In vivo evaluation of a rat model for diabetic neuropathies

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II. **ABBREVIATIONS**

AGE = advanced glycation end products

AD = Aqua Destillata

AP-1 = transcription factor activated protein1

AR = aldose reductase

ARTN = artemin

ATF3 = activating transcription factor 3

ATP = adenosine triphosphate

BDNF = brain derived neurotrophic factor

BG = blood glucose

BSA = bovine serum albumin

BW = body weight

CNS = central nervous system

CREB = cyclic monophosphate responsive element binding protein

DAG = diacylglycerol

DM = Diabetes Mellitus

DHAP = dihydroxyacetone phosphate

DRG = dorsal root ganglion

ETC = electron transfer chain

GDNF = glial cell-line derived neurotrophic factor

GFL = glial cell-line derived neurotrophic factor family of ligands

GlcNAc = *N*-acetylglucosamine

GSH = gluthathione

Hsp27 = heat shock protein 27

HPS = Hematoxylin Phloxin Saffrane

IEGs =immediate early genes

IENF = intraepidermal nerve fibre

IP3 = inositol triphosphate

JNK = c-Jun N-terminal kinase

LC = Langerhans cell

NADPH-oxidase = nicotinamide adenine dinucleotide phosphate-oxidase

NeuN = neuronal nuclei

NGF = nerve growth factor

NRTN = neurturin

NT = neurothrophin

$O_2^{-\cdot}$ = superoxide

PBS = phosphate buffered saline

PGP9.5 = protein gene product 9.5

PKCβ = protein kinase C-β

PP = paw pressure

PSPN = persephin

ROS = reactive oxygen species

RET = receptor tyrosine kinase molecule

SAPK = stress-activated protein kinase

SDH = sorbitol dehydrogenase

S.E.M. = standard error of the mean

STZ = streptozotocin

Trk = tropomyosin-related kinase

VF = von Frey

YFP = yellow fluorescent protein

III. <u>PREFACE</u>

IV. __ABSTRACT__

Diabetic peripheral neuropathy is considered to be a long-term complication of Diabetes Mellitus. This neuropathy is the most common form of peripheral neuropathy in the Western world and develops in about 50% of diabetes patients affected with either type I or type II diabetes. Despite advances in understanding metabolic causes of diabetic peripheral neuropathy, specific treatments against this complications are far from being used in therapy options.

In this study we have evaluated the diabetic peripheral neuropathy in the streptozotocin (STZ)-induced diabetic animal model. For years, this model has been used in an attempt to provide information of the underlying processes and to evaluate potential therapies. Therefore, as a primary objective of the present study, a quantification over time of the neuropathic behavior (sixteen weeks post STZ-injection) of diabetic rats was compared to normoglycemic controls. The neuropathy was assessed by quantifying mechanical hyperalgesia and allodynia and by evaluating the rat's global clinical condition. Simultaneously, skin biopsies and dorsal root ganglions (DRGs) were taken at different time points after STZ-injection in order to create an idea of the histological alterations of nerves during diabetic peripheral neuropathy. This diabetes-associated neuropathic behavior and the comparison with the histological alterations of nerves during neuropathy can be important for the development of a disease modifying treatment for diabetic peripheral neuropathy. Therefore the second objective of this study was to evaluate the diabetic peripheral neuropathy after treatment with neurotrophic factors. After subplantar injection of GDNF and NGF in one hind paw of diabetic animals, the neuropathy was again evaluated by quantifying mechanical hyperalgesia and allodynia. While GDNF improved the hyperalgesic effect in rats resulting from diabetes, NGF increased the nociceptive responses. After evaluating skin biopsies of these rats, no difference in epidermal thickness was shown, but an increased inflammatory response resulted after growth factor injection compared to rats injected with vehicle.

This study indicates that GDNF has an advantage effect on the neuropathic behavior observed in the *in vivo* model of diabetic peripheral neuropathy, while NGF increased the hyperalgesia and allodynia following injection. Therefore we can conclude that GDNF can be considered as a possible candidate for the treatment of diabetic peripheral neuropathy. Further research is necessary for improving the treatment options and to investigate whether GDNF could also be effective in the treatment of neuropathy in humans.

Een lange termijn complicatie van Diabetes Mellitus is de ontwikkeling van diabetische perifere neuropathie. Deze neuropathie is de meest voorkomende vorm van perifere neuropathie in de Westerse wereld. Het komt voor bij ongeveer 50% van de patiënten met diabetes type I of type II. Ondanks de vele onderzoeken naar de onderliggende processen van diabetische perifere neuropathie bestaat er nog geen specifieke behandeling tegen deze complicatie.

In deze studie evalueren we de diabetische perifere neuropathie in het streptozotocin (STZ)-geïnduceerde diabetes diermodel. Sinds jaren wordt dit diermodel gebruikt in een poging om nieuwe informatie te verkrijgen over de onderliggende processen en om potentiële therapieën te ontwikkelen. Een eerste doel van deze studie was dan ook om een inzicht te verkrijgen van de duur van het neuropathisch gedrag in diabete ratten na zestien weken STZ-injectie, vergeleken met normoglycemische controle dieren. De neuropathie werd geëvalueerd door het nagaan van de mechanische hyperalgesie en allodynie en door het scoren van de klinische conditie van de diabete ratten. Tegelijkertijd werden ook plantaire huidbiopten en dorsale ganglia van oude diabeten geanalyseerd om een idee te verkrijgen van de zenuwbeschadigingen tijdens diabetische perifere neuropathie. De vergelijking tussen het neuropathisch gedrag en de histologische zenuwbeschadigingen kan belangrijk zijn in de ontwikkeling van een behandeling voor diabetisch perifere neuropathie. In het tweede deel van de studie kijken we dan ook naar een mogelijke behandeling van neurotrofe factoren tegen diabetische perifere neuropathie. Na een subplantaire injectie van GDNF en NGF in één achterpoot van diabetisch dieren, werd het neuropathisch gedrag opnieuw geëvalueerd door het nagaan van de mechanische hyperalgesie en allodynie. Er werd aangetoond dat GDNF het hyperalgetisch effect verbeterde tijdens de injectie periodes. In tegenstelling, NGF zorgde voor een verhoging van de hyperalgesie en allodynie tijdens de injecties. De huidbiopten van deze ratten toonden aan dat er geen verschil was in epidermisdikte, maar dat er wel een verhoogde inflammatoire response volgde na groeifactor injectie.

Deze studie toonde aan dat GDNF een verbetering van het neuropathisch gedrag veroorzaakte, terwijl NGF de hyperalgesie en allodynie na injectie enkel verhoogde. Daarom kunnen we besluiten dat GDNF een mogelijke kandidaat is voor de behandeling van diabetische perifere neuropathie. Verder onderzoek is nodig om deze behandelingsmogelijkheid te verbeteren en om te na te gaan of GDNF even effectief is in de behandeling van neuropathie bij mensen.

1. __INTRODUCTION__

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a very complex disease and forms a worldwide health problem. The number of people suffering from DM is increasing due to population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity. Across the world, it appears that the most important demographic change to diabetes prevalence is the increase in proportion of people over 65 years of age. It has been estimated that the prevalence of diabetes for all age groups worldwide was 2.8% in the year 2000 (171 million in 2000). The urban population in developing countries is projected to double between 2000 and 2030 (1). This indicates that the research towards an effective treatment for diabetes and diabetic complications are of great importance for public health and consequently a challenge for the scientific community.

1.1.1 The pathogenesis of diabetes mellitus

Diabetes mellitus is a common metabolic disease in humans. The hallmark of diabetes is the existence of hyperglycaemia due to an inefficient processing of glucose. Diabetes is not one disorder, but can arise as the result of a number of defects in regulation of the synthesis, secretion and efficacy of insulin. Normally, the blood glucose levels are tightly controlled by insulin. This anabolic hormone is produced by the beta cells of the islets of Langerhans, located in the pancreas. After eating a meal, the level of glucose in the blood rises and insulin is released from the pancreas in order to normalize the blood glucose levels. Insulin reduces the amount of blood glucose by allowing glucose to enter muscle cells and by stimulating the conversion of glucose to glycogen (glycogenesis) as a carbohydrate store. In diabetes patients, the pancreas is unable to produce sufficient insulin or insulin is produced but isn't recognized by the insulin receptors. The resulting hyperglycaemia (>120 mg/dl) and the inadequate amount of sugar in the cells, produce the symptoms and complications of diabetes.

There are two major forms of DM. In type I diabetes, also called insulin dependent diabetes or juvenile-onset diabetes, more than 90% of the insulin producing cells of the pancreas are progressively destroyed. The pancreas, therefore, produces little or no insulin. In type 2 diabetes, also called non-insulin dependent diabetes or adult-onset diabetes, the pancreas continues to produce insulin, sometimes even at higher than normal levels. However, the body develops resistance to the effects of insulin, apparently there is not enough insulin to meet the body's needs. Of all the patients with diabetes, approximately 10% of the patients suffer from type I diabetes and the remaining 90% have type II diabetes (2-3).

1.1.2 Symptoms and complications of diabetes mellitus

Diabetes is often undiagnosed because many of its symptoms are neglected by the patient or seem harmless. The difficulty in diagnosing diabetes is that the symptoms begin gradually and diagnosis in the late stage is due to long-term complications. Most of these begin only after a few months after the onset of the disease and will persist in a progressive manner.

The first clinical symptoms of diabetes mellitus are related to the direct effects of high blood sugar levels. The capacity to reabsorb glucose from the renal tubuli does not suffice, and glucose is lost in the urine, accompanied by additional water. Consequently, these people begin to urinate more frequently (polyuria) and this in turn will create an increase in thirst. The patient also experiences a marked weight loss, because excessive calories are lost in the urine, which might be compensated by a hungry feeling. Other symptoms include blurred vision, drowsiness, nausea and decreased endurance during exercise.

