.

288. De Angelis K., Irigoyen M.C., and Morris M. Diabetes and cardiovascular autonomic dysfunction: application of animal models. Autonomic neuroscience : basic & clinical, 2009. 145(1-2): p. 3-10.

289. Rowan W.H., 3rd, Campen M.J., Wichers L.B., and Watkinson W.P. Heart rate variability in rodents: uses and caveats in toxicological studies. Cardiovascular toxicology, 2007. 7(1): p. 28-51.

290. Perlstein I., Sapir N., Backon J., Sapoznikov D., Karasik R., Havlin S., and Hoffman A. Scaling vs. nonscaling methods of assessing autonomic tone in streptozotocin-induced diabetic rats. American journal of physiology. Heart and circulatory physiology, 2002. 283(3): p. H1142-1149.

CITED LITERATURE (continued)

291. Kuwahara M., Yayou K., Ishii K., Hashimoto S., Tsubone H., and Sugano S. Power spectral analysis of heart rate variability as a new method for assessing autonomic activity in the rat. Journal of electrocardiology, 1994. 27(4): p. 333-337.

292. Kurata C., Okayama K., Wakabayashi Y., Shouda S., Mikami T., Tawarahara K., and Sugiyama T. Cardiac sympathetic neuropathy and effects of aldose reductase inhibitor in streptozotocin-induced diabetic rats. Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 1997. 38(11): p. 1677-1680.

293. Ganguly P.K., Beamish R.E., Dhalla K.S., Innes I.R., and Dhalla N.S. Norepinephrine storage, distribution, and release in diabetic cardiomyopathy. The American journal of physiology, 1987. 252(6 Pt 1): p. E734-739.

294. Berkowitz B.A., Head R., Joh T., and Hempstead J. Experimental diabetes: alterations in circulating dopamine-beta-hydroxylase and norepinephrine. The Journal of pharmacology and experimental therapeutics, 1980. 213(1): p. 18-23.

295. Ganguly P.K., Dhalla K.S., Innes I.R., Beamish R.E., and Dhalla N.S. Altered norepinephrine turnover and metabolism in diabetic cardiomyopathy. Circulation research, 1986. 59(6): p. 684-693.

296. Yoshida T., Nishioka H., Yoshioka K., Nakano K., Kondo M., and Terashima H. Effect of aldose reductase inhibitor ONO 2235 on reduced sympathetic nervous system activity and peripheral nerve disorders in STZ-induced diabetic rats. Diabetes, 1987. 36(1): p. 6-13.

297. Fushimi H., Inoue T., Matsuyama Y., Kishino B., Kameyama M., Funakawa S., Tochino Y., Yamatodani A., Wada H., Minami T., and et al. Impaired catecholamine secretion as a cause of diabetic autonomic neuropathy. Diabetes research and clinical practice, 1988. 4(4): p. 303-307.

298. Kuncova J., Slavikova J., and Sviglerova J. Norepinephrine release in the heart atria of diabetic rats. General physiology and biophysics, 2003. 22(3): p. 397-410.

299. Kuncova J., Sviglerova J., Tonar Z., and Slavikova J. Heterogenous changes in neuropeptide Y, norepinephrine and epinephrine concentrations in the hearts of diabetic rats. Autonomic neuroscience : basic & clinical, 2005. 121(1-2): p. 7-15.

CITED LITERATURE (continued)

300. Akiyama N., Okumura K., Watanabe Y., Hashimoto H., Ito T., Ogawa K., and Satake T. Altered acetylcholine and norepinephrine concentrations in diabetic rat hearts. Role of parasympathetic nervous system in diabetic cardiomyopathy. Diabetes, 1989. 38(2): p. 231-236.

301. Gallego M., Setien R., Izquierdo M.J., Casis O., and Casis E. Diabetes-induced biochemical changes in central and peripheral catecholaminergic systems. Physiological research / Academia Scientiarum Bohemoslovaca, 2003. 52(6): p. 735-741.

302. Chottova Dvorakova M., Kuncova J., Pfeil U., McGregor G.P., Sviglerova J., Slavikova J., and Kummer W. Cardiomyopathy in streptozotocin-induced diabetes involves intra-axonal accumulation of calcitonin gene-related peptide and altered expression of its receptor in rats. Neuroscience, 2005. 134(1): p. 51-58.

303. Katona M., Boros K., Santha P., Ferdinandy P., Dux M., and Jancso G. Selective sensory denervation by capsaicin aggravates adriamycin-induced cardiomyopathy in rats. Naunyn-Schmiedeberg's archives of pharmacology, 2004. 370(6): p. 436-443.

304. Richardson R.J., Grkovic I., and Anderson C.R. Immunohistochemical analysis of intracardiac ganglia of the rat heart. Cell and tissue research, 2003. 314(3): p. 337-350.

305. Shoba T. and Tay S.S. Nitrergic and peptidergic innervation in the developing rat heart. Anatomy and embryology, 2000. 201(6): p. 491-500.

306. Takanaga A., Hayakawa T., Tanaka K., Kawabata K., Maeda S., and Seki M. Immunohistochemical characterization of cardiac vagal preganglionic neurons in the rat. Autonomic neuroscience : basic & clinical, 2003. 106(2): p. 132-137.

