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**Schwann cells mediate the impairment of neurite  
growth by increased VEGF secretion in DRG neurons  
exposed to hyperglycemia**

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## TABLE OF CONTENTS

CHAPTER 1 .....	4
1. Diabetic Polyneuropathy.....	4
1.1 Patophysiological features.....	6
1.2 Biochemical features.....	7
1.2.1 Polyol pathway.....	10
1.2.2 AGE pathway.....	11
1.2.3 Hexosamine pathway.....	11
1.2.4 PKC Activation.....	13
1.2.5 Additional effects of hyperglycemia.....	16
2. Sensory neurons.....	19
3. Schwann cells.....	23
4. Vascular Endothelial Growth Factor.....	25
4.1 VEGF in Peripheral Nervous System.....	29
5. Aim of the thesis.....	32
References.....	33
CHAPTER 2: Schwann cells mediate the impairment of neurite growth by increased VEGF secretion in DRG neurons exposed to hyperglycemia .....	43
CHAPTER 3: In vivo and ex vivo studies on peripheral neuropathy in Zucker rats: a type 2 rat model. Preliminary results.....	72
CHAPTER 4: Summary, future perspectives and conclusions.....	89

# **CHAPTER 1**

## **INTRODUCTION**

### **1. DIABETIC POLINEUROPATHY**

Diabetes is a serious systemic disease that carries the risk of multiorgan impairment triggered by sustained hyperglycemia. The rate of complications and their relationship with the metabolic control make it a challenging condition in clinical practice with significant impact on patients' morbidity and mortality (Cusick, et al., 2005; Stratton, et al., 2000). Type 2 is the most common form of diabetes and its prevalence is estimated to significantly rise worldwide over the next 2 decades mainly in the developing countries as a result of the increasing rate of obesity. Indeed, 60% of all cases of type 2 diabetes can be directly attributed to weight gain, and the relationship between familial behavior, obesity, and diseases is so strong that social interventional policies have been advocated (Yach, et al., 2006).

Microvascular complications involving retina, kidney, and peripheral nerves affect the majority of diabetic patients. In particular, the peripheral nervous system is a primary target of hyperglycemia. Somatic and autonomic neuropathy represents one of the most frequent and potentially invalidating complications of diabetes. Its prevalence ranges between 13% and 58% according to the definition of neuropathy, the methods used to assess it, the

duration of diabetes, and the presence of other complications, particularly nephropathy (Dyck et al. 1993; Dyck et al 1997; Melton et al. 1999; Partanen et al. 1995). The most common presentation is that of a length-dependent diabetic polyneuropathy (DPN). Primary axonal impairment mainly affects the longest nerves leading to chronic sensory disturbances in the feet including neuropathic pain. Autonomic dysfunctions such as impaired sweating, impotence, and gastrointestinal dysfunctions can occur since in the early stage of DPN though most commonly progress causing life threatening cardiovascular instability. About 20% of patients with autonomic neuropathy experiences clinically silent complications including orthostatic hypotension, myocardial infarction, gastroparesis, and increased bladder capacity with urinary retention.

DPN has a chronic course with late waste and weakness of muscles in the distal extremities. Foot ulcers are late complications mainly responsible for the four-fold increase of yearly health care costs of patients with diabetic neuropathy comparing with those without (Le et al., 2006). Indeed, 2-3% of diabetic patients develop a foot ulcer per year and nearly 15% of patients have at least one chronic ulcer in the lifetime. Disease modifying treatments would certainly reduce the impact of neuropathy in diabetes. Unfortunately, no drug have shown in randomized trials to restore DPN and only the intensive control of hyperglycemia proved to slow but not to halt its progression (Partanen et al., 1995) (group, 1998; Ismail-Beigi, et al., 2010; Martin, et al., 2006; Navarro, et

al., 1997; Ohkubo, et al., 1995). Moreover, the complex and still partly obscure mechanisms triggered by hyperglycemia reduces also the ability of axons to regenerate (Polydefkis et al., 2004). The dying back of peripheral nerves causes the chronic denervation of target organs (e.g. skin, muscles, and bones) and, most importantly, of Schwann cells which lose their ability to support axon regeneration. This could hinder the effect of potentially neuroprotective drugs in patients with longstanding DPN. Therefore, the effects of disease modifying treatments should be assessed in the very early stage of DPN when nerves potentially retain the ability to restore their functions.

### 1.1 Pathophysiological features

The pathology of diabetic neuropathy is characterized by progressive nerve fiber loss. Sensory deficits usually exceed motor nerve dysfunction and appear first in the distal portions of the extremities and progress proximally in a “stocking-glove” distribution with increasing duration or severity of diabetes (Edward et al., 2008). The nerve fiber degeneration is length-dependent, and its progression gives rise to positive and negative signs and symptoms. Positive symptoms include burning and paroxysmal pain, pins and needles, paresthesia, and aberrant, exaggerated sensitivity to normally painless or moderately painful stimuli (allodynia and hyperalgesia). Negative symptoms consist of loss of sensory perception in one or several modalities as

thermal hypoalgesia, loss of vibration, or pain sensation and numbness (Dobretsov et al., 2007). Motor symptoms, manifest as muscle weakness, occurs only in the most severe cases (Vinik et al., 2000). Small fibers are preferentially affected in early stages of diabetic in patients followed by the involvement of large fibers related to reduced nerve conduction velocity (NCV) (Yagihashi et al., 2007).

In diabetic nervous system beyond variable amount of axon degeneration there is evidence of segmental remyelination and demyelination. Fibre loss is primary and demyelination with remyelination is secondary (Feldman et al., 2004). In human sural nerve and skin biopsy samples it has been observed regenerating axons, but over the course of the disease, regeneration fails (Said et al., 2008). Reduced blood flow through loss of autonomic nerve functions may contribute to the progression of diabetic neuropathy and alterations in microvessels, similar to the pathogenic neovascularization described in diabetic retinopathy and nephropathy, also are observed in peripheral nerves (Zent et al., 2007).

### 1.2 Biochemical features

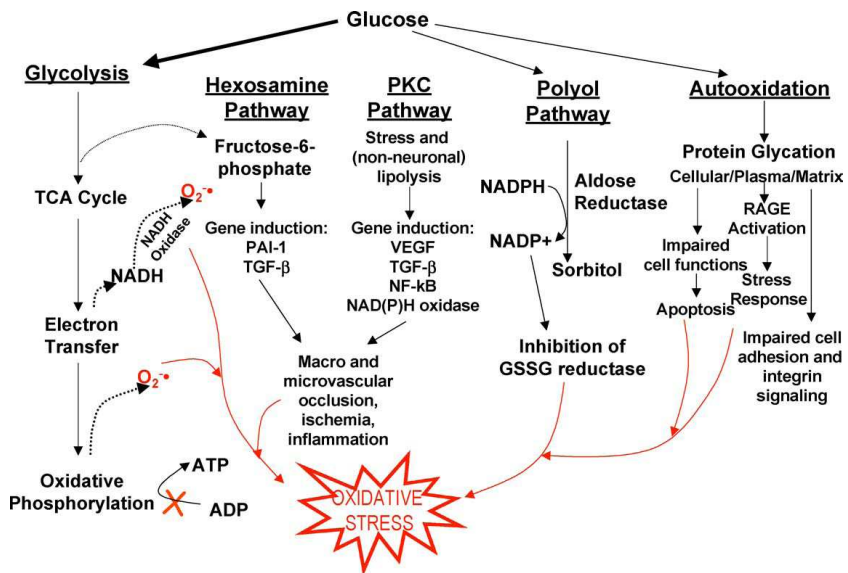
Although the pathogenesis of DPN is complex, hyperglycemia clearly plays a key role in its development and progression. DPN follows both type 1 and type 2 diabetes, and systemic hyperglycemia is the most obvious symptom that these types of the disease have in common (Vinik et al., 2004): both direct

glucose measures and levels of glycated hemoglobin correlate with the occurrence of this disease; insulin treatment to control hyperglycemia reverses some symptoms of DPN and delays its progression in general (Skyler et al., 2004, Sima et al., 2000). Furthermore data show that strict control of hyperglycemia in type 1 diabetes patients without clinical neuropathy decreased development of DPN in 60% of cases over 5 years of follow up study (Lasker et al., DCCT 1993). The Diabetes Control and Complications Trial (1995) established that hyperglycemia underlie neuropathy. Therefore investigations into the molecular and biochemical pathophysiology of diabetic neuropathy have focused on glucose metabolic pathways.

Intracellular glucose is principally removed through the process of glycolysis, which generates pyruvate for mitochondrial catabolism to form ATP. Excess pyruvate from glycolysis is thought to injure neurons. The overload of metabolites to the mitochondrial electron transfer chain leads to increased generation of reactive oxygen species, which inhibit the activities of key mitochondrial components such as aconitate hydratase and complex I, resulting in mitochondrial dysfunction. Excess pyruvate is shunted to the lactate pathway when oxygen is limiting, as this pathway yields NAD<sup>+</sup>, which is required for continued glycolysis. However, the lactate pathway is a temporary response to low oxygen: lactate cannot be further metabolized and must be converted back to pyruvate when oxygen levels recover. If lactate accumulates and NAD<sup>+</sup> is depleted, glycolysis is inhibited and neuronal functions

are impaired (Vincent et al., 2011).

If glycolysis does not adequately dispose of intracellular glucose, a number of alternative pathways are activated and they can be implicated in pathogenesis of diabetic complications: glucose flux through the polyol pathway; the hexosamine pathway; inappropriate activation of protein kinase C (PKC) isoforms; accumulation of advanced glycation endproducts.



**Fig. 1** Metabolic pathways favoured by raised glucose levels.

The normal end-point of glucose metabolism is the generation of ATP from ADP. In the presence of excess glucose ADP becomes rate-limiting, and the pathway becomes clogged. ROS then result at several points, including escape of electrons in the mitochondrial transfer chain to generate superoxide, and NADH oxidase is activated also producing superoxide. When glycolysis slows, fructose 1-6-bisphosphate is shunted into the hexosamine pathway that produces oxidative stress. Excess glucose is diverted to the polyol pathway as well as activating intracellular and extracellular glycation reactions.



### 1.2.1 Polyol pathway

Polyol pathway is a simple metabolic pathway that consist in reduction of glucose to sorbitol by the enzyme aldose reductase (AR), then oxidation of sorbitol to fructose by sorbitol dehydrogenase (SDH). Both of these enzymes are abundantly expressed in tissues inclined to diabetic complications. Under normoglycemic condition, glucose is metabolized to oxidative glycolytic pathway with phosphorylated oxidation through mitochondria, producing ATP energy by citric acid cycle. Under hyperglycemia, excessive glucose increased flux through the AR pathway causes increased intracellular sorbitol, that can also result in cellular osmotic stress that may alter the anti-oxidant potential of the cell and increase ROS, and compensatory efflux of other osmolytes such as myo-inositol (MI, important in signal transduction) and taurine (an antioxidant) (Nakamura et al., 1999; Vincent et al., 2004). Since NADPH is consumed by aldose reductase-mediated reduction of glucose to sorbitol (Jermendy et al., 1991; Brownlee, 2005) and NADPH is required for regeneration of reduced glutathione (GSH), this too contributes to oxidative stress. The second step in the polyol pathway oxidizes sorbitol to fructose via sorbitol dehydrogenase (Feldman et al., 1997). Formation of fructose promotes glycation as well as depletes NADPH. Thus, early after the induction of diabetes, metabolic defects lead to loss of NADPH, which limits the nerve's ability to scavenge reactive oxygen species, promoting a

vicious cycle of oxidative stress. NADPH in turn elicits impaired synthesis of NO or reduced glutathione, resulting in vascular insufficiency and overproduction of free radicals. Activation of aldose reductase may also increase formation of diacylglycerol, which activates the deleterious PKC pathway (Yamagishi et al., 2003; Uehara et al., 2004).

The aldose reductase pathway has been the target of multiple experimental and clinical studies. Aldose reductase inhibitors have been successful in experimental diabetes, however, they have not proven effective in clinical diabetes (Feldman et al., 2001; Obrosova et al., 2003).

Recent genetic studies showed that in humans, expression levels of AR in tissues are determined in part by gene polymorphism, gene promoter function or other epigenetic regulations, indicating the presence of complication-prone subjects related to AR gene (Demaine, 2003; Donaghue et al., 2005; Thamothersampillai et al., 2006).

### 1.2.2 AGE pathway

Advanced glycation is a nonenzymatic chemical modification of proteins, lipids and nucleic acids via attachment of glucose or other saccharides to exposed sites (Tattersall et al., 1993). While formation of a Schiff base and an Amadori product are reversible, formation of AGEs is irreversible.

In diabetes, the oxidizing environment and increased carbohydrate accumulation accelerate the formation of advanced

glycation end products (AGEs). Furthermore, clearance of AGEs from plasma is reduced in diabetic patients with renal impairment (Balakumar et al., 2010). AGE formation in cells leads to intra and extracellular cross linking of proteins and protein aggregation (Duran-Jimenez et al., 2009). These reactions require transition metal and ions as catalysts and depletion of the transition metal capacity in diabetes may lead to further AGE formation. Advanced glycation tends to decrease the biological function of proteins, thus inhibiting neuronal activity. Extracellular lipid and protein AGEs bind to a number of receptor proteins receptors, including the receptor for advanced glycation end products (RAGE) (Vincent et al., 2011). RAGE has multiple definite downstream signaling targets and is the main receptor through which AGE signaling is mediated. RAGE-AGE interaction activates the transcription factor nuclear factor kappa B (NF- $\kappa$ B) that regulates a number of activities including inflammation and apoptosis (Ramasamy et al., 2005). Activation of neuronal RAGE induces oxidative stress through NADPH oxidase activity (Vincent et al., 2007), and also increases nitrosative stress. Increased levels of AGE and RAGE are found in human diabetic tissue (Tanji et al., 2000). In experimental diabetes in rats and mice, the expression of RAGE is elevated in peripheral epidermal axons, sural axons, Schwann cells and dorsal root ganglia neurons. If AGE accumulation is blocked and/or AGE RAGE interaction is prevented there is a decrease in cellular oxidative stress and a restoration of nerve conduction deficits in the streptozotocin rat. It has also been showed that

genetic deletion of RAGE significantly reduces diabetic neuropathy in mice. This result suggest a new therapeutic target in humans, although the physiological role of RAGE is not known.