One of the complications resulting from high blood glucose levels is development of atherosclerosis, responsible for narrowing both the small and the large blood vessels. Poor circulation to the skin can lead to ulcers and infections, especially of the feet and the legs. Wound healing is compromised because the white blood cells cannot effectively fight infections or participate in physiological healing processes. Eye problems can also be the result of the high blood glucose concentrations. A high amount of sugars in the lens will attract water and cause osmotic imbalance, which eventually lead to cataract formation. Metabolically, ketoacidosis can develop, which may be life threatening. Damage to the nerves can be the result of the high blood glucose levels. It can manifest in several ways, depending on the type of nerve or how many nerves are damaged by the hyperglycaemia. The latter condition is called diabetic neuropathy and will be discussed in more detail in the next chapter (1, 4).

1.2 Diabetic peripheral neuropathy

Diabetic neuropathy is the most common form of peripheral neuropathy in the Western world. Most studies suggest that 50% of patients with a 20-year history of diabetes, of both type 1 and type 2 have neuropathy. The incidence of neuropathy increases with duration of diabetes and is accelerated by poor blood glucose control. It has been documented that it involves similar functional, morphological and metabolic changes in peripheral nerves in both human and animal models of I and type II diabetes (5-7).

Diabetic peripheral neuropathy can affect both the autonomic and the somatic nervous systems, however sensory neuropathy is the most prevalent one and is often simply referred to as diabetic neuropathy. The cell bodies of the primary sensory neurons are located within the dorsal root ganglion (DRG). Their unique characteristics have made them very vulnerable for diabetes-induced damage. Sensory neurons have a relatively leaky blood barrier, high metabolic requirements and as a result need higher levels of local blood flow. Because of these characteristics, they are the primary target of diabetic neuronal complications.

Although the pathogenesis of diabetic neuropathy is still poorly understood, several observations suggest that hyperglycaemia is a major cause of the abnormal nerve function and the pathophysiology of painful neuropathy. In addition, increasing attention has been paid to the pathogenic role of neurotrophic factor deficiency in diabetic peripheral neuropathy.

1.3 Primary sensory nerve fibres innervating the skin

The relatively unspecialized nerve cell endings that initiate the sensation of pain are called nociceptors (*noci* = 'hurt'). Primary afferent sensory neurons are the gateway by which sensory information from peripheral tissues is transmitted to the spinal cord and brain, and innervate peripheral tissue such as the skin. Nociceptors, like other somatic sensory receptors arise from cell bodies in dorsal root ganglia (DRG) (Figure 1-1). Primary afferent axons have widely varying diameters and their size correlates with the type of sensory receptor to which they are attached. There are three major classes of nociceptors in the skin, including Aδ mechanosensitive nociceptors, Aδ mechanothermal nociceptors and polymodal nociceptors, the latter being specifically associated with C fibres. The Aδ fibres are thin, poorly myelinated fibres, whereas C fibres are unmyelinated axons that are arranged in Remak bundles. These bundles are multiple unmyelinated C-fibre axons that are ensheated within a single basal lamina of a Schwann cell. The number of unmyelinated axons in a single Remak bundle may differ from one to more than ten. Nociceptors have the remarkable ability to detect a wide range of stimulus modalities, including those of a physical or chemical nature. To do this, nociceptors express an extremely diverse repertoire of receptors and transduction molecules that can sense noxious stimuli including thermal, mechanical and chemical, albeit with varying degrees of sensitivity (8).
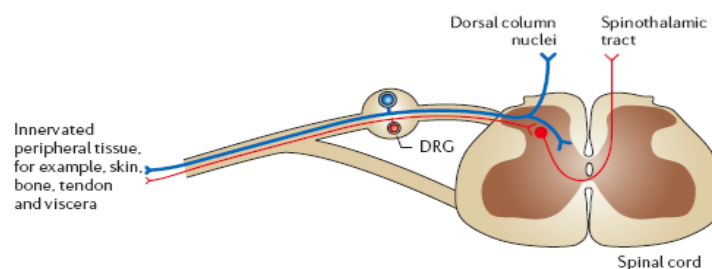
Figure 1-1: Primary afferent sensory nerve fibres innervating peripheral tissue, such as the skin.

The perception of nociceptive stimuli begins with sensory receptors in the skin with unmyelinated sensory nerves terminating in the epidermis. Sensory neuropathies can be classified as large-fibre type and small-fibre type neuropathy, according to the clinical signs. The selective impairment of unmyelinated C and small-myelinated Aδ nerve fibres is referred to as small fibre sensory neuropathies. Diabetic neuropathy is conceptually considered a mixed neuropathy of both large- and small fibre types. Spontaneous and evoked pain, burning, and hyperalgesia (extreme sensitivity to pain) dominate the clinical picture of this type of neuropathy. The simultaneous involvement of large diameter nerve fibres are responsible for paraesthesiae (abnormal skin sensations) and allodynia (exaggerated response to otherwise non-noxious stimulus) (9-11).

1.4 Hyperglycaemia

Diabetic patients exhibit high levels of glucose circulating in their blood plasma (hyperglycaemia). Research indicates that this increased blood glucose level is the most important pathway for eliciting diabetic peripheral neuropathy. Neurons have a high demand for adenosine triphosphate (ATP) to support synthetic and transport activities. Neurons obtain energy through the aerobic breakdown of glucose, and do not maintain glycogen reserves. As a result, these cells are totally dependent on the oxygen and glucose delivered by the circulation, and any interruption in the circulatory supply may damage or destroy neurons.

Diabetic neuropathy is thought to occur through direct hyperglycaemia-induced damage to the peripheral nerves (oxidative stress) or from neuronal ischemia brought about indirectly by hyperglycaemia-induced decreases in neurovascular flow. Hyperglycaemia can produce oxidative stress by four mechanisms, including production of advanced glycation end products (AGEs), overactivation of the polyol pathway, increased production of diacylglycerol (DAG), promoting the overproduction of protein kinase C-β (PKCβ), and the increased hexosamine pathway activity. These pathways will contribute to an increase in oxidative stress, which is a key mediator in the development of diabetic peripheral neuropathy (10, 12).

1.4.1 Intracellular production of advanced glycation end products

Advanced glycation end products (AGEs) are formed at a constant but slow rate during physiological normal conditions, starting in early embryonic development, but accumulate over time. However, their formation is markedly accelerated in diabetes because of increased availability of glucose. Glucose at elevated concentrations undergoes nonenzymatic reactions with primary amino groups of proteins to form glycated residues, called Amadori products. After a series of dehydration and fragmentation reactions, the Amadori products are converted to stable covalent adducts known as AGEs.

One important feature of certain reactive or precursor AGEs is their ability to covalent cross-link proteins. This can alter the structure and function of proteins, in e.g. cellular matrix, basement membranes, and vessel-wall components. Other major features of AGEs are related to their interaction with a variety of cell-surface AGE-binding receptors, followed by their endocytosis. Previous studies have shown that interaction of AGEs with their cell surface binding sites leads to oxidative stress (13-14).

1.4.2 Increased flux through the polyol pathway

The polyol pathway, also called sorbitol-aldose reductase pathway, was found to be an alternative pathway for producing fructose from glucose. (Figure 1-2) Later, the polyol pathway came under suspicion of causing damage in diabetic peripheral nerves by producing pathogenic accumulations of sorbitol, a glucose metabolite.

In the polyol pathway two oxido-reductases are active: aldose reductase (AR) and sorbitol dehydrogenase (SDH). AR normally has the function of reducing toxic aldehydes in the cell to

inactive alcohols. Interacting with their appropriate coenzyme, AR can transform glucose into sorbitol, and SDH can convert sorbitol into fructose. Because AR has a low affinity for glucose, the polyol pathway has only minimal importance in humans with normal glucose levels. In patients with hyperglycaemia, however, the excessive availability of glucose pushes this reaction in favour of aldose reductase activity. As a result, the sorbitol concentration within the cell increases and more fructose will be generated through SDH. When the increased aldose reductase activity is able to deplete reduced gluthathione (GSH), oxidative stress becomes prominent (10, 12, 15).
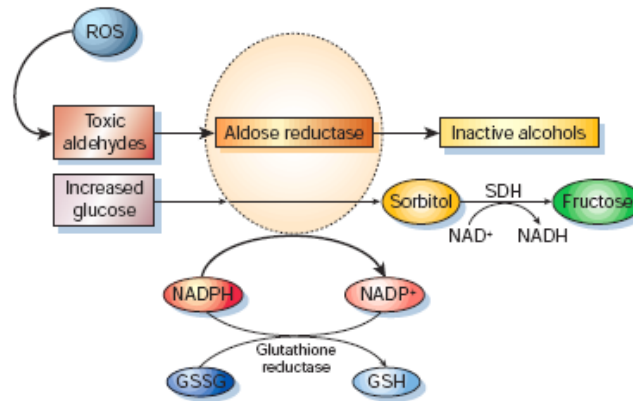
Figure 1-2: Aldose reductase and the Polyol pathway.

1.4.3   Increased production of diacylglycerol and protein kinase C activation

Protein kinase C, with the C referring to its Ca^{2+}-dependency, is a cyclic nucleotide-independent enzyme that can phosphorylate serine and threonine in many target proteins. It is involved in many biochemical processes, including development, memory, differentiation, proliferation and carcinogenesis. Once thought to be a single protein, PKC is now known to comprise a large family of enzymes that differ in structure, cofactor requirements and function.

The PKC family consists of ten isoforms, which vary in tissue expression and cellular compartmentalization. Hyperglycaemia induces an elevation of intracellular glyceraldehyde-3-phosphate. Increased levels of this intermediate can stimulate the synthesis of diacylglycerol, which in turn activates PKC, primarily the β- and δ-isoforms. When PKC is activated by increased intracellular glucose levels it has a variety of effects on gene expression, including an increase in NAD(P)H oxidases which increases reactive oxygen species (ROS) formation (4, 12, 14).