307. Felten S.Y., Peterson R.G., Shea P.A., Besch H.R., Jr., and Felten D.L. Effects of streptozotocin diabetes on the noradrenergic innervation of the rat heart: a longitudinal histofluorescence and neurochemical study. Brain research bulletin, 1982. 8(6): p. 593-607.

308. Zhang X., Fuller R.R., Dahlgren R.L., Potgieter K., Gillette R., and Sweedler J.V. Neurotransmitter sampling and storage for capillary electrophoresis analysis. Fresenius' journal of analytical chemistry, 2001. 369(3-4): p. 206-211.

CITED LITERATURE (continued)

309. Boomsma F., Alberts G., van Eijk L., Man in 't Veld A.J., and Schalekamp M.A. Optimal collection and storage conditions for catecholamine measurements in human plasma and urine. Clinical chemistry, 1993. 39(12): p. 2503-2508.

310. Quaiserova-Mocko V., Novotny M., Schaefer L.S., Fink G.D., and Swain G.M. CE coupled with amperometric detection using a boron-doped diamond microelectrode: validation of a method for endogenous norepinephrine analysis in tissue. Electrophoresis, 2008. 29(2): p. 441-447.

311. Neubecker T.A., Coombs M.A., Quijano M., O'Neill T.P., Cruze C.A., and Dobson R.L. Rapid and selective method for norepinephrine in rat urine using reversed-phase ion-pair high-performance liquid chromatography-tandem mass spectrometry. Journal of chromatography. B, Biomedical sciences and applications, 1998. 718(2): p. 225-233.

312. Koller M. Results for 74 substances tested for interference with determination of plasma catecholamines by "high-performance" liquid chromatography with electrochemical detection. Clinical chemistry, 1988. 34(5): p. 947-949.

313. Holcapek M., Kolarova L., and Nobilis M. High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. Analytical and bioanalytical chemistry, 2008. 391(1): p. 59-78.

314. Anton A.H. and Sayre D.F. A study of the factors affecting the aluminum oxide-trihydroxyindole procedure for the analysis of catecholamines. The Journal of pharmacology and experimental therapeutics, 1962. 138: p. 360-375.

315. Kim S.D., Beck J., Bieniarz T., Schumacher A., and Piano M.R. A rodent model of alcoholic heart muscle disease and its evaluation by echocardiography. Alcoholism, clinical and experimental research, 2001. 25(3): p. 457-463.

316. Kirino Y., Sato Y., Kamimoto T., Kawazoe K., Minakuchi K., and Nakahori Y. Interrelationship of dipeptidyl peptidase IV (DPP4) with the development of diabetes, dyslipidaemia and nephropathy: a streptozotocin-induced model using wild-type and DPP4-deficient rats. The Journal of endocrinology, 2009. 200(1): p. 53-61.

317. Fushimi H., Inoue T., Kishino B., Nishikawa M., Tochino Y., Funakawa S., Yamatodani A., and Wada H. Abnormalities in plasma catecholamine response and

CITED LITERATURE (continued)

tissue catecholamine accumulation in streptozotocin diabetic rats: a possible role for diabetic autonomic neuropathy. Life sciences, 1984. 35(10): p. 1077-1081.

318. Schmid H., Forman L.A., Cao X., Sherman P.S., and Stevens M.J. Heterogeneous cardiac sympathetic denervation and decreased myocardial nerve growth factor in streptozotocin-induced diabetic rats: implications for cardiac sympathetic dysinnervation complicating diabetes. Diabetes, 1999. 48(3): p. 603-608.

319. Dincer U.D., Bidasee K.R., Guner S., Tay A., Ozcelikay A.T., and Altan V.M. The effect of diabetes on expression of beta1-, beta2-, and beta3-adrenoreceptors in rat hearts. Diabetes, 2001. 50(2): p. 455-461.

320. Neubauer B. and Christensen N.J. Norepinephrine, epinephrine, and dopamine contents of the cardiovascular system in long-term diabetics. Diabetes, 1976. 25(1): p. 6-10.

321. Giordano A., Calcagni M.L., Verrillo A., Pellegrinotti M., Frontoni S., Spallone V., and Gambardella S. Assessment of sympathetic innervation of the heart in diabetes mellitus using 123I-MIBG. Diabetes, nutrition & metabolism, 2000. 13(6): p. 350-355.

322. Mihm M.J., Seifert J.L., Coyle C.M., and Bauer J.A. Diabetes related cardiomyopathy time dependent echocardiographic evaluation in an experimental rat model. Life sciences, 2001. 69(5): p. 527-542.

323. Subbalakshmi N.K., Adhikari P.M., Rajeev A., Asha K., and Jeganathan P.S. Independent predictors of cardiac parasympathetic dysfunction in type 2 diabetes mellitus. Singapore medical journal, 2008. 49(2): p. 121-128.

324. Chen L.S., Zhou S., Fishbein M.C., and Chen P.S. New perspectives on the role of autonomic nervous system in the genesis of arrhythmias. Journal of cardiovascular electrophysiology, 2007. 18(1): p. 123-127.

325. Hoit B.D., Castro C., Bultron G., Knight S., and Matlib M.A. Noninvasive evaluation of cardiac dysfunction by echocardiography in streptozotocin-induced diabetic rats. J Card Fail, 1999. 5(4): p. 324-333.