### 1.2.3 Hexosamine pathway

Excess glucose can also be shunted to the hexosamine pathway, in which the glycolytic intermediate fructose-6-phosphate is converted, via glucosamine-6-phosphate, to uridine diphosphate-Nacetylglucosamine (Brownlee et al., 2005). This molecule modifies serine and threonine residues of specific transcription factors, such as Sp1. These transcription factors are implicated in hyperglycemic inflammatory injury in endothelial basement membranes and pancreatic  $\beta$ -cells.<sup>37</sup> Further evidence suggests that Sp1 is activated in sural nerves of patients with diabetes.<sup>38</sup> Modulation of the hexosamine pathway can redirect glycolytic flow away from subsequent deleterious pathways.

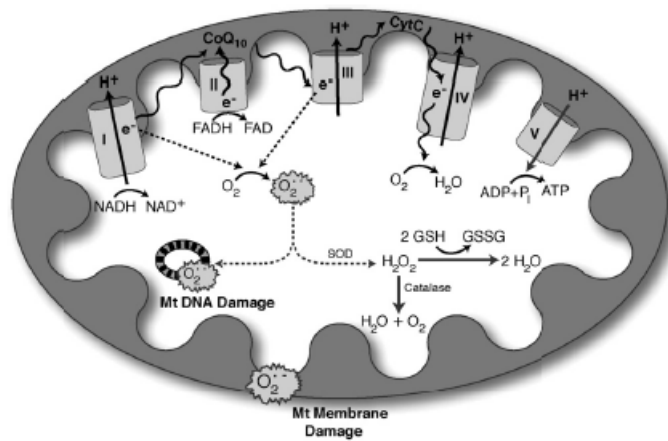
### 1.2.4 PKC Activation

It is generally held that the potential effects of the PKC pathway on the pathogenesis of diabetic neuropathy is more likely due to its contribution to vascular blood flow (Nishikawa et al., 2000). Activity of PKC is increased in the retina, kidney, and microvasculature of diabetic rats, but there is no evidence for altered activity of any of the PKC isoforms in the peripheral

neurons (Craven et al., 1989, Vincent et al., 2004). PKC is sensitive to a cell's redox status. Prooxidants react with the regulatory domain to stimulate PKC activity, but antioxidants react with the catalytic domain of PKC and inhibit its activity (Mullarkey et al., 1990). Once activated, PKC activates the MAPKs that phosphorylate transcription factors and thus alter the balance of gene expression of multiple cellular stress related genes such as c-Jun kinases and the heat shock proteins (Pugazhenti et al., 2003) These in turn can damage the cell. In streptozotocin diabetic rats, inhibition of PKC can normalize blood flow and nerve conduction deficits. There is currently an ongoing trial of a PKC blocker in the treatment of a separate microvascular complication of diabetes, retinopathy (Conn et al., 2001).

Each of these pathway may be injurious alone, collectively they cause an imbalance in the mitochondrial redox state of the cell and lead to excess formation of reactive oxygen species (ROS) (Kong et al., 1999; Vinik et al., 2003). The AGE and polyol pathways directly alter the redox capacity of the cell either through direct formation of ROS or by depletion of necessary components of glutathione recycling. The hexosamine and PKC pathways exhibit damage through expression of inflammation proteins. ROS, such as superoxide and hydrogen peroxide, are produced under normal conditions through the mitochondrial electron transport chain and are normally removed by cellular detoxification agents such as superoxide dismutase, catalase, and glutathione (Leininger et

al., 2006). Hyperglycemia leads to increased Mt activity, raising ROS production in the Mt. Excessive ROS formation eventually overloads the natural antioxidant capacity of the cell (generation of ROS may initiate a feed-forward cycle in which oxidative stress itself impairs antioxidative defense mechanisms), resulting in injuries to lipids, proteins, DNA. This damage ultimately compromises cellular function and integrity.



**Fig. 2** Oxidative stress and mitochondrial dysfunction (Leininger et al., 2006). Hyperglycemia increases production of reactive oxygen species (ROS) in mitochondria. NADH and FADH<sub>2</sub> produced from the tricarboxylic acid cycle transfer to the mitochondria, where they serve as electron donors to the mitochondrial membrane-associated redox enzyme complexes. The electrons (e<sup>-</sup>) are shuttled through oxidoreductase complexes I, II, III and IV (cytochrome c), until they are donated to molecular oxygen, forming water. The electron transfer into complexes I, III and IV by NADH (and FADH<sub>2</sub> via complex II to complex III) produces a proton gradient at the outer mitochondrial membrane, generating a potential between the inner mitochondrial membrane and outer mitochondrial membrane. This potential drives ATP synthesis, and is crucial for mitochondrial viability, function, and normal metabolism. As electrons are passed from complex II to complex III, however, ROS are produced as byproducts. The levels of ROS produced during normal oxidative phosphorylation are minimal, and they are detoxified by cellular antioxidants such as glutathione, catalase and superoxide dismutase. The hyperglycemic cell, on the other hand, shuttles more glucose through

the glycolytic and tricarboxylic acid cycles, providing the cell with an over-abundance of NADH and FADH<sub>2</sub> electron donors. This produces a high proton gradient across the inner mitochondrial membrane, which increases the turnover of the initial complexes, and thereby produces increased levels of radicals. Accumulation of these radicals, or ROS, is severely detrimental to mitochondrial DNA, mitochondrial membranes and the whole cell. Abbreviations: Cyto-c, cytochrome c; CoQ10, coenzyme Q10; e<sup>-</sup>, electrons; GSH

However mechanisms leading to diabetic neuropathy are more complex than increase oxidative stress. As a matter of fact antioxidants alone do not prevent this disorder and even if there are data from preclinical and clinical studies showing that in diabetes, oxidative and nitrosative stress are increased in plasma and tissues (Cheng et al., 2003; Lupachyk et al., 2011), others studies suggest that hyperglycemia does not increase oxidative stress in the dorsal root ganglia (Vincent et al., 2011).

#### 1.1.4 Additional effects of hyperglycemia

Besides metabolic alteration, hyperglycemia is thought to induce neurotoxicity through others pathways. Hyperglycemia lead to neuron injury also by depletion of myoinositol, an important membrane constituent. Coupled with oxidative injury to membrane lipids secondary to increased reactive oxygen species, the decrease in myoinositol could lead to membrane composition changes altering the function of membrane-bounds proteins, such as protein kinase C, or a disturbance in ion channel

function.

Microvascular insufficiency is also thought to play a role in diabetic neuropathy. This is based on histopathologic changes noted in endoneurial and epineurial blood vessel walls, as well as functional changes, such as altered blood flow or permeability of the microvasculature of the nerves in diabetic patients.

An immunologic pathogenesis for DPN has also been suggested by the occurrence of various autoantibodies, including antiphospholipid antibodies, more commonly in diabetic patients with neuropathy than those without neuropathy (Gomes et al., 2003). The presence of endothelial inflammatory infiltrates, noted on biopsy of the intermediate femoral cutaneous nerve, may correlate with inflammatory vasculopathy as a part of the neural injury in that syndrome.

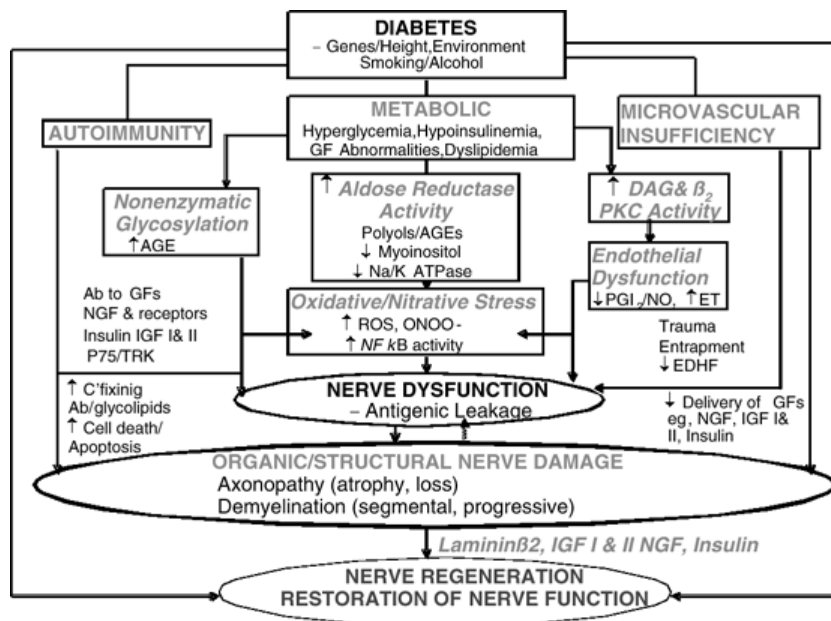
Alterations in the synthesis and expression levels of growth factors in the peripheral nervous system may account for the vulnerability of neurons and Schwann cells to the diabetic state, contributing in the pathogenesis of diabetic neuropathy (Leininger et al., 2004). Growth factors are critical mediators that direct the interplay of molecular signals operative in neuronal and glial differentiation and in re-establishing appropriate axon-glial interactions necessary for neuronal regeneration. Neurotrophins promote neuronal survival by inducing morphological differentiation and stimulating neurotransmitter expression (Apfel et al., 1999). Many of the changes seen in DN mimic those seen with depletion of endogenous growth factors. It has actually been observed a



decrease of availability of neurotrophins and an altered production of other growth factors such as insulin-like growth factor 1 (IGF-1), glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor in DPN. Studies in the last decade indicated that Nerve growth factor (NGF) that is a well characterized neurotrophin that plays an important role in the adult peripheral nervous system, was significantly reduced in the peripheral nerves of streptozotocin (STZ)-induced diabetic rats but in diabetic patients results are somewhat contradictory: although dermal NGF protein levels are reduced in patients with diabetes, sensory fiber dysfunction, skin mRNA NGF and NT-3 are increased. It has also been observed an increase of expression of TrkA (NGF-receptor) and trkC (NT-3 receptor) in the skin of patients with diabetes (Malik et al., 2007), whereas a phase II clinical trial of recombinant human nerve growth factor demonstrated some relief of the sensory deficits of distal sensorimotor neuropathy, a phase III trial failed to demonstrate a significant benefit in patients receiving recombinant human NGF versus placebo (Apfel et al., 2000). Some of the limited success in the clinical trials of NGF therapy for DPN is attributed to our poor understanding of how diabetes-induced changes in the neuronal or glial proteome may alter the predicted response to growth factor administration (Dobrowsky et al., 2000). Furthermore it must be considered that number of neuronal growth factors share downstream signaling cascades that promote survival and outgrowth, but any presumed efficacy in treating DPN would depend on whether the

involved neurons express relevant receptors. For example, in diabetes it is unlikely that a single growth factor such as NGF protects all types of neurons against damage because most cases of DPN involve both large- and small-fiber involvement (Zochodne, 2007).

To aid in predicting cellular responses to growth factors and enhancing their potential clinical effectiveness in DPN, it is important to determine whether diabetes alters normal signal transduction events in neurons and but also in glia. Neurons are often interdependent on Schwann cells (SCs), which also undergo substantial degenerative changes in DPN and respond to many growth factors.



**Fig. 3** Theoretic framework for the pathogenesis and treatment of diabetic neuropathy (Vinik et al., 2004)

## 2. SENSORY NEURONS

The peripheral nervous system (PNS) by definition includes sensory, motor and autonomic nerves that lie outside brain and spinal cord. Peripheral nerves are complex structures consisting of motor and sensory neurons, their axons, and the cells that support them, like Schwann cells, vascular cells (endothelial and smooth muscle cells) and endoneurial macrophages. The afferent branch, sensory nerves that originate from DRG neurons, has unipolar neurons with a single axon stem bifurcating into a peripheral branch that goes to the distal tissues, to periphery and a central branch that goes to dorsal horn, differing from the typical axon-dendrite structure of other neurons. The efferent arms of the PNS include motor neurons and autonomic neurons that originate in the spinal cord or autonomic ganglia and send branches to the skeletal muscle and internal organs, respectively. All components of the PNS can be affected by diseases of the PNS, commonly known as peripheral neuropathies; but the sensory neurons have anatomic and structural characteristics that make them particularly vulnerable to toxic conditions, being affected early and more severely. This is often evident in the constellation of symptoms that the patients experience; sensory symptoms such as pain, paresthesias, loss of perception and gait imbalance often dominate patients'

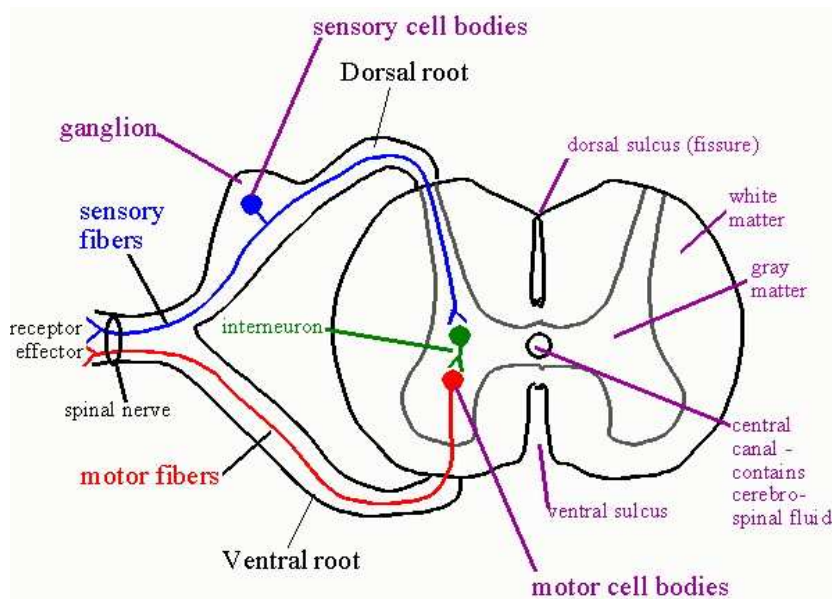
complaints and occur either in isolation or before weakness and autonomic symptoms (Melli et al., 2009).