1.4.4   Increased hexosamine pathway activity

When intracellular glucose is high it is metabolised through glycolysis, through which glucose-6 phosphate is converted into fructose-6-phosphate, to complete the glycolytic pathway. However, some fructose-6-phosphate gets diverted into a signalling pathway, which converts it into glucosamine-6 phosphate and finally to uridine diphosphate (UDP) N-acetyl glucosamine. In the nucleus, UDP N-acetyl glucosamine gets put onto serine and threonine residues of transcription

factors. Overmodification by this glucosamine often results in pathologic changes in gene expression and protein function, which together contribute to the pathogenesis of diabetic complications (Figure 1-3) (4, 12).
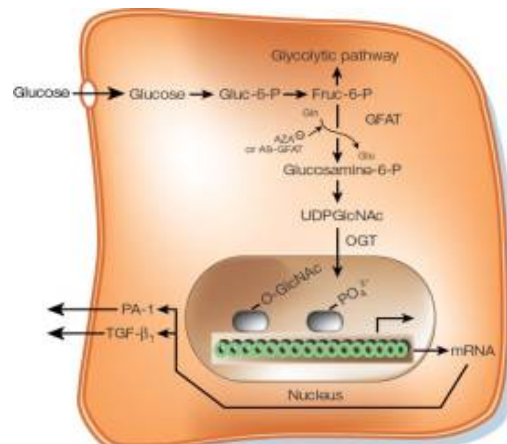
Figure 1-3: The hexosamine pathway.

1.5   Oxidative stress

Each of the four different pathogenic mechanisms results in a single hyperglycaemia-induced process: the overproduction of superoxide by the mitochondrial electron transport chain (ETC).

1.5.1   The mitochondrion electron transport chain

The mitochondrial respiratory chain is located in the inner membrane of the mitochondrion. It is composed of more then 80 peptides, which are organized in four inner membrane enzymatic complexes (I-IV) and the ATP synthase complex (Figure 1-4). The overall action of the mitochondrial respiratory chain is the transformation of electron carriers NADH and $FADH_2$ through the four complexes for the production of ATP from the reduction of oxygen. Superoxide ($O_2^{-\cdot}$) is formed at complex three from electrons released from the electron transfer chain (ETC). Under normal conditions $O_2^{-\cdot}$ is immediately eliminated by natural defence mechanisms. But in a hyperglycaemic state, there is an increased production of electron donors derived from the Krebs cycle (NADH and $FADH_2$). These donors will induce an increased electron transport through the different complexes of the electron transport chain. In turn, this will lead to an overproduction of free radicals and eventually to an increase in oxidative stress. (4, 16-17).
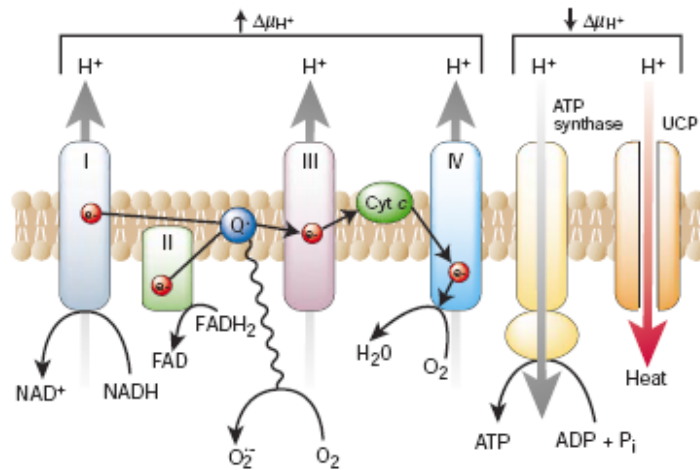
Figure 1-4: Production of superoxide by the mitochondrial electron-transport chain.

The potential mechanism by which hyperglycaemia-induced mitochondrial $O_2^{.-}$ overproduction activates the four pathways of hyperglycaemic damage originates in the glycolysis. Excess $O_2^{.-}$ Partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose over utilization. This will result in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, the activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-*N*-acetylglucosamine increases modification of proteins by *O*-linked *N*-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH (4).

1.5.2   <u>Neuronal damage through increased production of reactive oxygen species</u>

Researchers indicated that glucose-mediated oxidative stress as an inciting event in the development of diabetic neuropathy. In normal conditions, dividing cells can defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases. Because free radicals are highly reactive, excess production can lead to intracellular damage, such as genomic DNA modifications, including genomic instability and mutations. Non-dividing cells, such as neurons, may experience less oxidative damage to their DNA. However, mitochondrial DNA is very susceptible to oxidative damage, which can induce changes in mitochondrial structure and even alter the function of the cell. This is critical for cell survival and energy regulation, an important feature in high energy-requiring neurons.

In addition to DNA damage, ROS can also lead to modifications of proteins and lipids in neurons and can alter their function, by which the normal activity of the neurons is hampered. This can cause a decreased axonal transport and a as consequence, reduced delivery of growth factors and intermediates from synapse to the cell body will result in the induction of apoptosis (16-18).

1.6   Neurotrophic factors

Recent evidence coming from animal models for diabetic neuropathies and human diabetic subjects suggests that the reduced availability of neurotrophic factors may also contribute to the pathogenesis of diabetic peripheral neuropathy. Of these proteins, nerve growth factor (NGF), appears to be important for the development and normal functioning of peripheral neurons, but others, such as glial cell-line derived neurotrophic factor (GDNF) may also be involved (19-20).

1.6.1   The role of neurotrophic factors

During ontogeny, primary sensory neurons are dependent on the presence of neurotrophic factors. The latter play a pivotal role in the regulation of axonal growth and guidance and are essential for the development and maintenance of the vertebrate nervous system. Neurotrophic factors are secreted by target tissue, such as muscle cells, and prevent the initiation of apoptosis of the neuron, thus signalling the neurons to survive. They also induce differentiation of progenitor cells, to form neurons (20-21).

1.6.2   The neurotrophic factor family

Historically, the first growth factor family discovered were the neurotrophins, consisting of NGF, brain-derived neurotrophic factor (BDNF), neurothrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Figure 1-5). A second family of factors is called the glial cell-line derived neurotrophic factor (GDNF) family of ligands (GFL). They consist of four neurotrophic factors: GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Figure 1-6). It has been shown that NGF and GDNF appear to be greatly involved in the processes of diabetes and its complications.

1.6.2.1   *Nerve growth factor*

Nerve growth factor is a neurotrophin that plays a crucial role in promoting growth, differentiation and function of sympathic nerve cells. NGF is involved in a variety of peripheral actions such as tissue inflammation, neuropeptide expression regulation, skin physiology and peripheral tissue regeneration. (Figure 1-5) The action of NGF begins when it is released from the target cells and binds to one of his receptors, TrkA receptor and p75 receptor. Binding of NGF to the TrkA receptor causes activation of the receptor tyrosine kinase and downstream signalling cascades. One of the downstream signalling pathways of NGF activates phospholipase C, releasing DAG and inositol triphosphate (IP3) and activating associated downstream pathways such as protein kinase C, promoting differentiation and survival of neurons. In addition, NGF can also interact with its low affinity receptor, p75, a member of the tumor necrosis factor receptor superfamily. Binding of proteins to the cytoplasmic domain of this receptor can exert effects on apoptosis, survival, neurite elongation and growth arrest. Also, stimulation of p75 can increase the affinity of TrkA for NGF and can enhance its specificity for cognate neurotrophins. As a result, increased ligand selectivity can be conferred on the Trk receptors by the p75 receptor (19, 22).
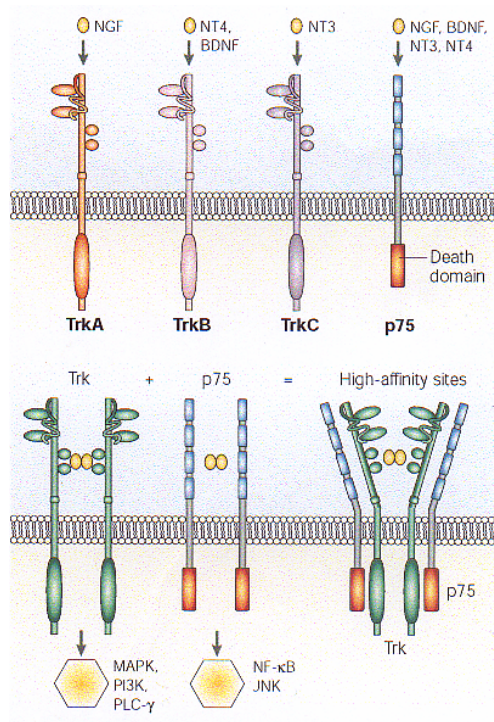
Figure 1-5: Neurotrophin family and their receptors.

1.6.2.2 _Glial cell-line derived neurotrophic factor_

Glial cell-line derived neurotrophic factor is vital to the development and maintenance of neural and other tissues. GDNF (Figure 1-6) was first identified as a survival factor for dopaminergic neurons of the mid-brain. Cells produce it as an inactive precursor molecule. At the cell surface of target cells, a signalling complex is formed, composed of a particular GFL dimer, a receptor tyrosine kinase molecule (RET), and a cell surface bound co-receptor (GFRα1). This binding will trigger trans-autophosphorylation of specific tyrosine residues within the tyrosine kinase domain of each RET molecule. Once phosphorylated, the tyrosine residues in the intracellular domain of activated RET serve as high-affinity binding sites for various intracellular signalling proteins in the target cells, initiating differentiation and survival of neurons (23-24).
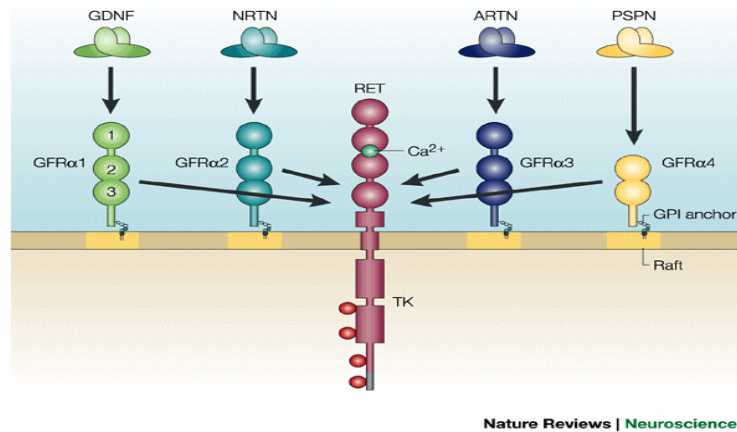
Figure 1-6: GDNF family of ligands and receptors interactions.