CITED LITERATURE (continued)

326. Morfini G., Stenoien D., and Brady S., 28:Axonal Transport, In: Basic Neurochemistry Molecular, Cellular and Medical Aspects, 2006, American Society for Neurochemistry,

327. Hoogenraad C.C. and Akhmanova A. Dendritic spine plasticity: new regulatory roles of dynamic microtubules. The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry, 2010. 16(6): p. 650-661.

328. Donelan M.J., Morfini G., Julyan R., Sommers S., Hays L., Kajio H., Briaud I., Easom R.A., Molkentin J.D., Brady S.T., and Rhodes C.J. Ca2+-dependent dephosphorylation of kinesin heavy chain on beta-granules in pancreatic beta-cells. Implications for regulated beta-granule transport and insulin exocytosis. The Journal of biological chemistry, 2002. 277(27): p. 24232-24242.

329. Vale R.D. The molecular motor toolbox for intracellular transport. Cell, 2003. 112(4): p. 467-480.

330. Lawrence C.J., Dawe R.K., Christie K.R., Cleveland D.W., Dawson S.C., Endow S.A., Goldstein L.S., Goodson H.V., Hirokawa N., Howard J., Malmberg R.L., McIntosh J.R., Miki H., Mitchison T.J., Okada Y., Reddy A.S., Saxton W.M., Schliwa M., Scholey J.M., Vale R.D., Walczak C.E., and Wordeman L. A standardized kinesin nomenclature. The Journal of cell biology, 2004. 167(1): p. 19-22.

331. Hirokawa N. and Takemura R. Molecular motors and mechanisms of directional transport in neurons. Nature reviews. Neuroscience, 2005. 6(3): p. 201-214.

332. Wozniak M.J. and Allan V.J. Cargo selection by specific kinesin light chain 1 isoforms. The EMBO journal, 2006. 25(23): p. 5457-5468.

333. Pfister K.K., Fisher E.M., Gibbons I.R., Hays T.S., Holzbaur E.L., McIntosh J.R., Porter M.E., Schroer T.A., Vaughan K.T., Witman G.B., King S.M., and Vallee R.B. Cytoplasmic dynein nomenclature. The Journal of cell biology, 2005. 171(3): p. 411-413.

334. De Vos K.J., Grierson A.J., Ackerley S., and Miller C.C. Role of axonal transport in neurodegenerative diseases. Annual review of neuroscience, 2008. 31: p. 151-173.

CITED LITERATURE (continued)

335. Zhao C., Takita J., Tanaka Y., Setou M., Nakagawa T., Takeda S., Yang H.W., Terada S., Nakata T., Takei Y., Saito M., Tsuji S., Hayashi Y., and Hirokawa N. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. Cell, 2001. 105(5): p. 587-597.

336. Fink J.K. The hereditary spastic paraplegias: nine genes and counting. Archives of neurology, 2003. 60(8): p. 1045-1049.

337. Lu S., Zhao C., Zhao K., Li N., and Larsson C. Novel and recurrent KIF21A mutations in congenital fibrosis of the extraocular muscles type 1 and 3. Archives of ophthalmology, 2008. 126(3): p. 388-394.

338. Puls I., Jonnakuty C., LaMonte B.H., Holzbaur E.L., Tokito M., Mann E., Floeter M.K., Bidus K., Drayna D., Oh S.J., Brown R.H., Jr., Ludlow C.L., and Fischbeck K.H. Mutant dynactin in motor neuron disease. Nature genetics, 2003. 33(4): p. 455-456.

339. El-Kadi A.M., Soura V., and Hafezparast M. Defective axonal transport in motor neuron disease. Journal of neuroscience research, 2007. 85(12): p. 2557-2566.

340. Sato-Yoshitake R., Yorifuji H., Inagaki M., and Hirokawa N. The phosphorylation of kinesin regulates its binding to synaptic vesicles. The Journal of biological chemistry, 1992. 267(33): p. 23930-23936.

341. Vagnoni A., Rodriguez L., Manser C., De Vos K.J., and Miller C.C. Phosphorylation of kinesin light chain 1 at serine 460 modulates binding and trafficking of calsyntenin-1. Journal of cell science, 2011. 124(Pt 7): p. 1032-1042.

342. Toda H., Mochizuki H., Flores R., 3rd, Josowitz R., Krasieva T.B., Lamorte V.J., Suzuki E., Gindhart J.G., Furukubo-Tokunaga K., and Tomoda T. UNC-51/ATG1 kinase regulates axonal transport by mediating motor-cargo assembly. Genes & development, 2008. 22(23): p. 3292-3307.

343. Guillaud L., Wong R., and Hirokawa N. Disruption of KIF17-Mint1 interaction by CaMKII-dependent phosphorylation: a molecular model of kinesin-cargo release. Nature cell biology, 2008. 10(1): p. 19-29.

344. Stagi M., Gorlovoy P., Larionov S., Takahashi K., and Neumann H. Unloading kinesin transported cargoes from the tubulin track via the inflammatory c-Jun N-terminal

CITED LITERATURE (continued)

kinase pathway. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2006. 20(14): p. 2573-2575.

345. Ichimura T., Wakamiya-Tsuruta A., Itagaki C., Taoka M., Hayano T., Natsume T., and Isobe T. Phosphorylation-dependent interaction of kinesin light chain 2 and the 14-3-3 protein. Biochemistry, 2002. 41(17): p. 5566-5572.