Primary sensory neurons have large, roughly round, cell bodies (somas) bundled together (with satellite cells) in dorsal root ganglia. These ganglia and their cell bodies lie parallel to, but outside of the spinal cord. DRG exist outside the protection of the blood nerve barrier, (Devor et al., 1999) and lack the added protection afforded by the choroid plexus. Choroid plexus protects central neurons generating cerebral spinal fluid. The cells of plexus control the constituents of cerebrospinal fluid and maintain the ionic and metabolic milieu necessary for neuronal activity (Redburn et al., 1998) Even under diabetic conditions glucose concentrations of cerebrospinal fluid are typically lower than those in either serum or peripheral extracellular fluid.

In addition, sudden alterations in serum and tissue bed pH, though reflected in cerebrospinal fluid, occur more gradually than in the periphery. Only the proximal axon that enters the dorsal horn lies within the blood nerve barrier. Thus, somas and peripheral axons of DRG neurons are exposed to osmotic, pH, and other imbalances caused by diabetes including high glucose concentrations that diffuse freely across vascular and tissue beds.

DRG neuron structure imposes high metabolic demands that may be easily underappreciated. The axons of these neurons can be over 1.5 m in length , they are mitochondria-rich and directly access the nerve blood supply. Cell bodies range between 25 and 50 um in diameter. DRG neurons with these dimensions have

99.8% of their cytoplasm in the axon and all nuclear genetic information and much of its synthetic machinery in the remaining 0.2% of total cell volume (McHugh et al., 2004). DRG neurons must regularly replace and recycle worn membrane bound structures and cytoplasmic proteins from throughout the cell. In addition, information from peripheral tissues must transit the length of the axons to the nucleus in order to ensure appropriate gene transcription and protein manufacture for long-term homeostasis. After signals are received in the nucleus and necessary genes are transcribed, newly synthesized proteins must be transported over these extreme distances and inserted into correct locations along axon membranes (McLean et al., 2007). This morphology places extreme spatial demands on DRG survival functions. Furthermore glucose uptake is less rapidly regulated in neurons than in endothelial cells, which may account for the high susceptibility of neurons to glucose-mediated injury (Vincent et al., 2011).



**Fig. 4** Spinal cord cross-section .The soma of sensory neurons are found in the dorsal root ganglia. Sensory axons enter the spinal cord through the dorsal root and often synapse on interneurons in the gray matter. Motor neuron cell bodies are located in the gray matter and the axons leave the spinal cord through the ventral root. Spinal nerves are formed when the dorsal and ventral roots join together.

### 3. SCHWANN CELLS

Schwann cells (SCs) are the main glial cells of the vertebrate peripheral nervous system. Along the entire length of peripheral nerves, axons of motor, sensory, and autonomic neurons are in close association with SCs, being covered in most of their surface by them. Axons and these glial cells are in intimate physical contact and in constant and dynamic communication. Development, function and maintenance of the peripheral

nerves are crucially dependent on controlled bi-directional signalling between axons and SCs. In the mature nervous system, Schwann cells can be divided into four classes: myelinating cells (MSCs), nonmyelinating cells (NMSCs), perisynaptic Schwann cells (PSCs) (also known as terminal Schwann cells), and satellite cells of peripheral ganglia. These classes are based on their morphology, biochemical makeup and the neuronal types (or area of their axons) with which they associate. All of them derive from immature SCs emanating from the neural crest via intermediate SC precursors (Bhatheja et al., 2006). MSCs, the best characterized SC, wrap around all large-diameter axons, including all motor neurons and some sensory neurons. Each MSC associates with a single axon and creates the myelin sheath necessary for saltatory nerve conduction. NMSCs associate with small-diameter axons of C-fibers emanating from many sensory and all postganglionic sympathetic neurons. Each NMSC wraps around several sensory axons to form a Remak bundle, keeping individual axons separated by thin extensions of the Schwann cell body. Located more peripherally, PSCs reside at the neuromuscular junctions (NMJ), where they cover, without completely wrapping around, the presynaptic terminal of motor axons. Satellite cells associate with neuronal cell bodies in peripheral ganglia (Corfas et al., 2004). Myelinating SCs ensheath individual large-caliber axons ( $>1 \mu\text{m}$ ) and deposit a myelin envelope, while non-myelinating SCs embed bundles of small-caliber axons. Peripheral nerve fibres (axon-SC units) are surrounded further by a coat made from collagen fibres

and a cellular tube, the perineurium. Recent studies revealed the existence of sensory and motor phenotype SCs, which define the modality of specific axonal regeneration (Hoke et al., 2006; Moradzadeh et al., 2008). SCs can react promptly and strongly to local environmental stimuli by secreting many different growth promoting factors. These multifunctional cells can also synthesize, secrete and express many neurotrophic, neurotropic, neurite promoting factors, major myelin glycoproteins, cell adhesive molecules (CAMs), basement membrane components as well as many receptors at various stages of life (Heumann et al., 1987; Houle, 1992; Shibuya et al., 1995). Abnormalities of SCs under various conditions can cause nerve dysfunctions, such as reduced nerve conduction velocity, axonal atrophy and impaired axonal regeneration (Dyck and Giannini, 1996; Song et al., 2003; Yasuda et al., 2003). SC defects may be responsible for abnormalities in peripheral neuropathies.

Evidence of Schwann cell involvement in diabetic neuropathy has been observed in experimental diabetes with ultrastructural studies: decrease in axon caliber, segmental demyelination and onion bulb formation (an index of excessive SC proliferation), as well as by expression of aldose reductase in these cells, suggest a role for SC in diabetic neuropathy.

In vitro studies showed apoptosis in Schwann cells after exposure to very high concentration (150mM glucose) of glucose (Delaney et al., 2001), but only some Schwann cells showed apoptotic responses in vivo (Kalichman et al., 1998).



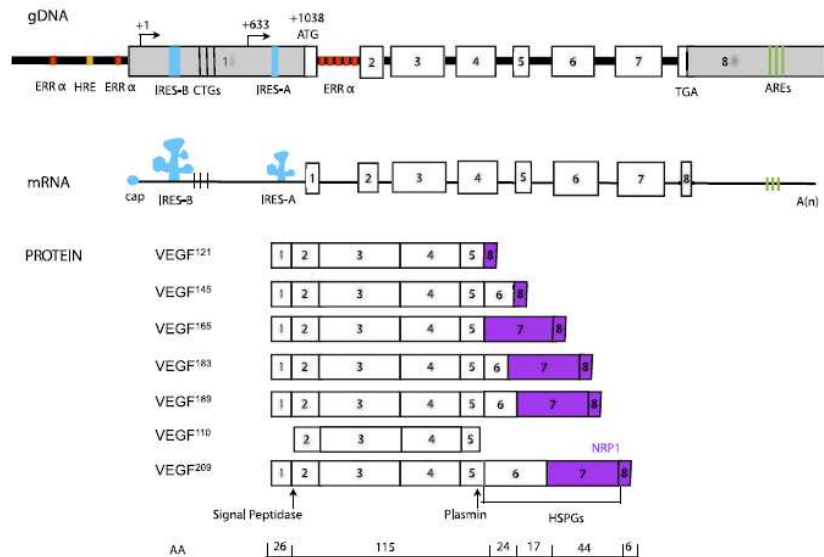
Few studies have been reported on how hyperglycaemia affects the supporting role of SC in regeneration of axons in cultured sensory neurons and it is still not well understood which cell type is predominantly affected by hyperglycaemia and if the effect on neuron and on Schwann cells is direct and cells autonomous.

#### 4. VASCULAR ENDOTHELIAL GROWTH FACTOR

Since its initial discovery in 1983 (Senger et al., 1984) and subsequent cloning of the gene in 1989 from pituitary cells (Leung et al., 1989; Keck et al., 1989), vascular endothelial growth factor (VEGF-A, VEGF or vascular permeability factor) has been established to be an essential regulator of developmental, physiological and pathological (in a variety of common chronic human diseases) angiogenesis (Ferrara et al., 2003), stimulating proliferation and migration of endothelial cells and enhancing vascular permeability.

VEGF-A is the founding member of a family of homodimeric glycoproteins that are structurally related to the platelet-derived

growth factors (PDGF) family, that is characterized by the presence of eight conserved cysteine residues and a cystine knot structure. Human VEGF gene is localized on chromosome 6p2q1.3 and contains eight exons. VEGF is highly conserved across species. Through alternative RNA splicing, several VEGF isoforms are generated. All VEGF isoforms are secreted as covalently linked homodimers but display differences in the basic amino rich domains encoded by exon 6 and 7. All transcripts contain exons 1– 5, encoding the signal sequence and core VEGFR-binding or VEGF/PDGF homology domain, and exon 8, with diversity generated through the alternative splicing of exons 6 and 7. Exon 6 encodes a heparin-binding domain, while exons 7 and 8 encode a domain that mediates binding to neuropilin-1 (NP1) and heparin. The VEGF<sub>121</sub> isoform (121 amino acids in humans and 120 in mice; VEGF isoforms have 1 less amino acid in mice) is freely diffusible, since it lacks the basic amino acid residues responsible for heparin binding and, therefore, does not, or only minimally, bind to the extracellular matrix (ECM).



**Fig. 5** Transcriptional and posttranscriptional regulation of vascular endothelial growth factor (VEGF). Transcription of VEGF can start from either the P1 TATA less promoter (at nucleotide position +1) or the internal promoter (at nucleotide position +633). Hypoxia and nutrient deprivation enhances VEGF transcription through hypoxia-inducible transcription factor (HIF)-1 $\alpha$  (by binding to the HRE, which is indicated with an orange box in the P1 TATA less promoter) or alternatively through PGC-1 $\alpha$  [by coactivating the orphan nuclear estrogen-related receptor- $\alpha$  (ERR $\alpha$ ) on conserved binding sites found within the promoter and the first intron (red boxes)]. VEGF mRNA stability is also regulated through stress-induced factors, that bind to adenylate-uridylate-rich elements (AREs) in its 3'-untranslated (UTR) region (green stripes). The 5'-UTR contains two IRES (A and B) (blue) that ensure efficient initiation of the translation under stress condition, thus bypassing the 5' cap recognition required for the assembly of the translation initiation complex. However, cap-dependent hypoxia-regulated translation mechanisms also exist. Three in-frame CUGs translation initiation codons can also initiate the synthesis of a long VEGF (L-VEGF). Normally, translation starts however from the canonical ATG. Maturation in the endoplasmic reticulum generates the different VEGF isoforms. Three coding exons are alternatively spliced and give rise to, at least, six VEGF isoforms that possess different affinity to heparin sulfate proteoglycans (HSPG) depending on the absence or presence of one or two HSPG binding sites. All VEGF isoforms can be cleaved by plasmin to generate a 110-amino acid-long VEGF. Exons 7 and 8 (indicated in purple) code for the regions described to bind NRP (Ruiz de Almodovar et al., 2009).

The other members of the VEGF family include placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (and others viral forms). Each of these family members is characterized by the presence of eight conserved cysteine residues, which form a typical cysteine-knot structure (Robinson et al., 2001). All VEGF family members are able to regulate angiogenesis, and in addition, VEGFs C and D are implicated as biologically important mediators of lymphangiogenesis; however, in contrast to VEGF-A, the precise biological roles of other VEGFs are not yet fully understood.

The expression of VEGF is tightly regulated. Molecular studies reveal that VEGF expression is regulated at the transcriptional and post-transcriptional levels. Regulatory factors for VEGF expression can be divided into two main categories: hypoxia through hypoxia-inducible factor (HIF)-1 and a group of many cytokines, growth factors and transcription factors other than HIF-1.

The biological functions of VEGF-A are mediated via the protein tyrosine kinase receptors, VEGFR2 fetal liver kinase receptor (KDR/Flk-1) and VEGFR1 fms-like tyrosine kinase receptor (Flt-1) (Zachary et al., 2003; Ferrara et al., 2003). VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF (Cross et al., 2007). The function of VEGFR-1 is less well-defined, although it is thought to modulate VEGFR-2 signaling. Certain VEGF isoforms bind to neuropilins (NPs), non-tyrosine kinase transmembrane receptors. Whereas binding of VEGF to

Nrps enhance VEGF's action, binding to VEGFR1 results in a diminution of VEGF availability. Therefore, it is possible that the tissue ratios of VEGFR1:VEGFR2 determine the tissue's angiogenic status. For instance, under hypoxic conditions, when VEGF is upregulated, concomitant upregulation of VEGFR1, may act to modulate VEGF signalling (Maharaj et al., 2007).

After binding, soluble VEGF dimmers induce VEGF receptor dimerization leading to homo- or heterodimers (Barrit et al., 2006). The juxtaposed cytoplasmic tyrosine kinase domains of the VEGFR molecules, transphosphorylate several tyrosine residues in neighbour molecule. Activated receptors in turn activate proteins of different signaling pathways by phosphorylation: phospholipase c (PLC), phosphatidylinositol 3'-kinase/AKT, Ras GTPase activating protein (GAP), Src family (Dufour et al., VEGF signaling)

#### 4.1 VEGF in PNS

Neural and glial cells express one or more of the VEGF receptors (e.g., VEGFR-1, -2 and neuropilin-1) and can thus directly respond to VEGF released by neighbouring neural cells. VEGF acts on Schwann cells via high-affinity binding to both membrane-spanning tyrosine kinase receptors, flk-1 andflt-1 (Williams et al., 1992).

Schwann cells themselves may be the principal source of VEGF in peripheral nerves. Neuropilin-1 is on the cell surface of both

neurons and endothelial cells, and is functionally implicated in both the guidance of neural growth cones and angiogenesis.

In the peripheral nervous system, some vessels and sensory nerves migrate along the same path and track alongside each other being intimately associated. Coculture experiments of endothelial cells with dorsal root ganglia (DRG) neurons and Schwann cells demonstrate that both cell types promote arterial differentiation of ECs through release of VEGF (Anderson et al., 2002 ). It has also been observed that both nerve- and Schwann cell derived VEGF is required to induce arterial differentiation of these vessels (Anderson et al., 2005) and vessels release guidance cues, such as VEGF, artemin, neurotrophin- 3, or endothelin-3 to attract axons to track alongside the vessels (Ginty et al., 2008). Recent observations indicate that VEGF in nervous system has significant nonvascular functions, with a direct effects on neurons and glial cells stimulating their growth, survival, and axonal outgrowth (Carmeliet et al 2002). It prolongs the survival and stimulates proliferation of Schwann cells in explants of superior cervical ganglia (SCG) and DRG (Isner et al., 2000). Even though Schwann cells express VEGFR-1, VEGFR-2, and NRP1, the effect of VEGF on their migration appears to be mediated predominantly by VEGFR-2. Nonetheless, the precise role of VEGF in peripheral innervation remains incompletely understood.