1.7 Neuronal stress factors

Previous research indicates that diabetes-induced peripheral nerve injury leads to the expression of several immediate early genes (IEGs) in sensory neurons. The rapid and transient activation of these IEGs occurs in response to a wide variety of cellular stimuli, including nerve damage. They initiate an organized cascade of molecules that have important functions in the neuronal response to injury. The most important IEGs expressed in response to nerve injury are activating transcription factor 3 (ATF3) and c-Jun (25-26).

1.7.1 Activating transcription factor 3

ATF3 is a stress-inducible gene that encodes a member of the ATF/cyclic monophosphate responsive element binding protein (ATF/CREB) family of transcription factors. ATF3 is strongly and selectively upregulated by injured sensory and motor neurons and following peripheral nerve injury. The ATF/CREB family of transcription factors can bind to a consensus ATF/CRE site. According to its counterpart, ATF3 has different transcriptional activities. Unlike the ATF3 heterodimer, the ATF3 homodimer binds to the ATF/CRE consensus site and act as a repressor for transcription. In contrast, the ATF3 heterodimer has been proven to activate specific promotors. Because ATF3 is known to be induced after a variety of stress signals, one suggests that it plays a key regulator in cellular responses (25-29).

1.7.2 C-Jun

C-jun is a transcription factor which expression has been most closely associated with axonal regeneration. It is upregulated by DRG and motor neurons following peripheral nerve injury. c-Jun is a family member of the basic region leucine zipper protein family of transcription factors which homodimerizes or heterodimerizes other proteins after activation of c-Jun. This activation is regulated by c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). After

dimerization the transcription factor activated protein1 (AP-1) is formed. AP-1 mediated gene expression is known to induce apoptosis in many cell types, including neurons (26, 29-30).

1.7.3   ATF3/c-Jun heterodimer

According to previous research, ATF3 and c-Jun are both upregulated following neuronal stress. When ATF3 and c-Jun are co-expressed, they are able to associate physically and form heterodimers. Stress-signals will stimulate the phosphorylation of c-Jun. Consequently, c-Jun will bind to the ATF promoter site and induces ATF3 expression. This expression will generate the activation of heat shock protein 27 (Hsp27). Both ATF3 and c-Jun are necessary for the activation of Hsp27, which directly or indirectly activates Akt. Akt can regulate the cellular survival and metabolism by binding and regulating many downstream effectors. Growth factor mediated cell survival could be promoted by Akt through the Bcl-2/Bcl-X complex and activation of NF-κB, a transcription factor that controls the expression of genes involved in immune responses, apoptosis, and cell cycle. The influence of Akt on the metabolism could be elicited through glycogen synthase kinase-3 (GSK-3) inhibition upon Akt phosphorylation. Possibly by these mechanisms, Akt could inhibit apoptosis and induce nerve elongation (26, 30-31).

1.8   Current therapies for diabetic peripheral neuropathy

Theoretically, the current goal of treating diabetic neuropathy is prevention, reversion of the progression of nerve damage, and reduction of the subjective symptoms. It has been demonstrated by the Diabetes Control and Complications trial (DCCT) that maintaining a tight glycaemic control reduces the risk of neuropathic changes.

In addition to glycaemic control, there are three major classes of medication which are commonly used in the treatment of neuropathic pain, including antidepressants, anticonvulsants, and sodium channel blockers. It needs emphasis that these therapies only treat symptoms of diabetic neuropathies and do not inhibit or reverse the processes underlying the pathology (10, 32).

1.9   Study objective

Despite advances in understanding metabolic causes of diabetic peripheral neuropathy, the use of drug that aim to interrupt these pathological processes have been limited by side effects or the lack of efficacy. Therefore, more research towards the developing mechanisms of diabetic neuropathy is necessary to discover a more specific treatment against this complication.

Before looking for putative treatments of diabetic polyneuropathy, this study evaluated the streptozotocin (STZ)-induced diabetic rat model. STZ is an antibiotic and is diabetogenic due to a selective cytotoxic action upon pancreatic beta cells. For years, this model has been used in an attempt to provide information on underlying processes, and to evaluate potential therapies.

Thereafter, as a primary objective of the present study, a quantification of the neuropathic behavior over time (16 weeks after STZ-injection) of diabetic rats was compared to normoglycaemic control rats. The neuropathy was assessed by quantifying mechanical hyperalgesia and allodynia and by evaluating the rat's global clinical condition. Simultaneously, skin biopsies and dorsal root ganglions (DRGs) were taken at different time points after STZ-injection in order to become an idea of the histological alterations of nerves during diabetic peripheral neuropathy.

The second objective of the present study was to evaluate the diabetic peripheral neuropathy after treatment with neurotrophic factors. After subplantar injection of GDNF and NGF in one hind paw of diabetic animals, neuropathy was evaluated by performing nociceptive tests. In addition, skin biopsies and DRGs are taken after the neurotrophin injections. To screen for an effect on diabetic neuropathy the results of nociceptive tests and skin biopsies of diabetic rats, receiving neurotrophic treatment, were compared to a control group.

2.   **MATERIALS AND METHODS**

2.1   Animal model

Adult male Sprague-Dawley rats (Sprague Dawley®™SD®™ - Harlan Netherlands), weighing 275-320g (controls = 320-350g), were used in all STZ-experiments. After arrival, the animals were allowed to acclimatize to the environment in groups of five, for a period of seven days. Thereafter, the animals were housed in individual ventilated cages at a room temperature of 22 ± 1°C in a 12hr light/dark cycle. All experiments were performed during the light phase. Food and water were available *ad libitum*. The experiment was reviewed and approved by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and the local ethical committee to ensure ethical standards of treatment.

2.2   Induction of Diabetes Mellitus

After overnight starvation, the animals were made diabetic by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, Inc. St-Louis, MO, USA; 65 mg/kg body weight; 1 ml/100g rat), freshly dissolved in MilliQ water, just prior to use. This naturally occurring chemical is known to be very toxic to the beta cells of the pancreas, inducing insulin deficiency. Control rats received an equal amount of vehicle (MilliQ water, 1ml/100 g rat). One week later, diabetes was confirmed in STZ-injected rats by measuring the concentration of glucose in a blood sample obtained by tail prick in the lateral tail vein. This was determined using a test strip and a reflectance meter (Lifescan OneTouch® Ultra® glucometer, Milpitar, California, USA). Glycaemia measurements were taken before and 1, 2 and 12 weeks after induction of diabetes in STZ-injected animals. Glycaemia measurements of control animals were done before and 1 and 12 weeks after injection of vehicle. STZ-injected animals with a blood glucose concentration higher than 300 mg/dl were considered diabetic and were included in the studies.

2.3   Experimental protocol

The goal of a first experiment was to monitor behavioural changes in the model of diabetic peripheral neuropathy in rats. The experiment included one diabetic group (N=16), which was observed for a period of twelve weeks. After two weeks, eight animals were sacrificed for histological analysis of skin biopsies and dorsal root ganglions (DRGs). Control animals were also included in the study and were observed for a period of twelve weeks (N=10). After twelve weeks the other half the diabetic group and control animals were sacrificed for further *in vitro* purposes. Besides this group, another group of diabetic animals were also included in the study in order to evaluate the diabetic nociceptive responses for a period of sixteen weeks. The nociceptive tests and clinical observations were performed on a weekly basis for both groups. This experiment will

document sensory changes associated with development of the diabetic peripheral neuropathy in rats.

In a second experiment, diabetic animals were given a subplantar injection of neuroptrophic factor in the left hind paw. The first group of animals (*N*=10) was given a subplantar injection of NGF. The second group of animals (*N*=10) received a subplantar injection of GDNF. Also, a control group of diabetic animals (*N*=8), which were given a subplantar injection of vehicle (phosphate buffered saline, PBS), was included in the study. By injecting these growth factors in the plantar surface of the left hind paw, an improvement or worsening of the neuropathy in diabetic animals can be observed by determining their nociceptive thresholds in the different tests (section 2.6).

2.4   Mechanical nociceptive testing

2.4.1   Experimental design

The plantar surface of the hind paws was tested for mechano-allodynia and mechano-hyperalgesia. The nociceptive flexion reflexes were quantified before and after STZ-injection for a period of 12 weeks. Both tests generate a mechanical force to the hind paw of the rat. In this way the sensory threshold can be determined. Prior to treatment with vehicle or STZ, two baseline sessions for each test were performed on separate days. Both performances were averaged to obtain baseline values. After treatment, nociceptive thresholds were measured twice a week and were averaged to obtain a weekly objective measure.

2.4.2   Hind paw mechano-allodynia

Mechanical allodynia of the hind paw was assessed using the von Frey test. Rats were placed in individual, elevated wire-mesh floored, plastic cages (20 x 12 x 15 cm) to allow insertion of the mechanical probe against the plantar surface of the hind paw. The diabetic and the control animals were allowed to habituate at least 30 minutes prior to behavioural testing. A mechanical force transducer and a metal probe (electronic von Frey transducer, SOMEDIC) were used to evoke a withdrawal response. The pressure applied with the von Frey probe (1.0 mm of diameter) is gradually increased perpendicular to the mid-plantar surface of both hind paws. The stimulus is continued until the hind paw is withdrawn or slowly elevated such that the force levels off. The peak force in gram is recorded, with a minimum requirement of 10.0g. Paw withdrawal due to locomotion or weight shifting were not counted and such trials were repeated. The mean force to evoke the withdrawal response was calculated from three measurements for both hind paws.