346. Vaughan P.S., Leszyk J.D., and Vaughan K.T. Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin. The Journal of biological chemistry, 2001. 276(28): p. 26171-26179.

347. Tsai M.Y., Morfini G., Szebenyi G., and Brady S.T. Release of kinesin from vesicles by hsc70 and regulation of fast axonal transport. Molecular biology of the cell, 2000. 11(6): p. 2161-2173.

348. Calcutt N.A. Tolerating diabetes: an alternative therapeutic approach for diabetic neuropathy. ASN neuro, 2010. 2(4).

349. Urban M.J., Li C., Yu C., Lu Y., Krise J.M., McIntosh M.P., Rajewski R.A., Blagg B.S., and Dobrowsky R.T. Inhibiting heat-shock protein 90 reverses sensory hypoalgesia in diabetic mice. ASN neuro, 2010. 2(4).

350. LaPointe N.E., Morfini G., Pigino G., Gaisina I.N., Kozikowski A.P., Binder L.I., and Brady S.T. The amino terminus of tau inhibits kinesin-dependent axonal transport: implications for filament toxicity. Journal of neuroscience research, 2009. 87(2): p. 440-451.

351. Lazarov O., Morfini G.A., Pigino G., Gadadhar A., Chen X., Robinson J., Ho H., Brady S.T., and Sisodia S.S. Impairments in fast axonal transport and motor neuron deficits in transgenic mice expressing familial Alzheimer's disease-linked mutant presenilin 1. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2007. 27(26): p. 7011-7020.

352. Morfini G., Pigino G., Beffert U., Busciglio J., and Brady S.T. Fast axonal transport misregulation and Alzheimer's disease. Neuromolecular medicine, 2002. 2(2): p. 89-99.

CITED LITERATURE (continued)

353. Pigino G., Morfini G., Pelsman A., Mattson M.P., Brady S.T., and Busciglio J. Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. The Journal of neuroscience : the official journal of the Society for Neuroscience, 2003. 23(11): p. 4499-4508.

354. Morfini G., Pigino G., and Brady S.T. Polyglutamine expansion diseases: failing to deliver. Trends in molecular medicine, 2005. 11(2): p. 64-70.

355. Du J., Wei Y., Liu L., Wang Y., Khairova R., Blumenthal R., Tragon T., Hunsberger J.G., Machado-Vieira R., Drevets W., Wang Y.T., and Manji H.K. A kinesin signaling complex mediates the ability of GSK-3beta to affect mood-associated behaviors. Proceedings of the National Academy of Sciences of the United States of America, 2010. 107(25): p. 11573-11578.

356. Williams S.K., Howarth N.L., Devenny J.J., and Bitensky M.W. Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. Proceedings of the National Academy of Sciences of the United States of America, 1982. 79(21): p. 6546-6550.

357. Medori R., Jenich H., Autilio-Gambetti L., and Gambetti P. Experimental diabetic neuropathy: similar changes of slow axonal transport and axonal size in different animal models. The Journal of neuroscience : the official journal of the Society for Neuroscience, 1988. 8(5): p. 1814-1821.

358. Macioce P., Filliatreau G., Figliomeni B., Hassig R., Thiery J., and Di Giamberardino L. Slow axonal transport impairment of cytoskeletal proteins in streptozocin-induced diabetic neuropathy. Journal of neurochemistry, 1989. 53(4): p. 1261-1267.

359. Medori R., Autilio-Gambetti L., Monaco S., and Gambetti P. Experimental diabetic neuropathy: impairment of slow transport with changes in axon cross-sectional area. Proceedings of the National Academy of Sciences of the United States of America, 1985. 82(22): p. 7716-7720.

360. Hoffman P.N., Griffin J.W., and Price D.L. Control of axonal caliber by neurofilament transport. The Journal of cell biology, 1984. 99(2): p. 705-714.

361. McLean W.G. The role of axonal cytoskeleton in diabetic neuropathy. Neurochemical research, 1997. 22(8): p. 951-956.

CITED LITERATURE (continued)

362. Gunes A., Ceylan A., Sarioglu Y., Stefek M., Bauer V., and Karasu C. Reactive oxygen species mediate abnormal contractile response to sympathetic nerve stimulation and noradrenaline in the vas deferens of chronically diabetic rats: effects of in vivo treatment with antioxidants. Fundam Clin Pharmacol, 2005. 19(1): p. 73-79.

363. Gallego M., Setien R., Izquierdo M.J., Casis O., and Casis E. Diabetes-induced biochemical changes in central and peripheral catecholaminergic systems. Physiol Res, 2003. 52(6): p. 735-741.

364. Sears C.E., Ashley E.A., and Casadei B. Nitric oxide control of cardiac function: is neuronal nitric oxide synthase a key component? Philos Trans R Soc Lond B Biol Sci, 2004. 359(1446): p. 1021-1044.

365. Mayer J.H. and Tomlinson D.R. Prevention of defects of axonal transport and nerve conduction velocity by oral administration of myo-inositol or an aldose reductase inhibitor in streptozotocin-diabetic rats. Diabetologia, 1983. 25(5): p. 433-438.