A transient increase in the transcriptional regulator hypoxia-inducible factor-1 alpha and a number of its target genes including VEGF and erythropoietin has been demonstrated recently in

diabetic rats (Chavez et al., 2005). Similarly in the STZdiabetic rat intense VEGF staining has been shown in cell bodies and nerve fibers compared with no or very little VEGF in controls and animals treated with insulin or NGF (Samii et al., 1999). A recent study showed that diabetic patients with DPN have a significant higher level of serum VEGF in comparison with those without DPN (Arimura et al., 2009).

## **AIM OF THE THESIS**

This project focuses on understanding how hyperglycemia and diabetes lead to cell body and axon injury in the peripheral nervous system and on discovering pathways potentially involved in counteracting the pathogenetic cascade. We used an in vitro model to identify the principal targets of glucose-induced neuronal or axonal damage through different approaches, including the quantification of apoptosis and axonal growth. We also investigated the effect of hyperglycemia on Schwann cells with the aim to clarify their role in the pathogenesis of neuronal and axonal damage.

In the second part of the thesis we evaluated if Zucker diabetic fatty (ZDF) rat is a valuable model for study diabetic peripheral neuropathy, in order to validate our in vitro findings in an in vivo model.



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## CHAPTER 2

Schwann cells mediate the impairment of neurite growth by increased VEGF secretion in DRG neurons exposed to hyperglycaemia

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### **Abstract**

Diabetic polyneuropathy (DPN) causes axonal injury and Schwann cells (SCs) might be crucial in this process. We investigated the direct interaction between SCs and dorsal root ganglion (DRG) neurons under hyperglycaemic condition and the effects of Schwann cell-conditioned media on neurite outgrowth of DRG neurons. We observed that high glucose impaired axonal growth in neuron-Schwann cells co-culture and in neuron monoculture exposed to the medium obtained from Schwann cells cultured in high glucose. We found that VEGF concentrations were significantly higher in Schwann cells-media under the high glucose condition than in those under control condition. The reduced neurite outgrowth observed in hyperglycaemic co-culture was inhibited by VEGF neutralizing antibody. Our results suggest that an increase of VEGF secretion by SCs under

the diabetic condition would cause a defect of axonal regeneration, resulting in the development of diabetic neuropathy.

### **Introduction**

Diabetic polyneuropathy (DPN) is a chronic complication of both type I and type II diabetes mellitus. It increases morbidity and mortality and severely impairs the quality of life (Vinik et al., 1995; Galer et al., 2000; Tesfaye et al., 2005). DPN can compromise ability to perceive tactile sensation and can induce abnormal sensitivity to nociceptive (hyperalgesia or hypoalgesia) and non-painful normal stimuli (allodynia). The pathogenesis of DPN likely involves different pathways including metabolic, vascular, and growth factor deficiency, and extracellular matrix remodelling. Hyperglycaemia (Dobrestov et al., 2007) is considered the trigger for DPN and its poor control is the main risk factor for DPN (Martin et al., 2006; Perkins et al., 2001; Trial, 1996). DPN is characterized by a distal axonopathy with dying back degeneration (Greenbaum et al., 1964; Sima et al., 1983; Zochodne et al., 2001; Kamiya et al., 2006) and reduced ability of nerve regeneration (Dyck et al., 1986; Ekstrom and Tomlinson, 1989; Kennedy and Zochodne, 2000, 2005; Sharma and Thomas, 1975; Sima et al., 1988). However, is still unclear if the

physiopathologic process primarily affects the axon or the cell body.

Diabetes is a complex and multifactorial disease and *in vitro* cell culture models are used to investigate the specific behaviour of neuronal cells and axons independently from systemic influences. Although this setting does not reproduce the physiological condition, it allows to examine specific pathways potentially involved in the damage caused by hyperglycemia. Dorsal root ganglion (DRG) sensory neurons are the main cellular target of diabetic neurotoxicity. They are particularly vulnerable to toxic agents because lack the blood brain barrier and are supplied by fenestrated capillaries that allow free passage of circulating substances. Furthermore they have long axons rich in mitochondria and are particularly susceptible to any interference of energy metabolism, mitochondrial function, oxidative stress, and axonal transport. Most of the studies that have investigated the effect *in vitro* of hyperglycaemia have used embryonic DRG neurons. They need neurotrophins for the survival in culture and an high basal concentration of glucose (25 mM). Embryonic DRG neurons are particularly vulnerable to the lack of trophic support since they are harvested during the period of programmed cell death (Patel et al., 2000; Snider and Silos-Santiago, 1996). Conversely, adult DRG neurons, mature and completely developed, are independent from neurotrophin and can survive in 5mM glucose because they develop mechanisms protective from apoptotic death (Benn et al., 2002; Vogelbaum et al., 1998). This can explain the

controversial results obtained using in vitro embryonic or adult models rather. The advantage of the embryonic model is that it allows to obtain a significantly higher number of cells than using adult DRG. Moreover they are relatively easy to obtain and to keep alive. The high amount of cells and their resistance make this model suitable for the study of neuronal death and axonal degeneration and the screening of potential neuroprotective agents. Most studies on in vitro diabetic neuropathy focused on DRG neurons and little is known on the role of Schwann cells, which are in close relationship with axons in a reciprocal interaction necessary for their normal functions, and possibly involved in endogenous neuroprotective pathways (Hoke et al., 2006). During regeneration, Schwann cells receive signals from the axons and regulate their function through the secretion of various factors (Chen et al., 2005; Hoke et al., 2006; Jessen and Mirsky, 1999; Nave and Salzer, 2006; Triolo et al., 2006). Only few studies have investigated if and how hyperglycaemia affects the supporting role of Schwann cells in cultured DRG neurons. In vitro monoculture of DRG neurons and Schwann cells could allow to establish the role of each cell type and the relationship between them in the presence of hyperglycaemia, in order to better understand the contribution to the pathogenesis of diabetic neuropathy. The present study aims to investigate if hyperglycaemia primarily affects DRG neurons or axons, and what is the role of Schwann cells. The identification of pathways specifically involved in the development of the neuronal damage could contribute to the

knowledge on the pathophysiology of diabetic neuropathy and reveal potential targets for its treatment in patients.

### **Methods**

All procedures involving animals were performed according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

**Cell culture.** Primary DRG culture were freshly isolated from embryonic age day-15 rats. Dissected embryonic DRG were enzymatically dissociated with 0.25% trypsin in L-15 medium. Dissociated cells were plated in 24-well plates on collagen-coated glass coverslips, pretreated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO). Culture prepared according to this protocol contain mainly sensory neurons and Schwann cells (Keswani et al., 2003). Mono-culture of DRG neurons were obtained after exposition to ARA-C (10  $\mu$ M). Embryonic culture were maintained in Neurobasal medium (Gibco Invitrogen, Grand Island, NY) (that contains 25mM glucose), supplemented with 1xB27, penicillin (1 U/L), streptomycin (1 U/L), and nerve growth factor (10 ng/ml). Schwann cells monocultures were obtained from 2 days-old rat sciatic nerves and purified using a modified Brockes' method. Purified Schwann cells were maintained in DMEM 10%

FBS, neuregulin (20 ng/ml) and forskolin (2  $\mu$ M). 24 hours before the experiments, medium were changed to neuronal medium.

High glucose condition was obtained (where is not specify) adding 20mM glucose to a final glucose concentration of 45mM for embryonic cultures. Co-cultures and monocultures were exposed to cisplatin or taxol (10  $\mu$ g/ml and 250 ng/ml for 24 hours, respectively) as positive control. Paclitaxel and cisplatin were purchased from Sigma-Aldrich (St.Louis, MO). Schwann cell conditioned media was collected after 24 hours treatment, than added to neuron monoculture or analyzed by cytokine array. Culture were exposed to rVEGF (50-100ng/ml) for 24 hours (R&D System, Minneapolis, MN), anti-VEGF antibody (0,25 mg/ml) (R & D Systems, Minneapolis, MN) and NGF (50-100ng/ml) in neuroprotection assays.

***Assessment of axonal outgrowth.*** After 24 hours exposure to treatments, cells were fixed in 4% paraformaldehyde and stained with fluorescent antibody anti-BIII tubulin (TuJ1, Berkley Antibody Company, Richmond, CA). Neurites elongation were assessed by measuring the longest neurite on each of at least 100 randomly selected neurons per condition and using an image analysis system on fluorescence microscope. (Image Pro-Plus, Media Cybernetics, Silver Spring, MD) on fluorescence microscope as previously described (Keswani et al., 2003 ). Each condition was assessed in duplicate for at least three time.



***Assessment of neuronal apoptosis.*** Cells were fixed and stained with In situ DNA nick labelling (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, WI). Cells were co-stained with DAPI and tuJ1 for neuron identification or with anti-gial fibrillary acidic protein (GFAP, Dako, Glostrup, Denmark) antibodies for Schwann cells identifications and apoptotic cells were counted using a fluorescence microscope and expressed as percentage of the total number of cells present in each culture. Apoptosis were also evaluated with Annexin V/PI assay (Immunostep, Salamanca, Spain), staining the cells according to manufacturer's instructions and analyzed by flow cytometry. Early apoptosis and late apoptosis were evaluated on fluorescence 2 (for propidium iodide) versus fluorescence 1 (for annexin) plots. The percentage of cells stained with annexin V only was evaluated as early apoptosis; the percentage of cells stained with both annexin V and propidium iodide was evaluated as late apoptosis.

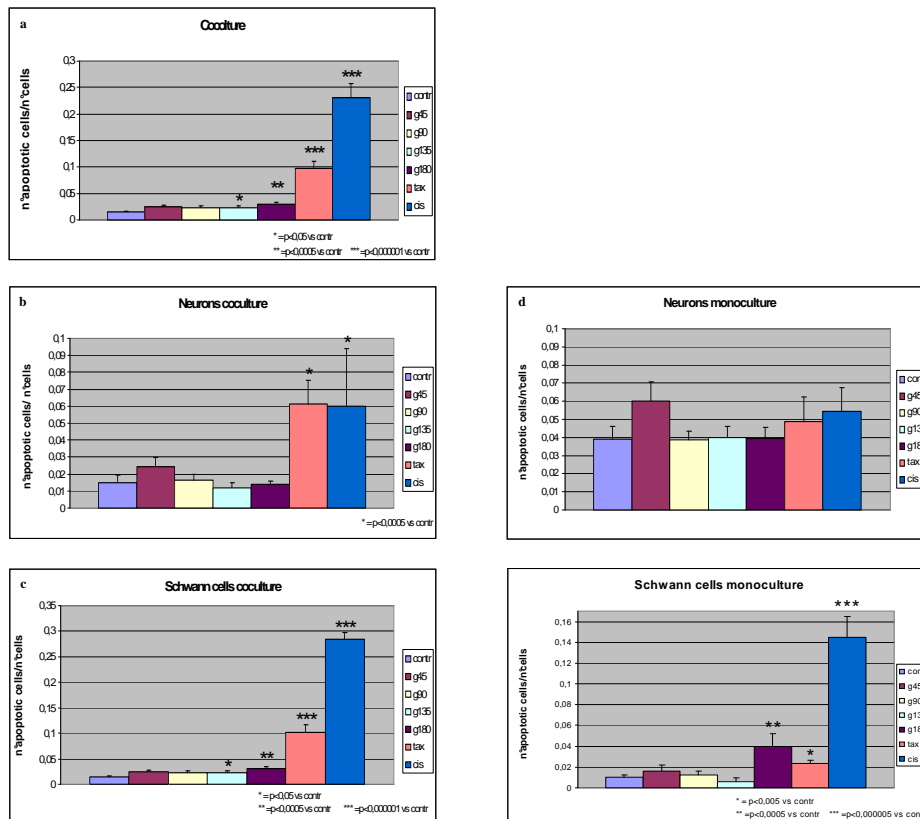
***Rat cytokine array.*** The cytokine profile expression in Schwann cells conditioned media were evaluated using the semiquantitative RayBio™ Rat Cytokine Antibody Array 1 (RayBiotech, Norcross, GA, USA), which detects 19 growth factors, cytokines, and chemokines, following the manufacturer's recommendations. The signals were visualized using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Spot densities were compared using Image J software

***Quantification of secreted VEGF protein levels by ELISA.***

Enzyme-linked immunosorbent assay (ELISA) were performed with a commercial VEGF ELISA kit (R&D Systems, Minneapolis, MN). Conditioned medium was collected from wells after 24 hours treatment. Assays were performed in duplicate, and values were compared with standard curves obtained with human recombinant VEGF165, provided by the kit.

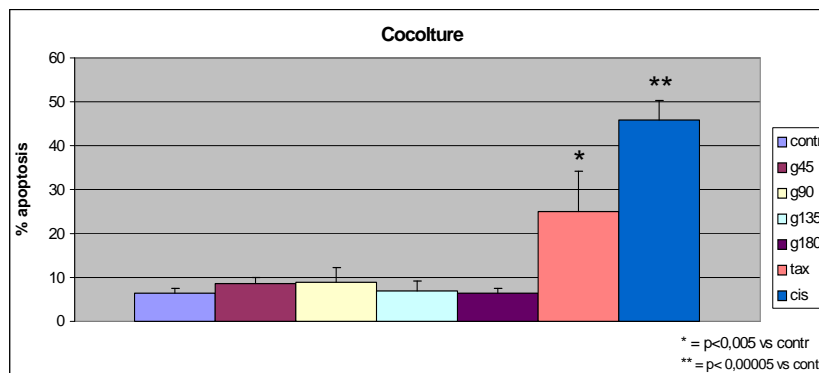
**Results**

***Hyperglycemia did not increase apoptosis of DRG neurons and Schwann cells.*** Embryonic (E15) DRG co-cultures and neuron and Schwann cell monocultures were exposed to increasing glucose concentrations (from 45 mM to 180 mM) in NB medium in presence of B27 without antioxidant. This medium allow the survival for some days of both neuron than Schwann cells. Co-cultures and monocultures exposed to cisplatin or taxol (10 µg/ml and 250 ng/ml respectively) were used as positive control (concentrations are in accordance with previous in vitro studies (Ta et al., 2006 and Wang et al., 2004). Apoptosis was investigated after 24 hours by means of TUNEL. Hyperglycemia did not affect either DRG neurons or Schwann cells at any of the concentration tested, whereas neurons and Schwann cells showed high rate of apoptosis after exposure to cisplatin and taxane, demonstrating that they are not intrinsically resistant to apoptosis.