2.4.3   Hind paw mechano-hyperalgesia

To assess mechano-hyperalgesia, the paw pressure test (Ugo Basile analgesymeter, Biological Research Apparatus, Varese, Italy) was used. This device generates a mechanical force that increases linearly with time. The mechanical pressure was applied to the dorsal surface on the left and right hind paw by a cone-shaped plunger (diameter 1.4mm). The nociceptive threshold is defined as the force, in grams, at which the rat attempts to withdraw its paw or until vocalization. The cut-off pressure was set up to 250g. The left and right flexion reflex for each animal was measured once during every test.

2.5   Clinical evaluation

To monitor the general health of the animals, body weights were recorded and clinical condition scores were obtained before and after STZ- or vehicle injections. Every animal was individually scored by looking at the different diabetic symptoms. These clinical symptoms include the level of polyuria, porphyrins on eyes and nose, desquamated skin, loss of body weight, diarrhoea and cataract. Next, a score was given as follows: 0, death; 1, heaving all the prescribed symptoms; 2, heaving 5/6 of the prescribed symptoms; 3, heaving 4/6 of the prescribed symptoms; 4, heaving 3/6 of the prescribed symptoms; 5, 2/6 of the symptoms and 6, heaving 1/6 of the prescribed symptoms. If the clinical condition of an animal extremely declined in which the experimental results are to be questioned, the animal was excluded from the study. Control animals were also evaluated for their clinical conditions. Due to the great difference in clinical health of diabetic versus normal healthy rats, the control rats were given a score of 10.

2.6   Neurotrophic factors

In a second experiment, another group of STZ-induced animals were used. Two weeks after STZ-injection, these diabetic rats received a subplantar neurotrophic factor treatment in the left hind paw. Before, the animals were tested twice a week with the von Frey and the paw pressure test to assess their mechanical nociceptive flexion reflexes. One group of animals (N=10) received a NGF subplantar injection of 50 μl in the left hind paw. Human-β NGF (PREPROTECH, INC. Rocky Hill, NJ) was administered at a concentration of 1mg/ml (1/1 PBS, +Ca +Mg). The second group (N=10) received a GDNF subplantar injection of 50 μl in the left hind paw. Human GDNF (PREPROTECH, INC. Rocky Hill, NJ) had a concentration of 1mg/ml (1/1 PBS, +Ca +Mg). A control group (N=8) was also included in this experiment. These animals received an injection of phosphate buffered saline (PBS + Ca + Mg; 50 μl) in the left hind paw. The NGF group received subplantar injections at day 19, 20 and 23 (no injections at day 21 and 22, due to an increased inflammatory response) after STZ injection, the GDNF group and the control group received injections at day 19, 20, 21, 22 and 23 after STZ injection. Twelve hours after every injection, the nociceptive flexion reflexes were quantified with the von Frey and the paw pressure test. At day 24

after STZ-injection, five animals of the NGF and the GDNF group and four animals of the PBS group were sacrificed to obtain skin biopsies. The other half of each group was further characterized with the von Frey and paw pressure test.

2.7   Analysis of skin biopsies

Skin biopsies of the plantar surface of the left hind paw of rats in the first experiment (N=4, 2 weeks; N=5, 16 weeks) and of both hind paws for the second experiment (N=5, GDNF & NGF; N=4, PBS) and DRGs (first experiment) are dissected and fixed in acidic formalin by immersion overnight at room temperature. Subsequently, tissue samples were dehydrated and paraffin embedded. Skin biopsies and DRGs were further analysed at HistoGeneX NV.

2.7.1   Immunohistochemistry for paraffin sections

All tissues were sectioned at 4 levels (100µm inter level distance) at 6 µm. Routine morphological evaluation and selection of appropriate levels (#3) of all tissue sections was performed on Hematoxylin Phloxin Saffrane (HPS) stained sections. All sections (3 levels per case) are deparaffinised and rehydrated prior to immunohistochemistry. To control for cross-reactivity caused by aspecific binding, negative controls were included by performing the immunostaining without adding primary antibodies to the sections. All reactions were negative confirming the non-interference of the secondary antibody. The epidermal thickness was measured on 3 levels, in 2 areas per level, on images made with a 40x objective.

2.7.2   Immunofluorescence single labeling

Immunofluorescence stainings require enzymatic pre-treatment (Trypsin, 10 minutes at 37°C), followed by heat mediated microwave antigen retrieval using citrate (15 minutes at 93°C). Incubation with the primary rabbit polyclonal anti-protein gene product 9.5 (PGP9.5) antibody (UltraClone Limited, England) (1/500 in PBS/BSA) was performed overnight at room temperature. Subsequently, sections were rinsed and incubated with Cy3 conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., PA, USA) (1/500) for 2 hours at room temperature and counterstained with Hoechst (Invitrogen Corporate, Molecular Probes, California, USA) (1/2000 in Aqua Destillata (AD)).

2.7.3   Double Labeling Immunofluorescence

Double immunofluorescence histochemistry was performed for ATF3 expression in neuronal nuclei (NeuN). Double labelling immunofluorescence requires enzymatic pre-treatment (Trypsin, 10 minutes at 37°C), followed by heat mediated microwave antigen retrieval using citrate (15 minutes at 93°C). Sections were incubated with primary mouse monoclonal anti-NeuN (Chemicon International, Serologicals Corp.)(1/100 in PBS/BSA) and rabbit polyclonal anti-ATF3 antibodies

(Santa Cruz Biotechnology, Inc) (1/5000 in PBS/BSA), overnight at room temperature. Sections were subsequently rinsed and incubated with biotin labelled goat anti-rabbit antibody (Vector Laboratories, Inc., USA) for 30 minutes at room temperature followed by Alexa Fluor 555 conjugated streptavidin antibody (Invitrogen Corporate, Molecular Probes, California, USA) (1/500) and Alexa Fluor 488 conjugated goat anti-mouse IgG (Invitrogen Corporate, Molecular Probes, California, USA) (1/100) for 2 hours at room temperature and counterstained with Hoechst (Invitrogen Corporate, Molecular Probes, California, USA) (1/500 in AD).

2.7.4   Morphological analysis and data acquisition

All morphological analysis and data acquisitions were performed on an Axioplan-2 system (Carl Zeiss, Germany) with the integrated image analysis software package, Axiovision Release 4.6. Virtual images were produced using a Zeiss Mirax system.

2.8   Data analysis

Data are presented as mean ± S.E.M., after normalisation to pre-values and/or body weight. When appropriate two-way ANOVA (Tukey post-hoc), Wilcoxon Mann-Whitney (Rank sums) or t-test was used to screen for statistical significance between control and treatment groups ($P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$). Histological data were analysed with the Wilcoxon/Kruskall-Wallis test (Rank Sums) to screen for significant differences between control and treatment groups.

3. __RESULTS__

3.1 Body weight and blood glucose level

As described by previous studies using this animal model (33-36), body weight decreased rapidly in STZ-treated diabetic rats. Blood glucose levels increased three days post-STZ injection. The present study evaluates these parameters over a period of twelve weeks.

Measurements of body weight and blood glucose levels for rats of the two experimental groups are shown in figure 3-1 and table 3-1. Body weight increased normally in control rats, while diabetic rats showed a significant decrease in body weight as soon as one week post-STZ injection (pre: 314,25 ± 3,20g to 309,63 ± 5,69g; $P<0,001$). A progressive loss of body weight was noted in the diabetic group during the next weeks (12w diabetes: 267,6 ± 15,97g; $P<0,001$). The maximum decrease in body weight (18%) was reached after 9 weeks STZ-injection (9w diabetes: 261,13 ± 15,86g). Blood glucose levels increased to the maximum measurable value of 600mg/dl as soon as the first week after diabetes induction, statistically significant from control animals ($p<0,001$). At the end of the twelfth week testing period, diabetic animals showed blood glucose levels significantly higher than the control rats ($p<0,001$). In contrast, control animals remained normoglycaemic during the entire testing period of 12 weeks post-vehicle injection.

As expected, diabetic animals showed a significant decrease in body weight as soon as the first week post STZ-injection and remained significant during the entire testing period. Blood glucose levels were strongly increased after one week and remained high at least twelve weeks after STZ-injection.
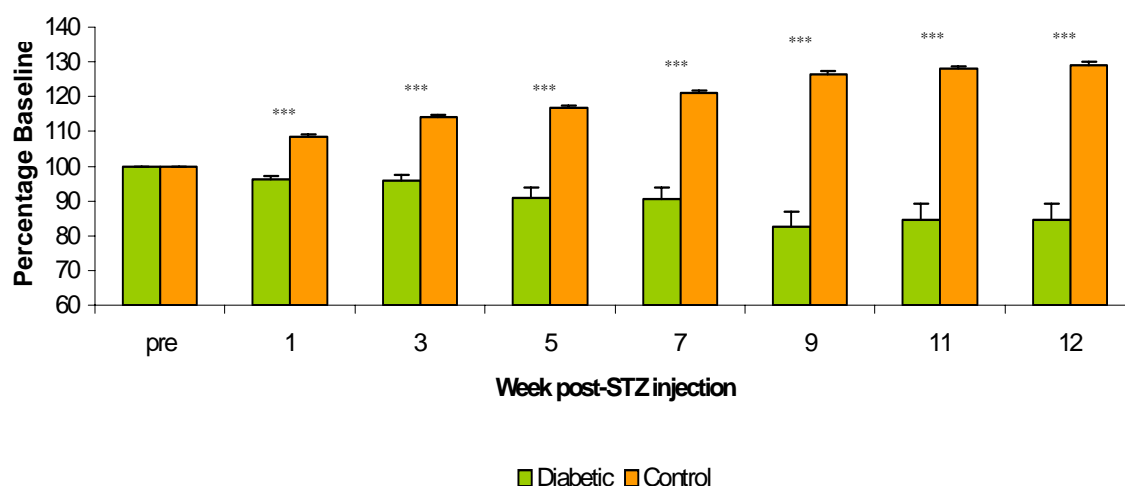
Figure 3-1: Body weight of diabetic rats (N=16) versus control animals (N=10) measured at different time points after STZ- or vehicle injection. Significant differences of body weight between diabetic and control animals was noted after one week STZ-injection and remained until the end of the twelfth week testing period. Body weight of control animals gradually increased over time. Data are represented as mean ± S.E.M., after normalisation to pre-values (%). Significant difference of each time point between diabetic and normoglycaemic control animals was determined using two-way ANOVA (Tukey post-hoc); $P <0,05^{*}$, $P<0,01^{**}$, $P<0,001^{***}$.