366. Di Giulio A.M., Lesma E., and Gorio A. Diabetic neuropathy in the rat: 1. Alcar augments the reduced levels and axoplasmic transport of substance P. J Neurosci Res, 1995. 40(3): p. 414-419.

367. Jiang Y., Nyengaard J.R., Zhang J.S., and Jakobsen J. Selective loss of calcitonin gene-related Peptide-expressing primary sensory neurons of the a-cell phenotype in early experimental diabetes. Diabetes, 2004. 53(10): p. 2669-2675.

368. Abdala A.P., Haibara A.S., and Colombari E. Cardiovascular responses to substance P in the nucleus tractus solitarii: microinjection study in conscious rats. Am J Physiol Heart Circ Physiol, 2003. 285(2): p. H891-898.

369. Robinson J.P., Willars G.B., Tomlinson D.R., and Keen P. Axonal transport and tissue contents of substance P in rats with long-term streptozotocin-diabetes. Effects of the aldose reductase inhibitor 'statil'. Brain research, 1987. 426(2): p. 339-348.

370. Cellek S. Point of NO return for nitrergic nerves in diabetes: a new insight into diabetic complications. Curr Pharm Des, 2004. 10(29): p. 3683-3695.

371. Odekunle A. Impairment of transneuronal traffic in Streptozotocin-induced diabetes, a WGA-HRP neurohistochemical study in the rat. Journal of biomedical science, 2006. 13(5): p. 675-680.

CITED LITERATURE (continued)

372. Middlemas A., Delcroix J.D., Sayers N.M., Tomlinson D.R., and Fernyhough P. Enhanced activation of axonally transported stress-activated protein kinases in peripheral nerve in diabetic neuropathy is prevented by neurotrophin-3. Brain, 2003. 126(Pt 7): p. 1671-1682.

373. Fernyhough P., Diemel L.T., and Tomlinson D.R. Target tissue production and axonal transport of neurotrophin-3 are reduced in streptozotocin-diabetic rats. Diabetologia, 1998. 41: p. 300-306.

374. Lee P.G., Hohman T.C., Cai F., Regalia J., and Helke C.J. Streptozotocin-induced diabetes causes metabolic changes and alterations in neurotrophin content and retrograde transport in the cervical vagus nerve. Exp Neurol, 2001. 170(1): p. 149-161.

375. Hellweg R., Raivich G., Hartung H.D., Hock C., and Kreutzberg G.W. Axonal transport of endogenous nerve growth factor (NGF) and NGF receptor in experimental diabetic neuropathy. Exp Neurol, 1994. 130(1): p. 24-30.

376. Lee P.G., Cai F., and Helke C.J. Streptozotocin-induced diabetes reduces retrograde axonal transport in the afferent and efferent vagus nerve. Brain Res, 2002. 941(1-2): p. 127-136.

377. Meiri K.F. and McLean W.G. Axonal transport of protein in motor fibres of experimentally diabetic rats--fast anterograde transport. Brain research, 1982. 238(1): p. 77-88.

378. Middlemas A., Delcroix J.D., Sayers N.M., Tomlinson D.R., and Fernyhough P. Enhanced activation of axonally transported stress-activated protein kinases in peripheral nerve in diabetic neuropathy is prevented by neurotrophin-3. Brain : a journal of neurology, 2003. 126(Pt 7): p. 1671-1682.

379. Schnell O., Hammer K., Muhr-Becker D., Ziegler A., Weiss M., Tatsch K., and Standl E. Cardiac sympathetic dysinnervation in Type 2 diabetes mellitus with and without ECG-based cardiac autonomic neuropathy. J Diabetes Complications, 2002. 16(3): p. 220-227.

380. Felten S.Y., Peterson R.G., Shea P.A., Besch H.R., Jr., and Felten D.L. Effects of streptozotocin diabetes on the noradrenergic innervation of the rat heart: a longitudinal histofluorescence and neurochemical study. Brain Res Bull, 1982. 8(6): p. 593-607.

CITED LITERATURE (continued)

381. Stevens M.J., Raffel D.M., Allman K.C., Schwaiger M., and Wieland D.M. Regression and progression of cardiac sympathetic dysinnervation complicating diabetes: an assessment by C-11 hydroxyephedrine and positron emission tomography. Metabolism, 1999. 48(1): p. 92-101.

382. Wendelschafer-Crabb G., Kennedy W.R., and Walk D. Morphological features of nerves in skin biopsies. J Neurol Sci, 2006. 242(1-2): p. 15-21.

383. Shun C.T., Chang Y.C., Wu H.P., Hsieh S.C., Lin W.M., Lin Y.H., Tai T.Y., and Hsieh S.T. Skin denervation in type 2 diabetes: correlations with diabetic duration and functional impairments. Brain, 2004. 127(Pt 7): p. 1593-1605.

384. McArthur J.C., Stocks E.A., Hauer P., Cornblath D.R., and Griffin J.W. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol, 1998. 55(12): p. 1513-1520.

385. Umapathi T., Tan W.L., Loke S.C., Soon P.C., Tavintharan S., and Chan Y.H. Intraepidermal nerve fiber density as a marker of early diabetic neuropathy. Muscle Nerve, 2007. 35(5): p. 591-598.

386. Mei Q., Mundinger T.O., Lernmark A., and Taborsky G.J., Jr. Early, selective, and marked loss of sympathetic nerves from the islets of BioBreeder diabetic rats. Diabetes, 2002. 51(10): p. 2997-3002.