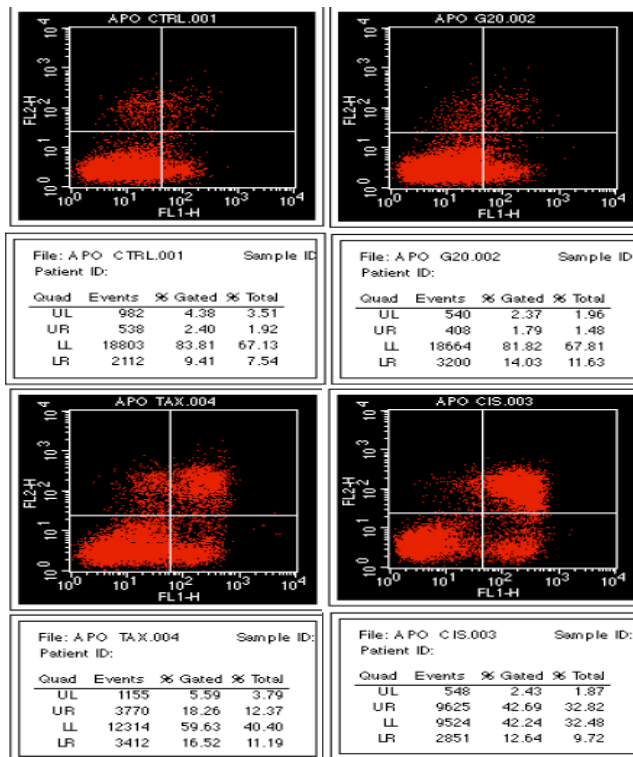


**Fig.1:** Embryonic DRG co-cultures (a) exposed to increasing glucose concentrations were associated to a modest increase of apoptosis at the highest concentration. As positive controls, co-cultures exposed to anti-neoplastic compounds shows a high increase of apoptosis that involved mainly Schwann cell (c) than DRG neuron. (b). In DRG neuron monoculture (d), we did not observe any significant apoptotic effect. Schwann cells monoculture (e) showed the same behaviour of that observed in co-cultures.

The apoptotic effect of hyperglycemia after 24 hours on embryonic DRG neuron and Schwann cell co-cultures were also evaluated using flow cytometric analysis in Annexin V/ PI assays. Cytofluorimetric studies confirmed the data obtained with the TUNEL assay. Indeed, there was not a significant increase in apoptotic death compared to control group and exposure to anti-neoplastic compounds.



**Fig. 2** Cytofluorimetric assay did not show increased apoptosis in co-culture of DRG neurons and Schwann cells after exposure to glucose compared with control group and anti-neoplastic compounds.

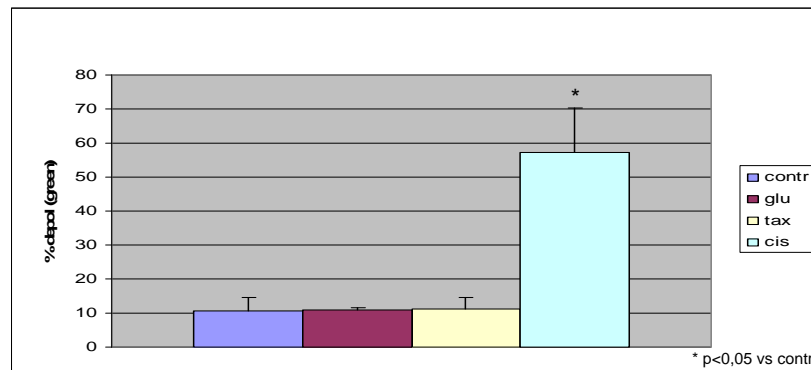


**Fig. 3** Representative cytogram of flow cytometry analysis showing apoptosis in DRG neuron and Schwann cell co-culture control (**a**) and after 24h of treatment with glucose 45mM (**b**), paclitaxel (**c**) and cisplatin (**d**). In each cytogram: Upper Left quadrant: necrosis; Upper Right quadrant: late apoptosis; Lower Right quadrant: early apoptosis; Lower Left quadrant: viable cells.

***Hyperglycemia did not cause the early loss of membrane potential differential in mitochondria***

Embryonic DRG co-culture were stained with JC1 24 hours after glucose or toxic compound exposure and membrane potential differential was analyzed by flow cytometry. Healthy mitochondria with intact membrane potential differential show high red-to-

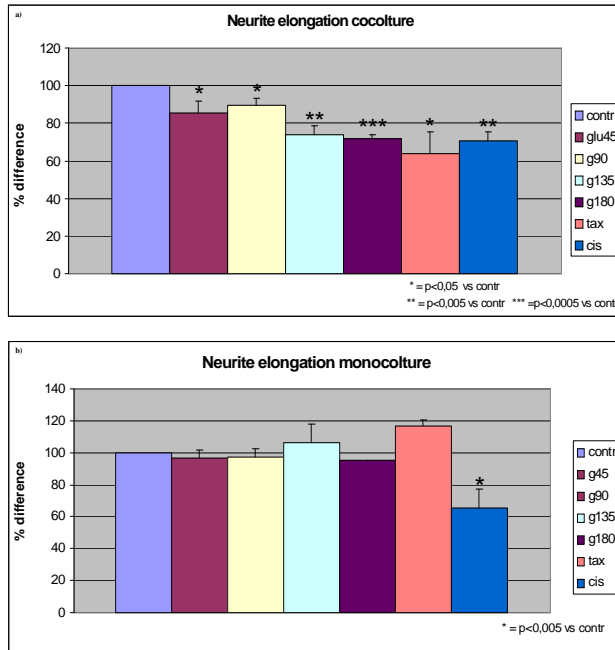
green fluorescence ratio, whereas in depolarized mitochondria the cationic dye remain in monomeric form and produces a lower red-to-green ratio. Only cisplatin induced an increase in green cell population consistent with impaired mitochondrial function.



**Fig. 4** JC-1 fluorescence emission measurements showed a reduction of red-to-green ratio in neurons exposed to cisplatin but not of those exposed to glucose 45mM or paclitaxel.

### ***Schwann cells mediate the impairment of neurite outgrowth in hyperglycemia***

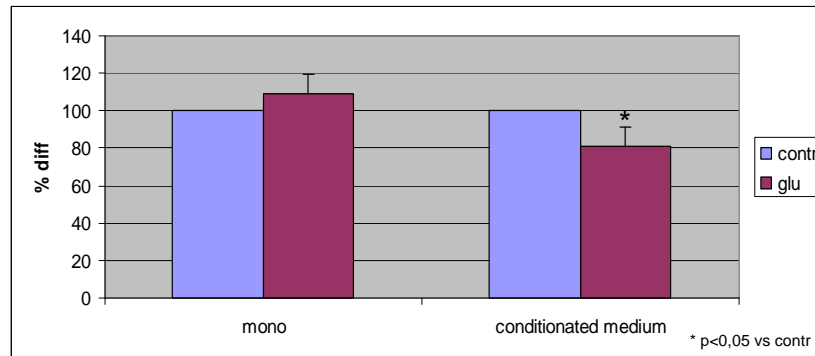
In embryonic DRG co-culture, 24 hours exposure to increasing glucose concentrations impaired axonal outgrowth in a dose dependent fashion compared to controls. In DRG neuron monoculture, high glucose concentrations did not affect neurite length, which was significantly reduced after exposure to cisplatin. This finding suggested that Schwann cells could mediate the toxicity induced by hyperglycemia.



**Fig. 5** Exposure to increasing glucose concentrations induced a significant reduction of axonal outgrowth in DRG neuron and Schwann cells co-culture (**a**) but not in neuron monoculture (**b**). The same behavior was found after taxol exposure, whereas cisplatin caused a reduction of the axonal length both in co-culture and mono-culture.

In order to confirm this hypothesis, we exposed DRG neuron monocultures to conditioned medium obtained from Schwann cell monocultures maintained in high glucose for 24 hours. After 24 hours, the neurite growth significantly decreased

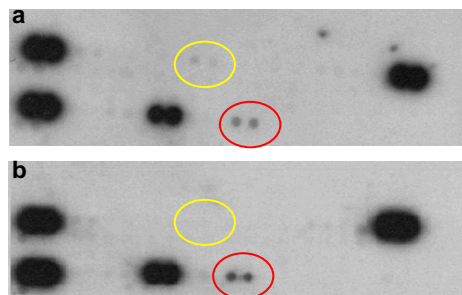
similarly to what observed in co-cultures.



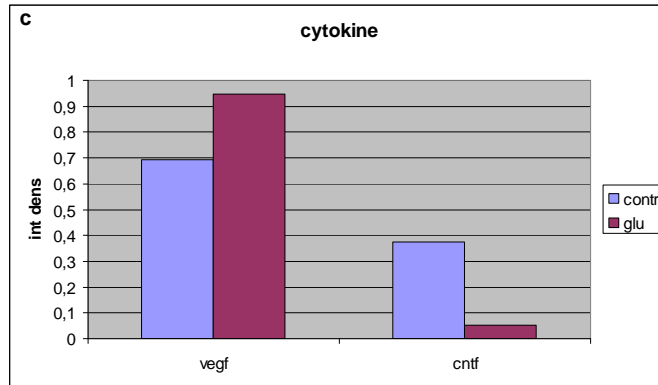
**Fig. 6** Exposure of DRG neuron monoculture to the medium obtained from Schwann cells cultured in high glucose caused a significant reduction of neurite outgrowth compared to controls.

***The medium of Schwann cells cultured in high glucose medium showed decreased CNTF and increased VEGF levels.***

We analyzed the medium from Schwann cells exposed to high glucose concentrations to evaluate if it induced any change in cytokine secretion. Cytokine profile array showed decreased ciliary neurotrophic factor (CNTF) that is a Schwann-cell-derived neurotrophic factor and increased vascular endothelial growth factor (VEGF) levels compared to control.







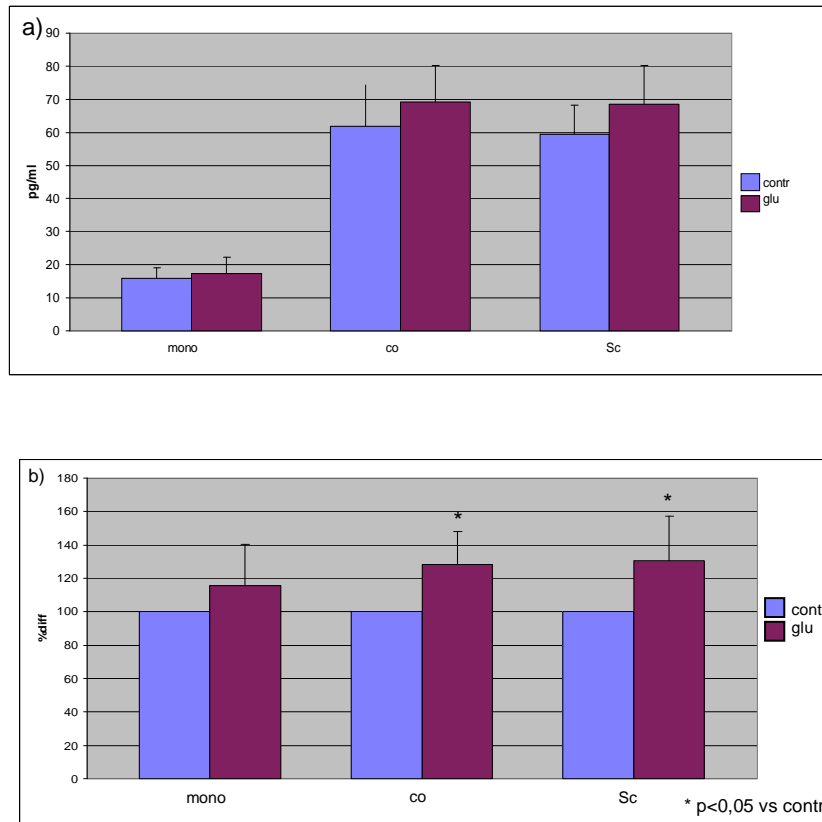
**Fig. 7** Cytokine array analysis of medium from control cells (a) and hyperglycemia (45mM) conditioned medium from Schwann cells after 24h (b). Data are expressed as relative levels of selected cytokines (c). Hyperglycemia conditioned medium showed increased VEGF whereas and decreased CNTF levels

***Increased VEGF secretion in DRG co-culture and Schwann cells monoculture exposed to hyperglycemia.***

To determine if hyperglycemia resulted in the induction of VEGF secretion, medium was collected for measurement of VEGF concentration by a commercially available ELISA.

VEGF concentration in control co-culture medium was  $61.9 \pm 12.6$  pg/ml. After 24 hours of exposure to hyperglycemia (45 mM), VEGF concentration significantly increased in DRG co-culture and Schwann cells monoculture (average increase of 30% in both conditions). Neuron monoculture showed an average increase of 15% in VEGF secretion, but the basal concentration of VEGF

was  $15.9 \pm 3.2$  pg/ml, 4-fold lower than in Schwann cells ( $59.5 \pm 8.8$  pg/ml) and in co-culture medium.