Table 3-1: Blood glucose measurements of diabetic rats (*N*=16) compared with control group (*N*=10). Blood glucose of STZ-induced animals increased significantly as soon as one week after diabetes onset. Control animals remained normoglycaemic during the entire testing period. Data are represented as mean ± S.E.M. Significant difference of each time point between diabetic and normoglycaemic control animals was determined using two-way ANOVA (Tukey post-hoc); $P < 0,05^{*}$, $P < 0,01^{**}$, $P < 0,001^{***}$.

	Blood glucose (mg/dl)			
Animals	**Pre**	**Week 1**	**Week 2**	**Week 12**
Control	116,7 ± 2,2	120,2 ± 2,7		112,2 ± 1,5
Diabetic	114,6 ± 2,1	591,9 ± 5,3 ***	596,4 ± 3,2	595,9 ± 3,6 ***

3.2   Clinical conditioning score

Other researchers (35-36) have reported that the STZ-induced diabetic model of peripheral neuropathy will produce severe illness in rats, displayed by a reduced growth, polyuria, diarrhoea, cataract and reduced activity. Our study uses these characteristics to score the individual health status of diabetic and control animals.

Animals treated with STZ, appeared chronically ill and displayed polyuria, and soft stool/diarrhoea. One animal from the diabetic group was removed from the study, due to severe clinical status. Criteria for removal were subjective, but parameters included the bad overall appearance, the severe lack of activity and the excessive weight loss. With these criteria in mind, animals were given a subjective score (Figure 3-2). Polyuria was already visible three days after STZ-injection. After two weeks, other symptoms also increased visibly and progressed over the following weeks. Already at week ten, two animals in the diabetic group displayed cataract at the left eye. After 12 weeks, 70% of the diabetic group suffered from severe cataract on both eyes. All symptoms were significantly ($P<0,001$) more apparent within the diabetic animals as compared to the control group. However, due to age, control animals also displayed some desquamated skin and increased red-brown porphyrines on the skin after 10 weeks STZ-injection.

Our results are consistent with previous reports suggesting that STZ-induced diabetes in rats induces a chronic illness. The STZ-induced diabetes resulted after three days in an excessive urination, followed by increased weight loss, diarrhoea, a severe lack of activity and cataract in the following weeks.
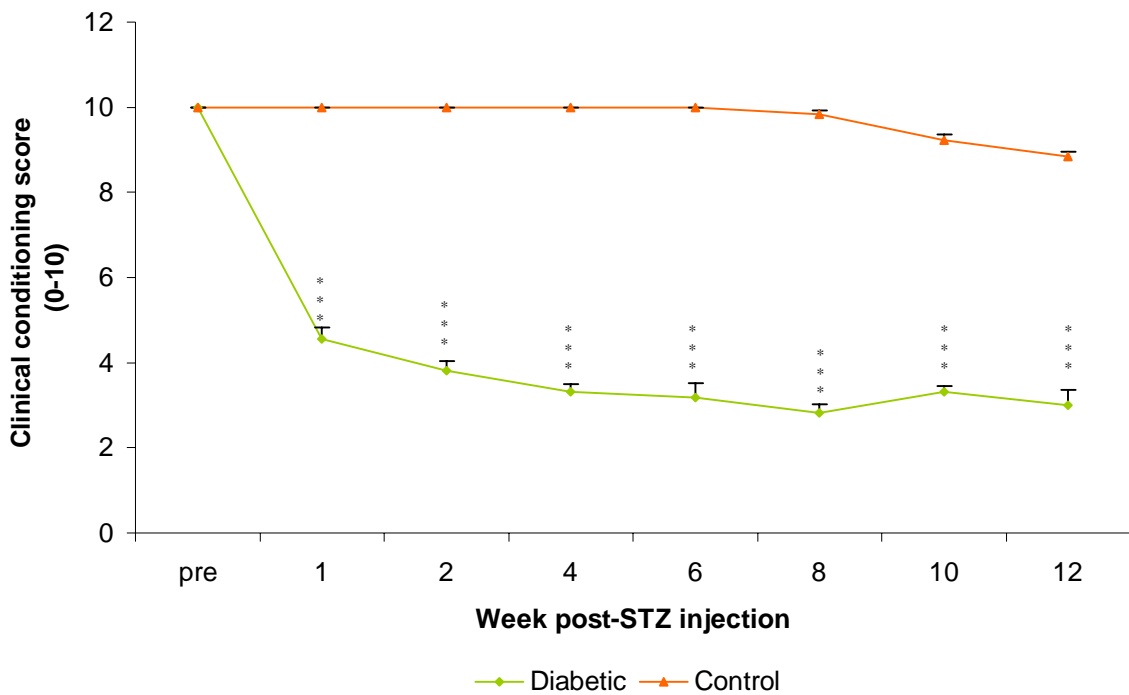
Figure 3-2: Clinical conditioning score of diabetic animals (*N*=16) versus control animals (*N*=10). Score was given ranging from 6 (1/6 diabetic symptoms) to 1 (6/6 diabetic symptoms). A significant decrease in general health of diabetic animals was reached one week post STZ-injection and remained until the end of the twelfth week testing period. Control animals were given a score of 10 at the beginning of the testing period. After eight weeks control animals displayed clinical signs of age and therefore were given a score of nine. Data are represented as mean ± S.E.M. Significant difference of each time point between diabetic and normoglycaemic control animals was determined using two-way ANOVA (Tukey post-hoc); $P < 0,05^*$, $P < 0,01^{**}$, $P < 0,001^{***}$.

3.3 Nociceptive testing 12 weeks after STZ-injection

After rats were rendered diabetic with STZ, mechanical thresholds were monitored. The nociceptive flexion reflexes of the diabetic and control animals were tested for a period of twelve weeks. The results of the mechanical nociceptive tests are shown in figure 3-3 and 3-4.

3.3.1 Hind paw mechano-allodynia

Lynch III *et al* (36) have demonstrated that streptozotocin diabetic rats showed a marked tactile allodynia when exposed to light touch on the plantar surface of the hind paw, within one week of the onset of diabetes and remaining for up to 14 weeks post-treatment. In our study, the nociceptive reflexes are observed for a period of twelve weeks post-STZ.

Figure 3-3 represents the mechanical allodynia in diabetic versus control animals as measured with the von Frey test. Both groups were tested on a weekly basis for a period of twelve weeks, beginning at one week post-STZ treatment. Before injection of STZ (diabetic group) or

vehicle (control group), there was no significant difference in baseline mechanical nociceptive thresholds observed between both groups (pre diabetic: 69,96 ± 5,31g, pre control: 63,14 ± 2,30g; *P*>0,05). After STZ-injection, a significant increase of hypersensitivity in diabetic rats was evident one week post-STZ injection (1w diabetic: 54,23 ± 3,95g, 1w control: 109,72 ± 4,59g; *P*<0,001). These significant allodynic responses remained at least eight weeks after onset of diabetes. Nociceptive threshold of control animals remained stable for at least eight weeks post-treatment. At nine weeks, control animals appeared to be hypersensitive, compared to baseline (9w control: 67,21 ± 2,23g; *P*=0,589). These slightly allodynic responses in control animals remained until the end of the twelfth week testing period. Because of this hypersensitive reaction in control animals, diabetic animals didn't display significant values of allodynia compared to the control group, with the exception of week ten (10w diabetic: 46,52 ± 2,52g; *P*=0,029). By comparing the results of the diabetic animals with the baseline level (100%), no significant allodynic response at nine weeks (9w diabetic: 52,20 ± 1,18g; *P*=0,887) was observed and remained so for at least twelve weeks, with the exception of week ten (*P*=0,015).