387. Rosa-e-Silva L., Troncon L.E., Oliveira R.B., Foss M.C., Braga F.J., and Gallo Junior L. Rapid distal small bowel transit associated with sympathetic denervation in type I diabetes mellitus. Gut, 1996. 39(5): p. 748-756.

388. Shotton H.R., Broadbent S., and Lincoln J. Prevention and partial reversal of diabetes-induced changes in enteric nerves of the rat ileum by combined treatment with alpha-lipoic acid and evening primrose oil. Auton Neurosci, 2004. 111(1): p. 57-65.

389. Brady S.T., Richards B.W., and Leopold P.L. Assay of vesicle motility in squid axoplasm. Methods in cell biology, 1993. 39: p. 191-202.

390. Brady S.T., Lasek R.J., and Allen R.D. Fast axonal transport in extruded axoplasm from squid giant axon. Science, 1982. 218(4577): p. 1129-1131.

CITED LITERATURE (continued)

391. Brady S.T., Lasek R.J., and Allen R.D. Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms. Cell motility, 1985. 5(2): p. 81-101.

392. Sharma S.K. and Carew T.J. Inclusion of phosphatase inhibitors during Western blotting enhances signal detection with phospho-specific antibodies. Analytical biochemistry, 2002. 307(1): p. 187-189.

393. Tubulin Polymerization with GTP/GMPCPP/Taxol. Available from: http://mitchison.med.harvard.edu/protocols/poly.html.

394. Desai A. MT Spindowns from Extracts. Available from: http://mitchison.med.harvard.edu/protocols/mt2.html.

395. Castoldi M. and Popov A.V. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. Protein expression and purification, 2003. 32(1): p. 83-88.

396. Jope R.S. and Johnson G.V. The glamour and gloom of glycogen synthase kinase-3. Trends in biochemical sciences, 2004. 29(2): p. 95-102.

397. Weigert C., Kron M., Kalbacher H., Pohl A.K., Runge H., Haring H.U., Schleicher E., and Lehmann R. Interplay and effects of temporal changes in the phosphorylation state of serine-302, -307, and -318 of insulin receptor substrate-1 on insulin action in skeletal muscle cells. Molecular endocrinology, 2008. 22(12): p. 2729-2740.

398. Lee Y.H., Giraud J., Davis R.J., and White M.F. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. The Journal of biological chemistry, 2003. 278(5): p. 2896-2902.

399. Tsakiridis T., Taha C., Grinstein S., and Klip A. Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. The Journal of biological chemistry, 1996. 271(33): p. 19664-19667.

400. Sweeney G., Somwar R., Ramlal T., Volchuk A., Ueyama A., and Klip A. An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. The Journal of biological chemistry, 1999. 274(15): p. 10071-10078.

CITED LITERATURE (continued)

401. Conejo R. and Lorenzo M. Insulin signaling leading to proliferation, survival, and membrane ruffling in C2C12 myoblasts. Journal of cellular physiology, 2001. 187(1): p. 96-108.

402. Lee Y.H. and White M.F. Insulin receptor substrate proteins and diabetes. Archives of pharmacal research, 2004. 27(4): p. 361-370.

403. Fukunaga M., Miyata S., Higo S., Hamada Y., Ueyama S., and Kasuga M. Methylglyoxal induces apoptosis through oxidative stress-mediated activation of p38 mitogen-activated protein kinase in rat Schwann cells. Annals of the New York Academy of Sciences, 2005. 1043: p. 151-157.

404. Askwith T., Zeng W., Eggo M.C., and Stevens M.J. Oxidative stress and dysregulation of the taurine transporter in high-glucose-exposed human Schwann cells: implications for pathogenesis of diabetic neuropathy. American journal of physiology. Endocrinology and metabolism, 2009. 297(3): p. E620-628.

405. Daulhac L., Mallet C., Courteix C., Etienne M., Duroux E., Privat A.M., Eschalier A., and Fialip J. Diabetes-induced mechanical hyperalgesia involves spinal mitogen-activated protein kinase activation in neurons and microglia via N-methyl-D-aspartate-dependent mechanisms. Molecular pharmacology, 2006. 70(4): p. 1246-1254.

406. Bastard J.P., Maachi M., Lagathu C., Kim M.J., Caron M., Vidal H., Capeau J., and Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. European cytokine network, 2006. 17(1): p. 4-12.

407. King G.L. The role of inflammatory cytokines in diabetes and its complications. Journal of periodontology, 2008. 79(8 Suppl): p. 1527-1534.

408. Wei R., Listwak S.J., and Sternberg E.M. Lewis hypothalamic cells constitutively and upon stimulation express higher levels of mRNA for pro-inflammatory cytokines and related molecules: comparison with inflammatory resistant Fischer rat hypothalamic cells. Journal of neuroimmunology, 2003. 135(1-2): p. 10-28.

409. Cizza G. and Sternberg E.M. The role of the hypothalamic-pituitary-adrenal axis in susceptibility to autoimmune/inflammatory disease. ImmunoMethods, 1994. 5(1): p. 73-78.

CITED LITERATURE (continued)

410. Fischer T.Z. and Waxman S.G. Neuropathic pain in diabetes--evidence for a central mechanism. Nature reviews. Neurology, 2010. 6(8): p. 462-466.