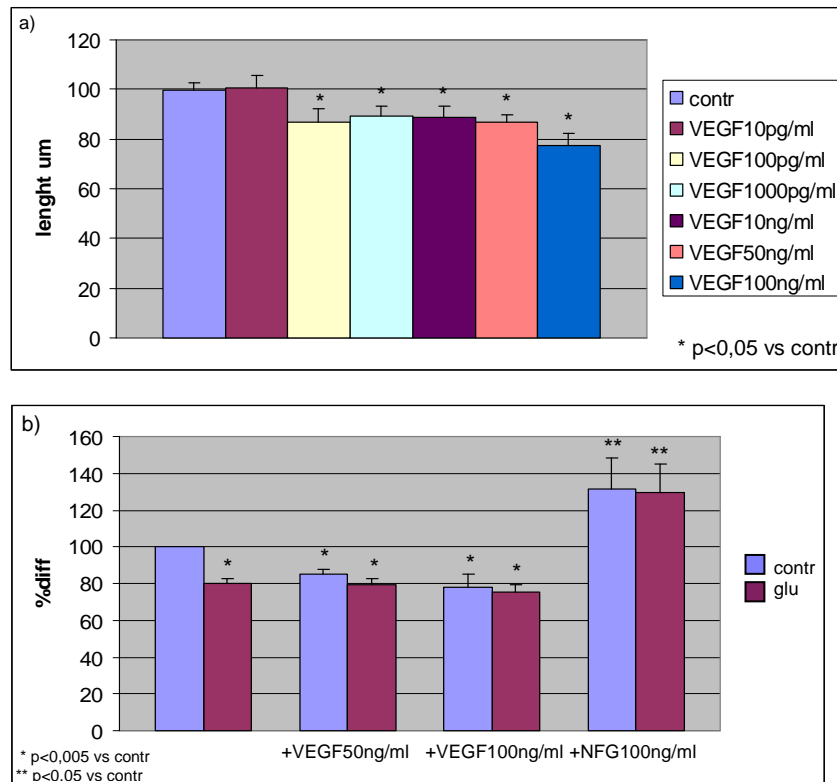


**Fig.8** Exposure to 45 mM of glucose for 24 hours induced an increase of VEGF secretion from Schwann cells. Data are express as concentration in a) and as percentage differential in b).

### ***VEGF impaired axonal outgrowth in DRG co-culture***

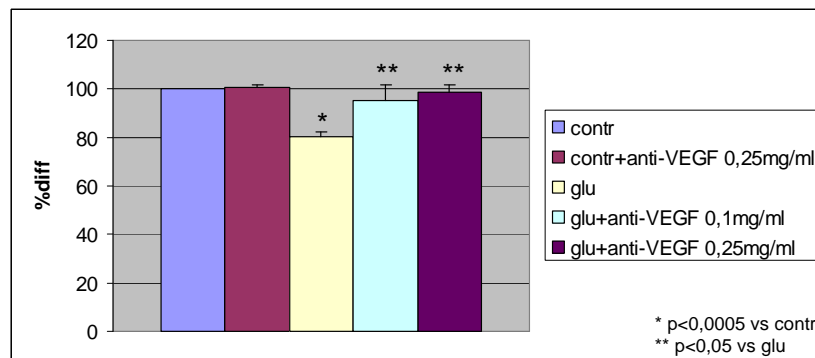
To evaluate the effect of VEGF increase on DRG neurons, DRG co-culture were exposed for 24 hours to VEGF (from 10pg/ml

to 100ng/ml) and compared to NGF (100 ng/ml) exposure as positive control. We observed a significant and dose-dependent reduction of neurite outgrowth in VEGF exposed co-cultures. Glucose effect was not worsened by a further exposure of VEGF.



**Fig. 9** VEGF induced a significant dose-dependent reduction in axonal outgrowth in neuron/Schwann cells coculture (a). An addition of VEGF did not further increased glucose effect. NGF increased axonal elongation in control and in hyperglycemic condition (b).

To confirm that toxicity induced by hyperglycemia on neurite outgrowth was mediated by VEGF increase, we exposed DRG co-culture to anti rat anti-VEGF antibody that neutralize rrVEGF164, rmVEGF120, rmVEGF164, rhVEGF121, or rhVEGF165, with a less than 2% cross-reaction with VEGF B, C (0.25mg/ml). Reduction of VEGF concentration in culture treated with anti-VEGF antibody was examined by ELISA assay. Anti-VEGF antibody did not affect control cells. In co-cultures exposed to hyperglycemia, anti-VEGF antibody normalized the ability of neurite outgrowth.



**Fig. 10** Anti-VEGF antibody did not significantly impaired axonal growth, but prevented the reduction of axonal outgrowth in high glucose DRG co-culture.

## **Discussion**

Diabetes is a chronic disease that causes several complications, among which neuropathy is one of the most frequent and disabling. The pathogenesis of diabetic neuropathy is unclear though longstanding hyperglycemia is considered the most important factor. In vitro cell culture models do not reproduce the systemic changes occurring in patients, but can elucidate some mechanisms by which hyperglycaemia affects the biological functions of DRG neurons and axons in a tightly controlled system. In this work, we used embryonic DRG neuron and Schwann cells mono- and co-culture, in order to evaluate the main target of glucose toxicity (e.g. cell body or axon) and the interaction between Schwann cells and neurons.

Data regarding experimental diabetic neuropathy are controversial. Most of the studies focused on neuronal survival, though the hypothesis that hyperglycemia primarily induces DRG neuron apoptosis is not supported by clear evidence. Some authors reported a higher susceptibility to apoptosis of DRG neurons exposed to hyperglycemia (Green et al,1999; Vincent et al., 2002; Zochodne et al., 2001; Kishi et al., 2002; Russell et al., 1999; Srinivasan et al., 2000). Several other studies described that the majority of DRG neurons underwent oxidative damage whereas only a small percentage of them showed apoptosis (Russell et al., 1999; Srinivasan et al., 2000; Kishi et al., 2002; Russell et al., 2002; Cheng and Zochodne, 2003; Schmeichel et al., 2003; Vincent et al., 2004; Sayers et al., 2003; Zherebitskaya et al.,

2009). In vitro studies from adult rats and mice (dissociated and explant) failed to demonstrate neuronal death over 1–8 days of exposure to hyperglycemic environment (Gumy et al., 2008) and suggested that DRG neurons may be particularly resistant to apoptosis (Cheng et al., 2003; Zochodne et al., 2001; Sima et al., 2005; Kamiya et al., 2005; Saito et al., 1999; Sango et al., 2002). Nevertheless, morphological changes of mitochondria (e.g. swelling, loss of cristae) and apoptotic markers (caspase 3 activation) have been described. One further issue is that all rodents develop the same degree of neuropathy or the same degree of neuronal injury. Moreover, different conditions of cell culture can affect the results. For example, it has been suggested that in order to achieve damaging effects from hyperglycaemia a serum-free medium should be used, because the serum can contain protective elements (Delaney et al., 2001) such as insulin and IGF-1. Moreover, not all the experimental designed used the same glucose concentration.

We optimized our serum-free culture conditions to guarantee surviving of DRG neurons and Schwann cells as closer as possible to in vivo condition. Embryonic sensory neurons have phenotypic differences from adult sensory neurons. Indeed, they are dependent on neurotrophic factors (Lynsday et al., 1988) and need an higher basal concentration of glucose (25 mM) than adult neurons for survival. In our experiments, we consider high glucose condition concentration starting from 45mM. . This was equivalent to a 1.8-fold above control, that is similar to the  $\geq 1.4$ -fold increase in

blood glucose concentration that is measured in a subject with diabetes (Mayfield et al., 1998).

Our primary aim was to investigate whether hyperglycemia caused a primary damage to the cell body or to the axon. We observed that high glucose concentrations did not affect DRG neuron or Schwann cell survival either in co-culture or in monoculture. Conversely, we found that DRG neurons showed a lower rate of neurite outgrowth when exposed to hyperglycemia after 24 hours as assessed by a decreased mean axonal length. These findings were in favour of a primary axonal impairment and reproduced what has been observed in animal models and diabetic neuropathy patients (Kennedy et al., 2005; Zochodne et al., 2007; Kamiya et al., 2006; Gummy et al., 2007). Intriguingly, the lower rate of neurite outgrowth was found in DRG co-culture but not in neuron monoculture, suggesting that Schwann cells could play an important role for axonal impairment. Little is known on the effect of hyperglycemia on Schwann cells, though a recent study showed that it can inhibit their proliferation and migration (Gummy et al., 2007), which are crucial events for axonal regeneration. In our model, Schwann cells both in co-culture and monoculture did not show an increased rate of apoptosis even at very high concentrations. Previous works reported similar findings after exposure to 60 mM glucose (Gummy et al., 2007), whereas Schwann cells apoptosis was observed after exposure to 150mM glucose (Delaney et al., 2001), although in vivo few Schwann cells showed apoptotic markers (Kalichman et al., 1998).

Interestingly, we observed that neurite outgrowth was impaired when DRG neurons in monoculture were exposed to Schwann cells conditioned medium obtained after exposure to high glucose concentrations. The lower rate of neurite outgrowth was similar to that observed in DRG neuron and Schwann cells co-culture. This finding strengthened the hypothesis that substances secreted by the Schwann cell exposed to hyperglycemia could mediate the axonal damage. Previous works showed that hyperglycemia can impair the secretion of cytokines and growth factors from Schwann cells and therefore affect the axonal growth. For example, immortalized Schwann cells (IMS32) under high glucose condition showed a reduced the secretion of NGF (Tosaki et al., 2008). We found that the conditioned medium from Schwann cells exposed to hyperglycemia had a lower level of CNTF compared to control. CNTF is a cytokine mainly secreted by the Schwann cells that enhances neurite regeneration in vitro (Shuto et al., 2001). It has also been found that mRNA level of CNTF in DRG was downregulated in the streptozotocin model of diabetic neuropathy (Nakamura et al., 2009) and that that the administration of CNTF prevented in a dose dependent fashion the slowing of nerve conduction velocity in galactose-fed diabetic rats (Mizisin et al., 2004).

The novel finding of our work is the evidence of increased levels of VEGF in the medium of Schwann cells exposed to hyperglycemia. ELISA assay on co-culture medium confirmed this finding. The synthesis of VEGF is stimulated by hypoxia,



hyperglycaemia, advanced glycation end products, and oxidative stress (Riddle et al., 1995; Kim et al., 1998). VEGF has been recognized as a key cytokine related to the development of microvascular diabetic complications such as retinopathy, (Stockert et al., 2001), nephropathy (Baik et al., 2004) and microangiopathy (Sakai et al., 2005). The relationship between VEGF and diabetic neuropathy has been poorly investigated. A recent study found significant higher levels of serum VEGF in diabetic patients with neuropathy compared to those without neuropathy (Arimura et al., 2009). Increased expression of VEGF has been also reported in DRG neurons and axons of the sciatic nerve in a rat model of type I diabetes (Samii et al., 1999). High glucose concentration seems to directly stimulate the secretion of VEGF in retinal Müller cells in vitro (Schrüfer TL 2010). To elucidate the role of VEGF in DRG axonal growth, we investigated the effect of recombinant VEGF and anti-VEGF neutralizing antibody. The exposure of DRG co-culture to increasing concentrations of VEGF (from 10pM to 100uM) induced a dose dependent impairment of the neurite outgrowth. Anti-VEGF neutralizing antibody added to hyperglycemic co-culture prevented the axonal growth impairment, confirming the involvement of VEGF in the pathogenesis of axonal damage induced by hyperglycemia.

In conclusion, our study suggests that axonal impairment is likely the main factor involved in the pathogenesis of diabetic neuropathy. This event seems mediated by an altered

regulation of Schwann cells exposed to hyperglycemia that induced a decreased secretion of CNTF and an increased secretion of VEGF. This latter seems directly involved as suggested by the effect of the neutralizing antibody that prevented the axonal impairment. In human beings, impaired microcirculation and local hypoxia are important pathogenetic factors for the development of diabetic neuropathy (Sugimoto et al., 2000; Cameron et al., 2001) and VEGF could play a critical role.

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## CHAPTER 3

In vivo and ex vivo studies on peripheral neuropathy in Zucker rats: a type 2 rat model. Preliminary results.

### Introduction

Neuropathy is one the major cause of morbidity among diabetic patients. Identification of underlying mechanisms is of greatest importance to better understand the failures with existing treatments and develop new approaches for diagnosis and therapy of neuropathies.

Diabetic rodents exhibit many disorders seen in patients with neuropathy including allodynia, nerve conduction slowing and progressive sensory loss (Hounsom, L. et al 1997; Dobretsov, M. et al. 2007). Murine models allow to investigate the involvement of insulin, glucose as well as polyol pathway and axonal neurofilaments on neuropathy development. Genetic models are also available. Zucker diabetic fatty (ZDF) rat is an inbred rat model of type 2 diabetes with a genetic mutation (obesity gene) and manageable with diet. *Fa* mutation results in shortened leptin receptor which does not effectively interact with leptin. This mutation is phenotypically expressed as obesity with high levels of leptin in the blood. Islets from ZDF rats are resistant to the lipopenic action of leptin.

When fed with a diet of Purina 5008, *fa/fa* males (homozygote recessive) develop obesity, hyperlipidemia, fasting



hyperglycemia, and type 2 diabetes. Fa/+ males lean genotypes remain normoglycemic. ZDF rats become hyperglycaemic by 8 weeks of age and glucose remains elevated through their lifespan (Peterson et al., 1990). Initially they are hyperinsulinemic, then by 22–42 weeks of age serum insulin levels decline to below the levels of insulin in age-matched lean control rats (Peterson et al., 1990). A similar characteristic is seen in human type 2 diabetes.

The ZDF rat is an accurate model for Type 2 diabetes because it shows impaired glucose tolerance leading to insulin resistance and the loss of response to glucose is associated with the disappearance of GLUT2 transporters on the beta cells in the islets after the onset of diabetes. Beta-cell mass in ZDF rats is significantly lower than in lean control rats. Diabetic neuropathy is a common complication in type 2 diabetes and Zucker rat could be a model to investigate the pathophysiological mechanisms. Few and limited studies have been previously performed on this topic. Aim of this work was to characterize development and course of neuropathy in this model also in order to understand its possible use in neuroprotective experimental trials.

### **Material and methods**

Obese Zucker diabetic fatty male (ZDF/crl-lepr/fa) and control male lean fa/+ rats were purchased from Charles River at six weeks of age. The onset of hyperglycemia was assessed by

weekly urine testing (Keto-diabur test, Roche) and blood glucose was measured using a glucose strip tester (One Touch Ultra, Lifescan, Johnson and Johnson). The rats were raised on Purina rat chow 5008.

For the study, 8 rats per group were treated as above starting when the rats reach 16 weeks of age, when they become diabetic, with stable hyperglycemia, and low plasma insulin and reduced nerve conduction velocity (NCV).