Our findings suggest that increased allodynic responses are still present eight weeks post-STZ injection, confirming the previous report. However, after eight weeks the diabetic animals tended to have decreased sensitivity responses for up to twelve weeks compared to control animals. Vehicle-treated rats showed normal withdrawal thresholds, but an increase in hypersensitivity was seen after 8 weeks testing period.
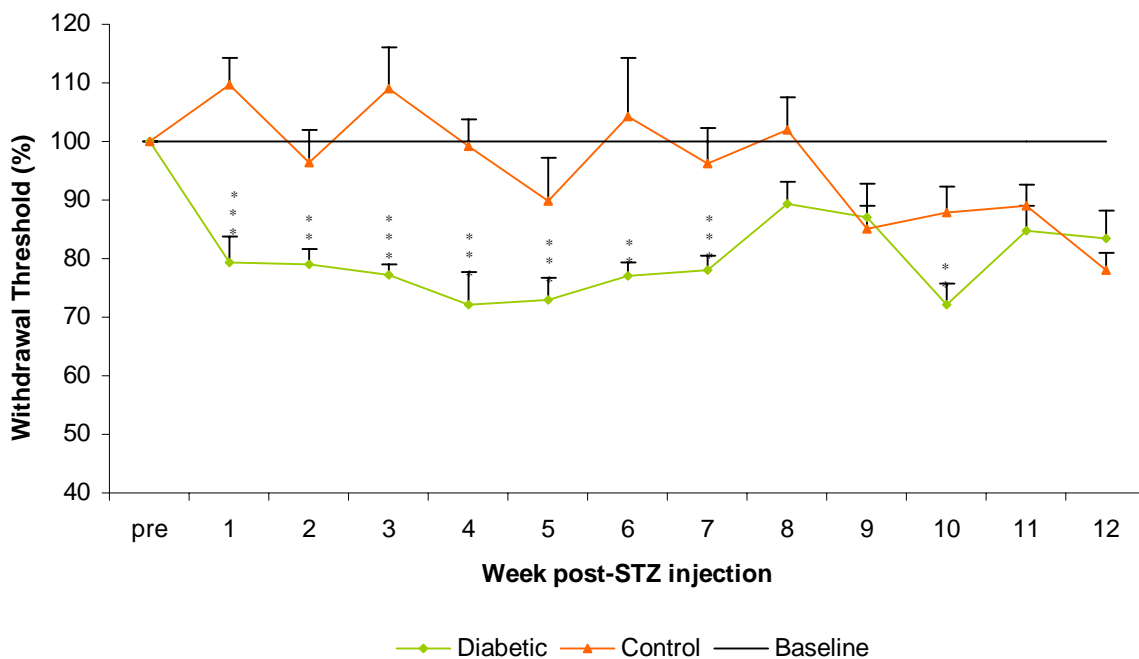
Figure 3-3: The percentage withdrawal threshold in STZ-induced diabetic rats using the von Frey test. Diabetic animals (*N*=16) showed a significant decrease in mechanical threshold as soon as one week post STZ-injection and remained below threshold level until 12 weeks after diabetes onset. Control animals (*N*=10) showed stable nociceptive responses for at least 8 weeks post-injection vehicle. Thereafter, an allodynic response was seen until the end of the testing period. Data are represented as mean ± S.E.M. (%), after normalisation to body weight and pre-values. Statistical analysis between diabetic and control animals for each time point was performed using two-way ANOVA (Tukey post-hoc); *P* <0,05[*], *P*0,01[**], *P*<0,001[***].

3.3.2 Hind paw mechano-hyperalgesia

In rats, a single systemic injection of streptozotocin induced a hyperalgesic reaction observed after seven days after the onset of diabetes as reported by Ahlgren *et al* (39). This present study evaluates this hyperalgesic reaction for a period of twelve weeks post-STZ.

Our results indicate a significant difference in hyperalgesic responses in diabetic versus control animals after one week post-STZ injection ($P<0,001$) (Figure 3-4). Pre-values between control and diabetic animals were not significantly different (diabetic: 103,13 ± 7,98g, control= 94,38 ± 6,63g; $P>0,05$). The hyperalgesia became more pronounced with time, reaching a maximum at 10 weeks with a 53% ± 8,95 reduction in paw withdrawal threshold. By comparing the post-values with the pre-value within the diabetic group, a significant reduction in mechanical threshold was first noticeable after four weeks post-STZ injection (4w diabetic: 51,57 ± 3,02g; $P=0,013$). Control animals displayed stable withdrawal responses for the remainder of 12 weeks post vehicle-injection. With the exception of the first week after vehicle-injection, at which control animals displayed a significant decrease in hyperalgesia compared to the diabetic group ($P=0,008$).

The present findings conclude that diabetic neuropathy leads to an increased hyperalgesic response for the period of twelve weeks post-treatment, compared to control animals.
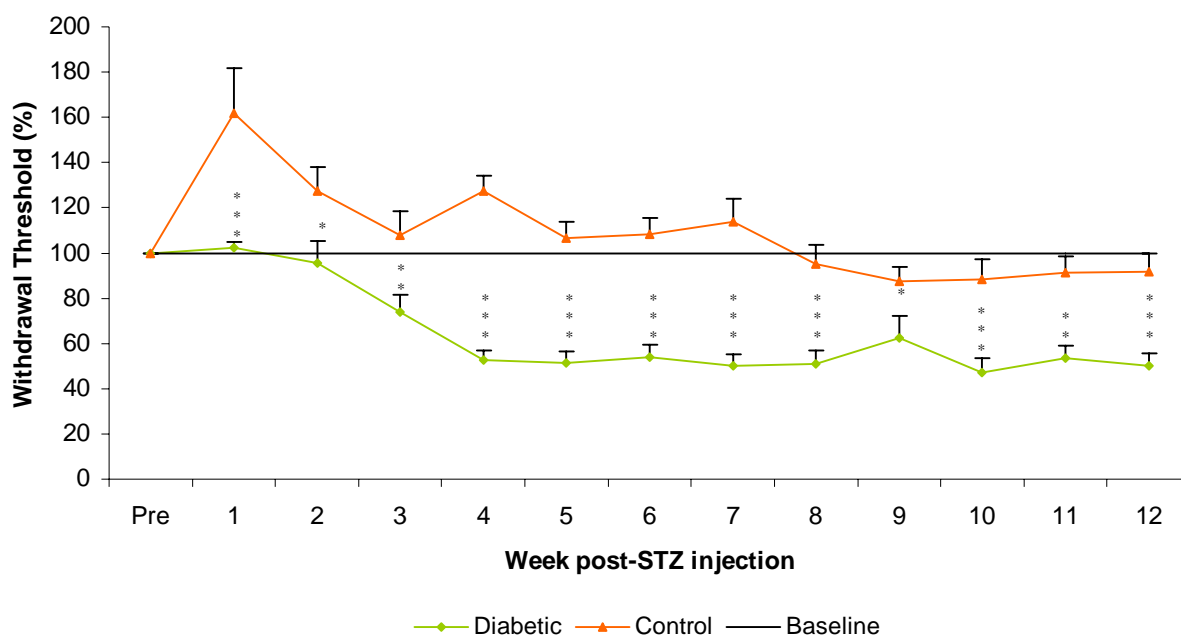
Figure 3-4: The percentage withdrawal threshold in STZ-induced diabetic rats using the paw pressure test. Compared to control animals, diabetic animals ($N=16$) showed a significant hyperalgesic response as soon as one week post-STZ injection and remained significant during the entire testing period of twelve weeks. In contrast, control animals ($N=10$) remained stable around threshold level during the entire testing period. Data are represented as mean ± S.E.M. (%), after normalisation to pre-values. Statistical analysis was performed between diabetic and control animals for each time point using two-way ANOVA (Tukey post-hoc); $P <0,05^{*}$, $P0,01^{**}$, $P<0,001^{***}$.

3.4   *In vivo* evaluation of peripheral neuropathy after 16 weeks STZ-injection

In humans, diabetic neuropathy is described as a long term complication of DM. Therefore, this study included another group of diabetic animals and evaluated them for a period of 16 weeks post-STZ injection.

Figure 3-5 resumes the behavioural results together with body weight and blood glucose of 16 week diabetic rats versus control animals. After 16 weeks, a slight increase in hypersensitivity was seen in the von Frey test (16w diabetic: 49,17 ± 3,45g, control: 65,91 ± 2,15g; *P*=0,082). In addition, the results of the paw pressure test show a significant increase in hyperalgesia as compared to control animals (16w diabetic: 56,39 ± 4,59g, control: 105,25 ± 5,48g; *P*<0,001). Next, body weight decreased and remained significantly lower than control animals (16w diabetic: 274,10 ± 9,35g; *P*<0,001). In the same way, blood glucose levels were significantly higher (16w diabetic: 591,80 ± 5,25mg/dl; *P*<0,001) compared to control animals.

These data suggest that peripheral neuropathy, illustrated by an increase in allodynic and hyperalgesic responses, remained present in diabetic animals after 16 weeks post-STZ-injection.
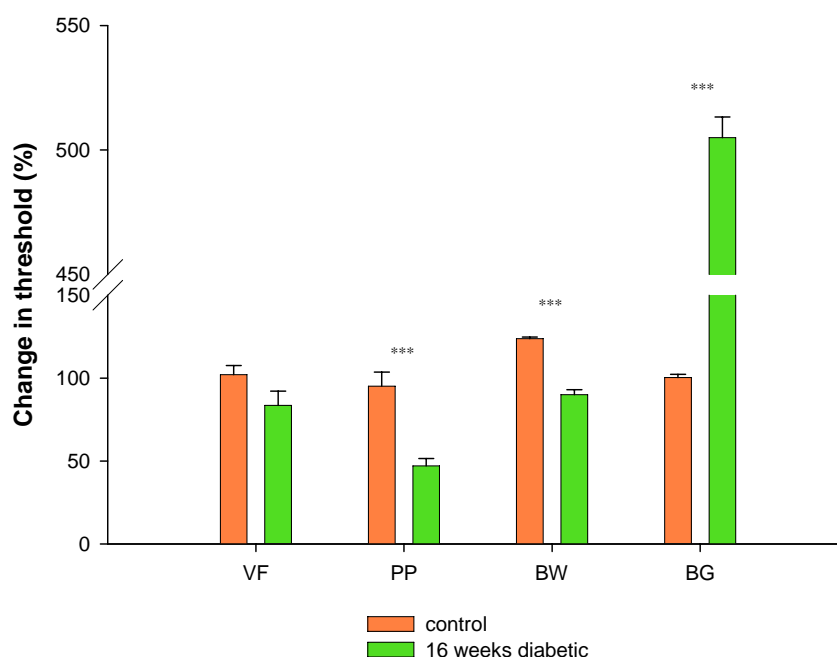
Figure 3-5: Long-term observation in 16 week diabetic animals (*N*=10) versus control animals (*N*=10). Data show a decrease in percentage mechanical nociceptive threshold for both behavioural tests at sixteen weeks post STZ-injection. The decrease in body weight and increased blood glucose level was still evident, sixteen weeks after diabetes onset. Data are represented as mean ± S.E.M. (%), after normalisation to pre-values and/or body weight. When appropriate t-test (VF & PP) or Mann-Whitney (Rank Sums) (BW & BG) was used to screen for statistical significance between diabetic and control animals. *P* <0,05[*], *P*0,01[**], *P*<0,001[***]. VF= von Frey; PP= paw pressure; BW=body weight; BG= blood glucose.