411. Calcutt N.A. and Backonja M.M. Pathogenesis of pain in peripheral diabetic neuropathy. Current diabetes reports, 2007. 7(6): p. 429-434.

412. Young R.J., Ewing D.J., and Clarke B.F. A controlled trial of sorbinil, an aldose reductase inhibitor, in chronic painful diabetic neuropathy. Diabetes, 1983. 32(10): p. 938-942.

413. Sweitzer S.M., Medicherla S., Almirez R., Dugar S., Chakravarty S., Shumilla J.A., Yeomans D.C., and Protter A.A. Antinociceptive action of a p38alpha MAPK inhibitor, SD-282, in a diabetic neuropathy model. Pain, 2004. 109(3): p. 409-419.

414. Mijnhout G.S., Alkhalaf A., Kleefstra N., and Bilo H.J. Alpha lipoic acid: a new treatment for neuropathic pain in patients with diabetes? The Netherlands journal of medicine, 2010. 68(4): p. 158-162.

415. Ho E.C., Lam K.S., Chen Y.S., Yip J.C., Arvindakshan M., Yamagishi S., Yagihashi S., Oates P.J., Ellery C.A., Chung S.S., and Chung S.K. Aldose reductase-deficient mice are protected from delayed motor nerve conduction velocity, increased c-Jun NH2-terminal kinase activation, depletion of reduced glutathione, increased superoxide accumulation, and DNA damage. Diabetes, 2006. 55(7): p. 1946-1953.

416. Purves T., Middlemas A., Agthong S., Jude E.B., Boulton A.J., Fernyhough P., and Tomlinson D.R. A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2001. 15(13): p. 2508-2514.

417. Price S.A., Agthong S., Middlemas A.B., and Tomlinson D.R. Mitogen-activated protein kinase p38 mediates reduced nerve conduction velocity in experimental diabetic neuropathy: interactions with aldose reductase. Diabetes, 2004. 53(7): p. 1851-1856.

418. Agthong S. and Tomlinson D.R. Inhibition of p38 MAP kinase corrects biochemical and neurological deficits in experimental diabetic neuropathy. Annals of the New York Academy of Sciences, 2002. 973: p. 359-362.

CITED LITERATURE (continued)

419. Sugimoto K., Murakawa Y., Zhang W., Xu G., and Sima A.A. Insulin receptor in rat peripheral nerve: its localization and alternatively spliced isoforms. Diabetes/metabolism research and reviews, 2000. 16(5): p. 354-363.

420. Sugimoto K., Murakawa Y., and Sima A.A. Expression and localization of insulin receptor in rat dorsal root ganglion and spinal cord. Journal of the peripheral nervous system : JPNS, 2002. 7(1): p. 44-53.

421. Wong Y.L. and Rice S.E. Kinesin's light chains inhibit the head- and microtubule-binding activity of its tail. Proceedings of the National Academy of Sciences of the United States of America, 2010. 107(26): p. 11781-11786.

422. Trinczek B., Biernat J., Baumann K., Mandelkow E.M., and Mandelkow E. Domains of tau protein, differential phosphorylation, and dynamic instability of microtubules. Molecular biology of the cell, 1995. 6(12): p. 1887-1902.

423. Lee K.D. and Hollenbeck P.J. Phosphorylation of kinesin in vivo correlates with organelle association and neurite outgrowth. The Journal of biological chemistry, 1995. 270(10): p. 5600-5605.

424. Matthies H.J., Miller R.J., and Palfrey H.C. Calmodulin binding to and cAMP-dependent phosphorylation of kinesin light chains modulate kinesin ATPase activity. The Journal of biological chemistry, 1993. 268(15): p. 11176-11187.

425. Hammond J.W., Griffin K., Jih G.T., Stuckey J., and Verhey K.J. Co-operative versus independent transport of different cargoes by Kinesin-1. Traffic, 2008. 9(5): p. 725-741.

426. Shumay E., Song X., Wang H.Y., and Malbon C.C. pp60Src mediates insulin-stimulated sequestration of the beta(2)-adrenergic receptor: insulin stimulates pp60Src phosphorylation and activation. Mol Biol Cell, 2002. 13(11): p. 3943-3954.

427. Rosenzweig T., Aga-Mizrachi S., Bak A., and Sampson S.R. Src tyrosine kinase regulates insulin-induced activation of protein kinase C (PKC) delta in skeletal muscle. Cell Signal, 2004. 16(11): p. 1299-1308.

428. Zhang B.B., Zhou G., and Li C. AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell metabolism, 2009. 9(5): p. 407-416.

CITED LITERATURE (continued)

429. Cameron N.E., Cotter M.A., Jack A.M., Basso M.D., and Hohman T.C. Protein kinase C effects on nerve function, perfusion, Na(+), K(+)-ATPase activity and glutathione content in diabetic rats. Diabetologia, 1999. 42(9): p. 1120-1130.

430. Eichberg J. Protein kinase C changes in diabetes: is the concept relevant to neuropathy? Int Rev Neurobiol, 2002. 50: p. 61-82.

431. Thornalley P.J. Glycation in diabetic neuropathy: characteristics, consequences, causes, and therapeutic options. International review of neurobiology, 2002. 50: p. 37-57.