Nerve conduction velocity (NCV) was the mark of the disease, so, diabetic groups were randomized on these bases and checked at the end of treatment. In each experimental paradigm, water and food intake were measured. Body weight was measured weekly. Mechanical nociceptive thresholds were measured every other week during the first months and weekly for the remaining period. Skin biopsies for IENF density determination were taken at 16 weeks and at the end of the experimental period (32 weeks). At sacrifice other tissue specimens (including sciatic nerve, tissue containing the epineurial arterioles, dorsal root ganglia, spinal cord...) were also collected for further biochemical, immunohistochemical, morphological and morphometrical analysis.

**Cell culture.** Spinal dorsal root ganglia (DRG) were dissected from rats at the end of experiments and enzymatically dissociated with 0.25% trypsin and collagenase 1% in L-15 medium. Neurons were maintained in DMEM low glucose medium, that contains 5mM glucose, supplemented with penicillin (1 U/L),

streptomycin (1 U/L) and 1xB27. Part of the cultures were exposed to a 20mM glucose overload.

***Assessment of neurite elongation.*** After 24 hours neurons were stained with fluorescent antibody anti-BIII tubulin and the longest axons of at least 100 neurons in each condition was measured using an image analysis system on fluorescence microscope.

***Assessment of neuronal apoptosis.*** To determine apoptotic cell death, the cells were fixed and stained with In situ DNA nick labelling ( DeadEnd Fluorometric TUNEL System). Cells were double-stained with DAPI and apoptotic cells were counted and expressed as percentage of the total number of cells present in each culture.

***Measurement of reactive oxygen species.*** Intracellular ROS was evaluated with the dichlorofluorescein (DCF) assay. The nonfluorescent fluorescein derivatives (dichlorofluorescein, DCFH), after being oxidized by various oxidants, become DCF and emit fluorescence. By quantifying the fluorescence, we were able to quantify the ROS.

***Tail NCV.*** NCV in the tail was measured by using a Myto EBNeuro electromiograph (EBNeuro, Firenze, Italy) for each animal by a method already used in experiments on neuroprotection (Cavaletti et al., 1994; Pisano et al., 2003).

***Nociceptive thresholds.*** The nociceptive thresholds was quantified using Randall-Selitto paw withdrawal test for mechanical sensitivity, as previously described (Bianchi et al., 2004).

***Immunohistochemistry of skin biopsies and IENF density.*** At the end of treatment, hind paws were collected, plantar glabrous skin including epidermis and dermis were separated from the metatarsal bones, and 3-mm round samples were taken, immediately fixed by immersion in 2% paraformaldehyde-lysine-periodate for 24h at 4°C, then cryoprotected overnight. Twenty-micron thick sections perpendicular to the epidermis were cut serially with a cryostat, sequentially labeled and stored at -20° C. Three sections from a single footpad were randomly chosen, treated with 0.5% Triton X-100 in 0.5 M Tris buffer at pH 7.6 for 1h and immunoassayed with rabbit polyclonal anti-protein gene product 9.5 antibodies (PGP 9.5; AbD Serotec, Oxford, UK), using a modified free-floating protocol (McCarthy et al., 1995). Sections were blocked with 4% normal goat serum in 0.5% non-fat dry milk/Tris for 1h, then incubated overnight with ant-PGP 9.5 antibodies (1:1000). After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit IgG for 1h (1:100), quenched in 30% methanol/hydrogen peroxide with 1% H<sub>2</sub>O<sub>2</sub> for 30 min and placed in avidin-biotin complex for 1h. The reaction product was visible as a blue chromogen/peroxidase substrate. The total PGP 9.5-positive IENFs were counted in each section under a light

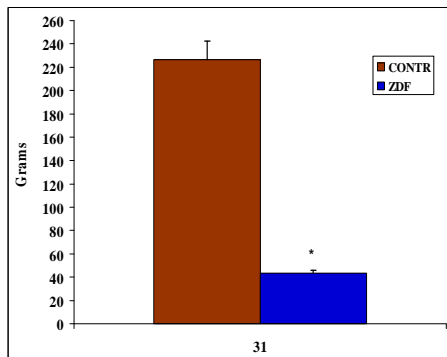
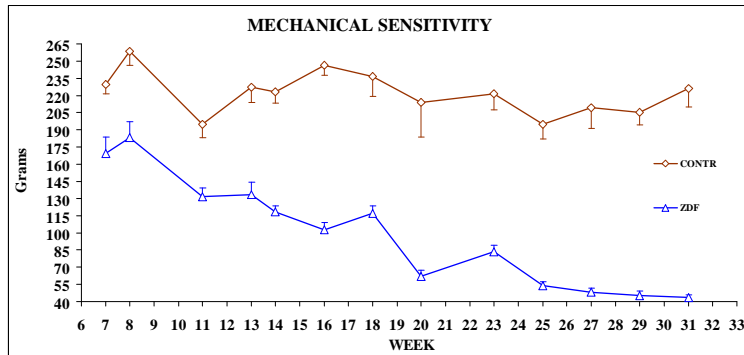
microscope (40X); the length of the epidermis was measured using a computerized system and the linear density of IENFs (IENFs/length of the epidermis) was obtained (Lauria et al., 2005).

***Statistical analysis.*** Differences in body weight, withdrawal latencies, NCV were compared by analysis of variance (ANOVA) and the Tukey-Kramer Multiple Comparison as post-test. Unpaired Student's t-test was applied to couples of independent variables

## **Preliminary results**

### ***Mechanical Nociceptive Thresholds***

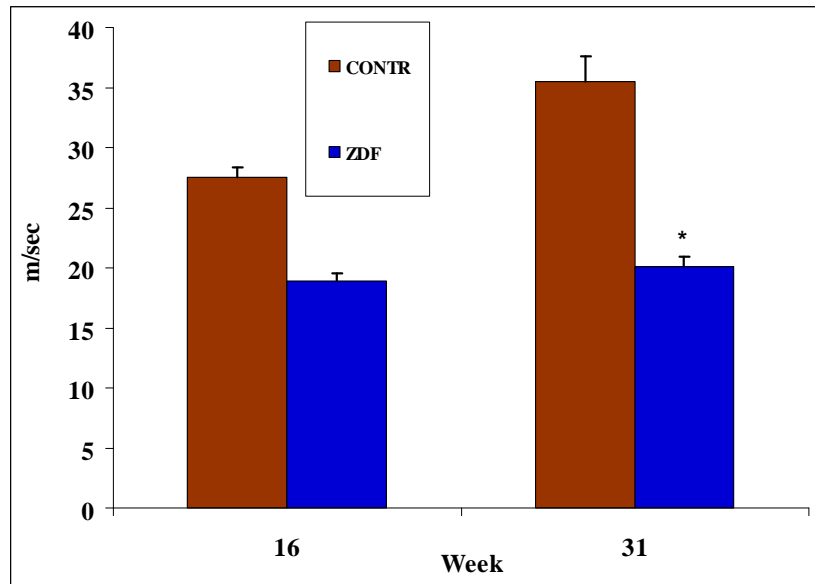
Mechanical hyperalgesia was measured during all the period of the experiment. It reached a statistical significance ( $p < 0.05$ ) at 1 month of diabetes and progressively increased up to 80% at the end of the study, consistent with the development of neuropathic pain.



**Fig. 1** The hind-paw force-withdrawal (mechanical) threshold in ZDF diabetic rat was decrease compared to control.

***Nerve conduction velocity***

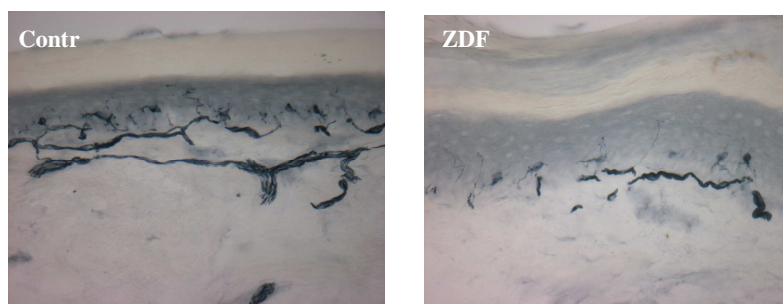
ZDF rats showed a progressive and significant slowing of NCV compared to controls that reached 40% decrease at 8 months but already present at 4months after only 10 weeks of hyperglycemia onset.



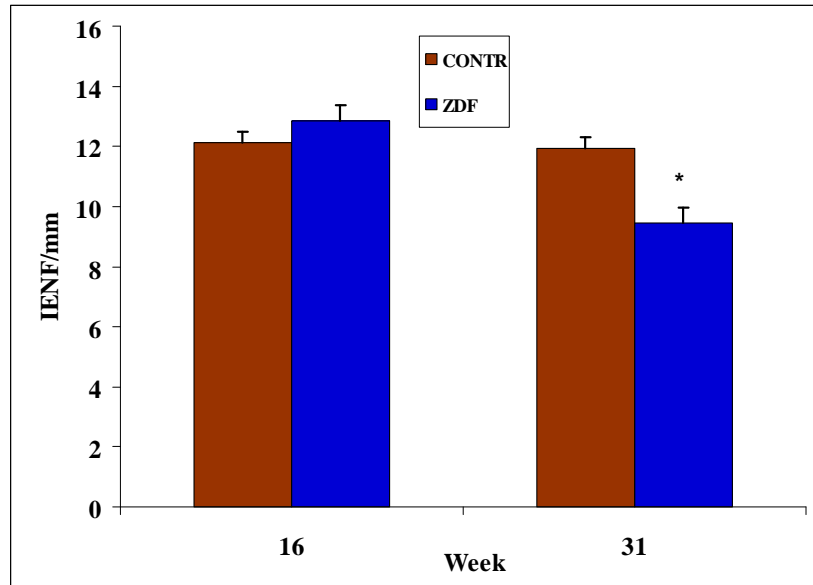
**Fig. 2** Tail NCV evaluated at 16 weeks and 31 weeks ( $p < 0.001$ ).

***Footpad Intraepidermal Nerve Fiber Density.***

Skin biopsy was taken from live animals at 16 week and at the end of the period experiments at 31 weeks. In diabetic rats, the IENF density is unchanged at 16 weeks of diabetes. At week 31 we measured a little, but significantly reduced density in diabetic rats. Decrease in cutaneous innervation density is not clearly establish.



**Fig. 3** Representative microphotograph of PGP 9.5 immunostaining of IENF in the footpad of control and diabetic rat.



**Fig. 4** Quantification of IENF density. At 4 months IENF density was still unaffected in ZDF rats and significantly reduced (-20%;  $p < 0.01$ ) only at 8 months compared to controls.

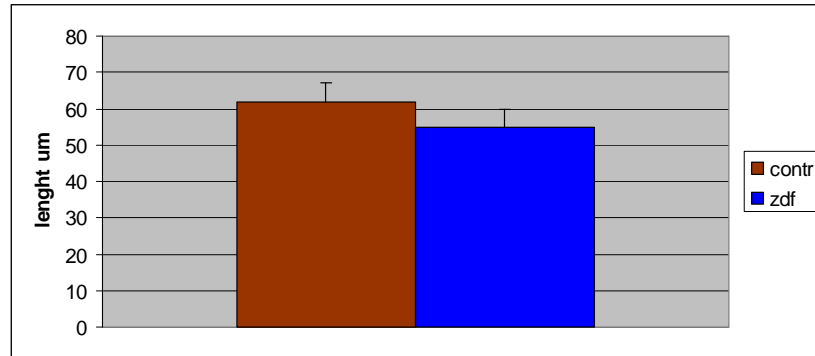
#### ***Neurite outgrowth evaluation***

Culture obtained after dissociation of DRG were allowed to growth for 5 days. After this time they were fixed and immunostained and axonal growth was evaluated.

An overload glucose exposure (+20mM) for 24 hours did not affect axonal regeneration both in DRG neuron control and in



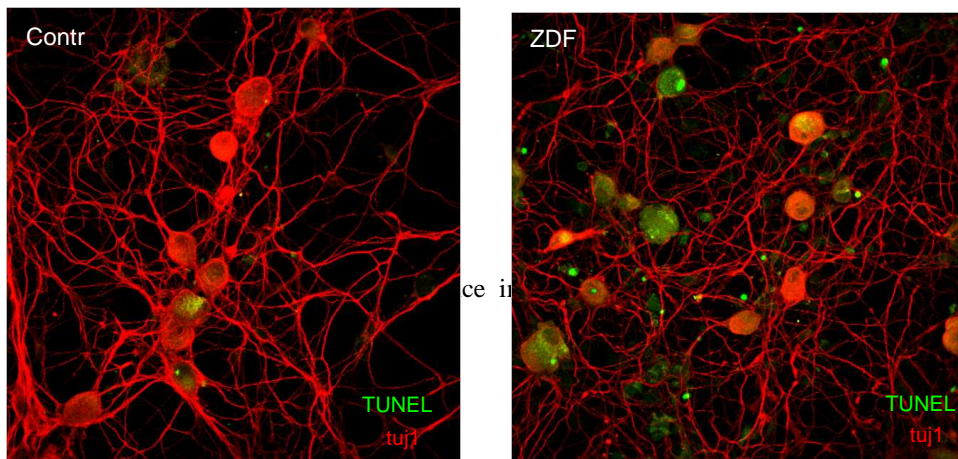
diabetic DRG neurons (data not show).