432. Medina M. and Castro A. Glycogen synthase kinase-3 (GSK-3) inhibitors reach the clinic. Current opinion in drug discovery & development, 2008. 11(4): p. 533-543.

433. Martinez A., Castro A., Dorronsoro I., and Alonso M. Glycogen synthase kinase 3 (GSK-3) inhibitors as new promising drugs for diabetes, neurodegeneration, cancer, and inflammation. Medicinal research reviews, 2002. 22(4): p. 373-384.

434. Eldar-Finkelman H. Glycogen synthase kinase 3: an emerging therapeutic target. Trends in molecular medicine, 2002. 8(3): p. 126-132.

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Natalia Marangoni, Mariann Piano, Scott Brady: Mechanism of fast axonal transport deficits in sensory and autonomic diabetic neuropathies. Society for Neuroscience. Chicago, Illinois, United States, 2009

Natalia Marangoni, Scott Brady: Mechanism of Fast Axonal Transport deficits in diabetic neuropathies. 68th American Diabetes Association. San Francisco, California, United States, 2008

Ramona Pufan, Natalia Marangoni, Sarah Pollema, Ray Chavez, Tamie Chilcote, Gerardo Morfini, Scott T. Brady: An Isoform of Protein Kinase C is Critical in Parkinson's Disease Pathogenesis. Society for Neuroscience, San Diego, CA, United States, 2007

M.N. Marangoni, G. Morfini, Y, You, C. Bagnato, D. Han, S. Brady: Impairment of fast axonal transport in diabetic neuropathies and p38 activation, 67th American Diabetes Association. Chicago, IL, United States, 2007.

VITA (continued)

Y. You, G. Morfini, G. Pigino, S. Pollema, N. Marangoni, S. Brady: Neuropathogenic forms of androgen receptor inhibit fast axonal transport through activation of JNK3. Chicago Chapter Society for Neuroscience. Chicago, IL, United States, 2005

PUBLICATIONS

M. Armanino, M. C. Gravielle, M. N. Marangoni, M. L. Fiszman: NMDA receptors contribute to the survival promoting effect of high potassium in cultured cerebellar granule cells. International Journal of Developmental Neuroscience (6): 545-8, 2005

Maria Natalia Marangoni
July 20th 2011

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A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,
Maria Natalia Marangoni
8 Oriole Ct, 60540, Naperville, IL, USA

Figure 1. General features of insulin signal transduction pathways (page 465)

The above request is approved.
Approved by:_____
Date:_____

Maria Natalia Marangoni
July 20th 2011

Scott Brady
808 S Wood St, 60612, Chicago, IL, USA
stbrady@uic.edu

I am writing to request permission to use the following material from your publication Fundamental Neuroscience, 1998 in my thesis. This material will appear as originally published. Unless you request otherwise, I will use the conventional style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,
Maria Natalia Marangoni
8 Oriole Ct, 60540, Naperville, IL

The figure used is a modified version by Scott Brady of original drawing by Scott Brady for Fundamental Neuroscience, 1998. the figure name is Fast Axonal Transport

The above request is approved.
Approved by:_____
Date:_____

Maria Natalia Marangoni
July 20th 2011

World Health Organization press
World Health Organization,
20 Avenue Appia, 1211 Geneva 27,Switzerland
(tel.: +41 22 791 3264; fax: +41 22 791 4806;
e-mail: permissions@who.int)

I am writing to request permission to use the following material from your publication Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia:report of a WHO/IDF consultation, 2006 in my thesis. This material will appear as originally published. Unless you request otherwise, I will use the conventional style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,
Maria Natalia Marangoni
8 Oriole Ct, 60540, Naperville, IL

Appendix 4. Disorders of Glycaemia-aetiological types and clinical stages (page 38)

The above request is approved.
Approved by:_____
Date:_____

Maria Natalia Marangoni
July 20th 2011

Aaron Vinik
Strelitz Diabetes Research Institutes
855 W Brambleton Avenue,
Norfolk, VA 23510, USA
vinikai@evms.edu

I am writing to request permission to use the following material from your publication Diabetic neuropathies: clinical manifestations and current treatment options, 2006 in my thesis. This material will appear as originally published. Unless you request otherwise, I will use the conventional style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,
Maria Natalia Marangoni
8 Oriole Ct, 60540, Naperville, IL, USA

Figure 1. a simplified view of the peripheral nervous system (page 271)

The above request is approved.
Approved by:_____
Date:_____

Maria Natalia Marangoni
July 20th 2011

Eva Feldman
Departmentof Neurology, University of Michigan,
4414 Kresge III, 200 Zina Pitcher Place,
Ann Arbor, MI 48109, USA.
Tel: +1-734-763-7274;
Fax: +1-734-763-7275; E-mail: efeldman@umich.edu

I am writing to request permission to use the following material from your publication The role of growth factors in diabetic peripheral neuropathy, 2004 in my thesis. This material will appear as originally published. Unless you request otherwise, I will use the conventional style of the Graduate College of the University of Illinois at Chicago as acknowledgment.

A copy of this letter is included for your records. Thank you for your kind consideration of this request.

Sincerely,
Maria Natalia Marangoni
8 Oriole Ct, 60540, Naperville, IL, USA

Figure 2. Neurotrophi-signaling pathways in neurons (page 35)

The above request is approved.
Approved by:_____
Date:_____