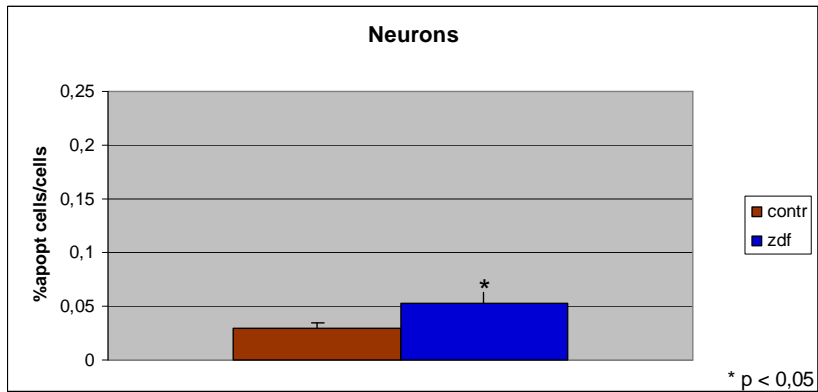
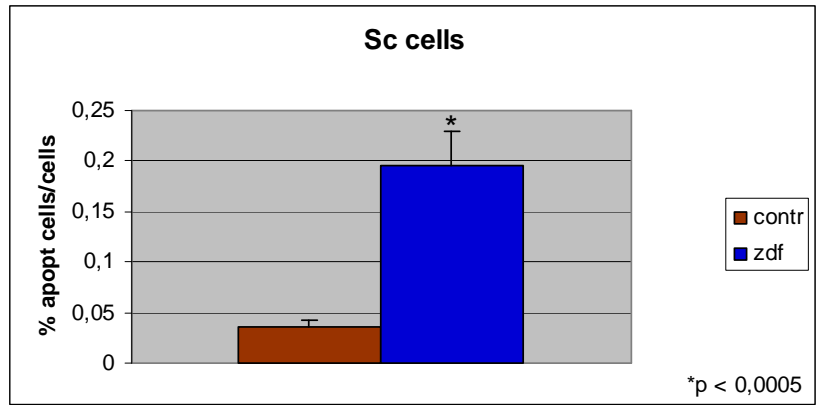


**Fig. 5** DRG neurons from ZDF rats did not show a significant impairment axonal outgrowth compared to control. Mean length was calculated considering at least 100 neurons for each animal culture.

### *Apoptosis evaluation*

After 5 days in culture, apoptosis was assessed by TUNEL assay. Both sensitive neurons and Schwann cells obtained from ZDF rats showed an increased apoptosis rate compared to cells from control. Schwann cell are greatly more affects than neuron



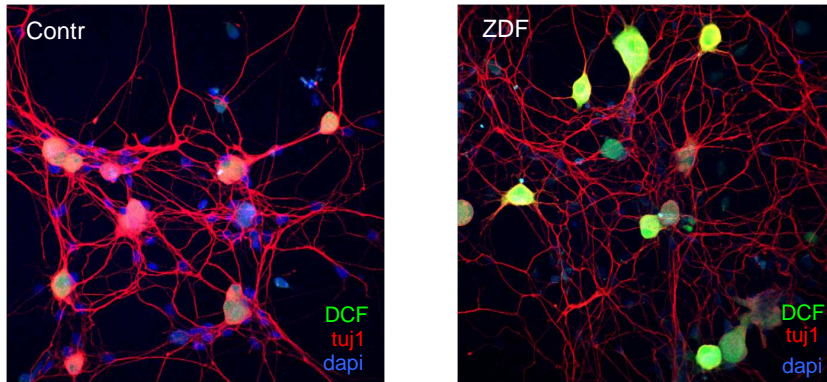


**Fig. 7** Cells culture from ZDF rats showed an higher rate of apoptosis than control. Schwann cells seem to be more affected than neurons.

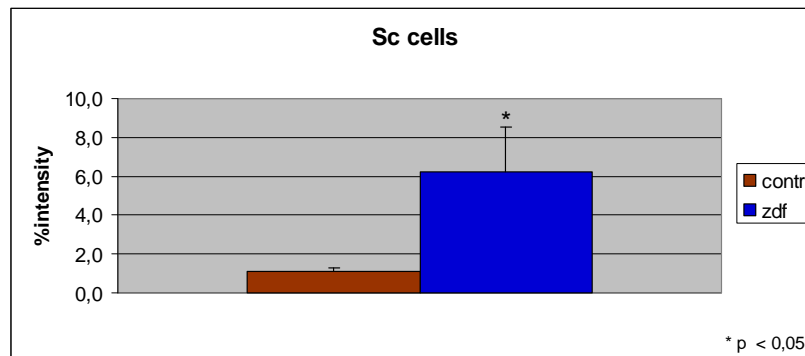
***Oxidative stress evaluation***

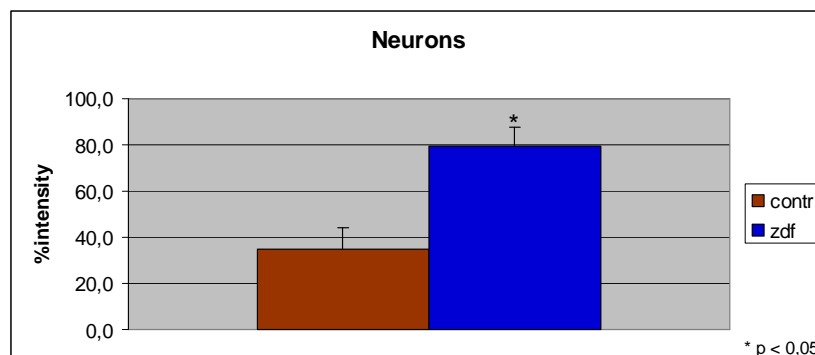
To estimate the cellular oxidative stress in DRG culture, levels of endogenous ROS were measured by DCF fluorescence. As

shown in fig. 9, compared with the control group, both neurons than Schwann cells from ZDF rat had a significant increase in DCF fluorescence.



**Fig. 8** Representative immunofluorescence image at 40x magnification of DRG culture





**Fig. 9** Both neuron and Schwann cells from diabetic rats showed an increased DCF intensity, that mean an increased of oxidative stress compared to control.

Studies of several others parameters that could confirm our preliminary findings (Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in sciatic nerve, level of expression of myelin proteins, magnetic resonance, G-ratio) are still in progress.

### **Discussion**

Diabetic peripheral neuropathy (DPN) associated with type 2 diabetes experimental models is less frequently studied than type 1 models. In this study we investigated the male Zucker diabetic fatty (ZDF) rat model which homozygous missense mutation causes a nonfunctional leptin receptor (*fa/fa*). ZDF rats

were compared with heterozygous lean males in a 8-month longitudinal study. We monitored the development of hyperglycemia and growth, and investigated the impact of type 2 diabetes on different neurological features, including nerve conduction velocity (NCV), quantification of intraepidermal nerve fibers (IENF) by repeated foot pad skin biopsy, and biochemical and morphometric analysis of sciatic nerve at sacrifice.

As expected growth rate at diabetes onset was faster in ZDF rats with respect to lean group, however after about 16-20 weeks of diabetes ZDF slower their growth rate and the lean group growth faster than ZDF rats. Confirming literature data, we observed that ZDF rats developed significant and progressive hyperglycemia from baseline that reached a plateau at 6 months of diabetes duration then stabilized up to the 8th month (data not shown).

We demonstrate that NCV progressively decreases in ZDF diabetic rats. Preliminary data on Na<sup>+</sup>K<sup>+</sup>-ATPase activity in sciatic nerves from ZDF rats suggest the involvement of fibres of large diameter in the development of peripheral neuropathy. Additionally, preliminary analysis of sciatic nerves disclosed axonal atrophy with signs of demyelination as demonstrated by the significant increase of the g-ratio.

Conversely, IENF density showed a much slower progression. Indeed, at 4 months IENF density was still unaffected in ZDF rats and significantly reduced (-20%; p<0.01) only at 8 months compared to controls.

From these observations we can conclude that ZDF rats develop a mild peripheral neuropathy with a slow progression. Indeed, NCV and Na<sup>+</sup>K<sup>+</sup>-ATPase activity are mainly linked to large myelinated fibers, and are affected earlier in this genetic model of diabetes, whereas intraepidermal fibre are small calibre fibres and their decrease progress slowly than those of large fibres.

Ex vivo, in DRG cells co-culture from ZDF rats we demonstrated a significantly higher rate of apoptosis in both neuronal (78%) and, in particular, in non-neuronal (mostly Schwann) cells (440%) compared to control. Similar findings were observed when oxidative stress was measured by fluorescent probes for intracellular presence of reactive oxygen species. Conversely, ZDF DRG neurons did not show impairment of neuritis elongation compared to control.

In conclusion, ZDF type 2 diabetic rats showed a milder and more gradual impairment of NCV and IENF density reflecting large- and small-calibre fibre function than STZ-induced type 1 diabetes model. The important role of Schwann cells is underline also by the great increased in apoptosis and in ROS intracellular level that we observed in culture from ZDF diabetic rats compared to control.

The characterization of ZDF model can be useful for the development of experimental neuroprotective trials with different anti-diabetic agents.

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## **CHAPTER 4**

### **SUMMARY, FUTURE PERSPECTIVE AND CONCLUSION**

Diabetic polyneuropathy (DPN) is the most common chronic complication of diabetes mellitus. It affects about 60% of individuals with type 1 or type 2 diabetes, but subclinical impairment of somatic and autonomic nerves may virtually occur in all patients. It is responsible for substantial morbidity, increased mortality and impaired quality of life. Currently no effective treatment is available for DPN beyond tight glycemic control. The pathophysiology of DPN remains unclear and it is not even known whether the process starts affecting the axons or the neuronal cell bodies, and to what extent Schwann cells are involved. In vitro studies offer the advantage to investigate apoptosis and axonal damage after the exposure to different glucose concentrations in culture medium. Most of the previous studies showing increased apoptosis were performed in neuron monocultures from embryonic dorsal root ganglia (DRG). Limited data on the changes of axonal growth in high glucose assays are available. Moreover, the contribution of Schwann cells to the neuronal damage has been scantily investigated.

This project aimed to investigate 1) if glucose primarily affected DRG cell body or axon; and 2) the pathways potentially involved in counteracting the events leading to axonal degeneration.



We used an in vitro model to identify the principal targets of glucose-induced neuronal or axonal damage through different approaches, including the quantification of apoptosis and axonal growth. We also investigated the effect of hyperglycemia on Schwann cells with the aim to clarify their role in the pathogenesis of neuronal and axonal damage.

In embryonic DRG co-cultures, exposure to high glucose was associated to a modest and non-significant increase of apoptosis as examined by TUNEL assay, compared to control groups. These results were confirmed by cytofluorimetry assay. Conversely, exposure to high glucose significantly impaired axonal growth compared to control group. After exposure of embryonic neuron and Schwann cell monocultures to high glucose we did not observe either increased apoptosis or changes in axonal growth compared to control groups. However, when neuron monocultures were exposed to the medium obtained from Schwann cell monocultures maintained in high glucose for 24 hours, we observed a significant decrease of axonal length compared to control group, similarly to what found in DRG co-cultures. Analysis of conditioned medium showed an increase in VEGF levels, both in hyperglycemic Schwann cells culture and in DRG co-culture. Therefore, we exposed DRG neurons co-culture to increasing concentration of VEGF and found a significant decrease of the axonal growth. Treatment with anti-VEGF neutralizing antibody prevented the impairment of neurite outgrowth induced by high glucose concentration in DRG co-culture, supporting the

hypothesis that VEGF is involved in the pathogenesis of hyperglycemia induced axonal damage.

Further studies are warranted to clarify the altered regulation of Schwann cell VEGF secretion induced by hyperglycemia in in vitro DRG co-culture. The expression of VEGF is regulated at multiple levels: transcriptional, posttranscriptional, and translational. VEGF release is mediated by a complex array of signaling pathways which integration results in the generation of a net signaling input (Xiaofeng Ye). We will use western blot and RT-PCR on Schwann cell culture to measure protein and mRNA level, in order to evaluate at which level regulation of VEGF is altered by hyperglycemia. Since any potential treatment for DPN would depend on whether involved neurons express relevant receptors, we will investigate the level of expression and the distribution of VEGF receptor (rVEGF) on DRG neuron in high glucose condition.

Diabetic neuropathy is a complex and systemic disease, leading to neuropathy likely through several different pathogenic mechanisms. Therefore, our findings need to be confirmed in an animal model. Streptozotocin (STZ) is widely used to create rodent models of type 1 diabetes (Rees, D.A et al 2005). This antibiotic directly affects the pancreatic beta cells and insulin secretion. The development of STZ induced diabetic neuropathy is mainly dependent on the level and duration of hyperglycemia. In the early course of diabetes in STZ rats, endoneurial blood flow and micro- and macrovascular reactivity were impaired

(Hounsom, L et al. 1997; Dobretsov, M. et al 2007). Slowing of sensory nerve conduction velocity (SNCV) and motor nerve conduction velocity (MNCV), degeneration of intraepidermal nerve fiber (IENF), hyperalgesia and allodynia were shown to develop within the first month from the onset of hyperglycemia in STZ rats (Bianchi R et al. 2004). After 8–12 weeks, signs of axonopathy, demyelination, nerve degeneration and hypoalgesia can also be detected (Sigaud-Roussela D. et al 2007). All these characteristics make this model suitable to our purpose.

Diabetic will be induced in rat by injection of STZ. For preventive studies rats will be treated with anti-VEGF neutralizing antibody just after STZ administration, while in the therapeutic protocol, treatment with anti-VEGF neutralizing antibody will start 6 weeks after diabetes induction, when neuropathy will be already established. Neuropathy will be monitored measuring NCV, thermal and mechanical nociceptive thresholds will be evaluated using respectively hot-plate test, that evaluates thermal pain reflexes due to footpad contact with a heated surface and Randal Selitto test that consists in an application of a uniformly increasing pressure on the paw to assess the threshold response to pain. Von Frey test will be assess to study the threshold for touch evoked sensations. Skin biopsies for IENF density determination will be collected from paw of live animals and at sacrifice. We will also evaluated Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the sciatic nerve as another clinical signs of the nerve degeneration, since it is responsible for the

maintenance of the potential difference throughout the nerves. Sample of blood will be collected during the experiment in order to evaluate the level of VEGF at different time points. It has been recently observed that VEGF blood level change with different stages of the disease (T. Deguchi 2009). At the end of treatment period tissue specimens (including sciatic nerve, dorsal root ganglia, spinal cord) will be collected for further biochemical, immunoistochemical, morphological and morphometrical analysis. In particular we will measure with RT PCR and Western blot techniques, mRNA and protein level of VEGF in DRG and sciatic nerve. Furthermore with immunofluorescence we will evaluated rVEGF distribution in DRG cell bodies and in nerve fibres.

Our in vitro studies showed that hyperglycemia mainly affects the axonal growth of DRG neurons and that an increased secretion of VEGF from Schwann cells is likely to give a major contribution. These findings will be further investigated in an animal model of diabetic neuropathy. If confirmed, they will provide new insight into the pathogenesis of DPN and the background for potential treatment strategies which could be translated in clinical trials.

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