3.5   Skin biopsies of different time-points after STZ-injection

After two and sixteen weeks post-STZ treatment, animals were sacrificed and skin biopsies (*N*=4, 2 weeks; *N*=5, 16 weeks) and DRGs (*N*=2, 16 weeks) were processed for immunohistochemistry. Consequently, histological evaluation of average epidermal thickness, amount of small and large neurons, ATF3-positive neurons and PGP9.5 quantification was performed on these tissues.

3.5.1   Average epidermal thickness

The perception of nociceptive stimuli begins with sensory receptors in the skin with unmyelinated sensory nerves terminating in the epidermis (11, 37-38). It had been noticed that diabetic rats showed a thinner skin as compared to control animals. It could be that the epidermal fibres are more located on the surface when thinning of the skin in diabetic animals occurs. This could signify the hypersensitivity effect in STZ-injected rats. Therefore, the average epidermal thickness was first measured to screen for differences among diabetic and control animals.

Figure 3-5 represents the epidermal thickness of diabetic (*N*=4, 2 weeks; *N*=5, 16 weeks) versus control rats (*N*=4). Significant reduction (2w diabetic: 69,96 ± 1,50µm, control: 78,25 ± 3,93µm; *P*=0,0304) in epidermal thickness was observed 2 weeks after STZ-injection. The reduction was more pronounced 16 weeks after the onset of diabetes (16w diabetic: 63,58 ± 2,64µm; *P*=0,0200) compared to control group. When comparing both diabetic groups, a slightly higher epidermal thickness in 2 weeks was noticed (*P*= 0,0662).

These results clearly indicate that reduced epidermal thickness is a result of STZ-injection and is apparent early in diabetes and is even more pronounced 16 weeks post-STZ injection.
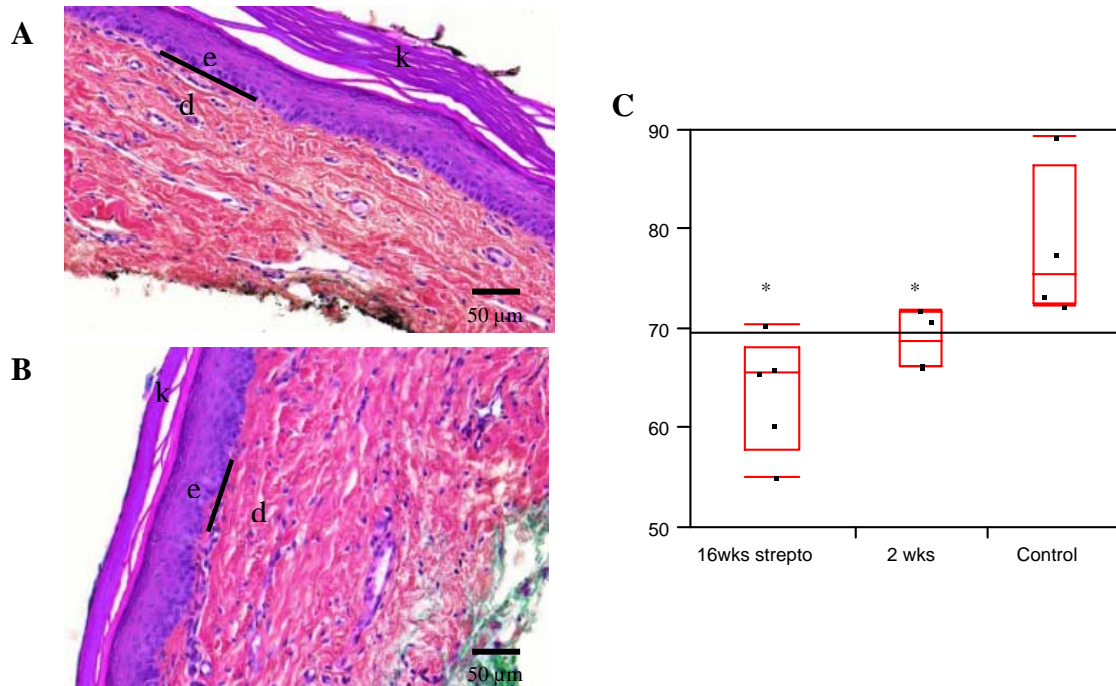
Figure 3-5: Epidermal thickness of the plantar surface of the hind paw of diabetic rats and control rats. Miramax images of HPS stained slides (40x objective; A, B). Epidermal thickness diabetic rats (**A**, 16 weeks) versus normoglycaemic control rats (**B**). The boundary between the epidermis (e) and the dermis (d) is marked by a line, layer of keratinocytes (k). **C**. Graphic presentation of the epidermal thickness in two weeks and sixteen weeks diabetic animals versus control rats. Statistical significance between diabetic and control group was determined using the Wilcoxon/Kruskal-Wallis test (Rank Sums). $P < 0,05^{*}$, $P 0,01^{**}$, $P < 0,001^{***}$.

3.5.2 Amount of small and large neurons

The *in vivo* results of the diabetic group 16 weeks post-STZ indicate a higher nociceptive threshold compared to control animals. Previous reports have stated that the hyperalgesia and allodynia of diabetic peripheral neuropathy is related to a decrease in DRG neurons (39). Therefore, this study evaluates the amount of neurons present in DRGs of long-term diabetic rats (16 weeks) compared to control group.

Figure 3-6 shows the amount of neurons present in the lumbar DRGs of 16 week diabetic rats (*N*=2) and normoglycemic controls (*N*=2). There was no significant difference seen in the amount of total neurons in diabetic versus control group (16w diabetic: 136,67 ± 13,17, control: 175,83 ± 2,77; *P*=0,1282). The percentage small and large neurons did not differed significantly between 16 weeks and control group. (*P*=0,128, *P*=0,173).

These results suggest that the amount of neurons present in these DRGs of diabetic rats is not significantly different from control group. Also, the amount of small and large neurons present in diabetic DRGs did not differ significantly compared to the DRGs of the control group.
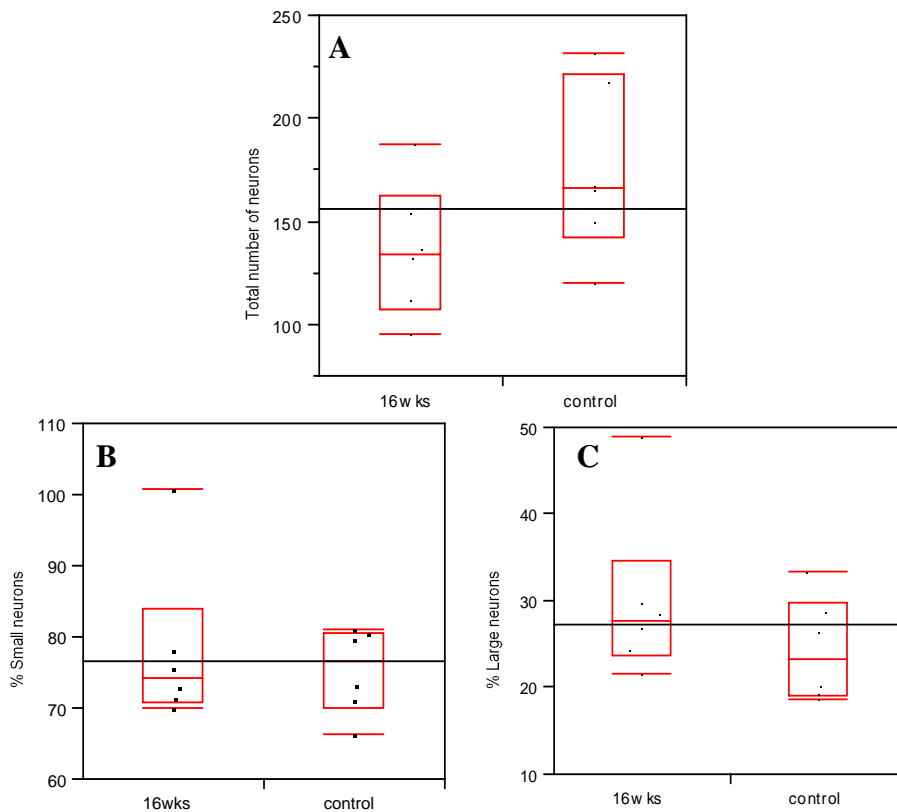
Figure 3-6: Total number of neurons present in DRGs of 16 week diabetic rats versus control rats. **A**. No significant difference was seen in the amount of total neurons between both groups. **B** Total number of small neurons per 10000 μm^2 (%). **C** Total number of large neurons per 10000 μm^2 (%). No difference in small and large neurons between 16 week diabetic rats versus control rats. Statistical significance between groups was assessed using Wilcoxon/Kruskal-Wallis test (Rank Sums). $P < 0.05^*$, $P 0.01^{**}$, $P < 0.001^{***}$.

3.5.3 ATF3 positive neurons in dorsal root ganglions

Although the pathogenic mechanism of diabetic peripheral neuropathy is not fully understood, reports have stated that the pathology of diabetic rats is marked by the loss of a population of neurons located in the lumbar dorsal root ganglia (39). Similar results were published, examining the level of ATF3 expression in DRG neurons isolated from diabetic mice, where an up-regulation of ATF3 expression was shown after onset of diabetes (25).

In our study, the lumbar dorsal root ganglia (L4, L5, L6) were dissected and the ATF3 expression of these DRGs isolated from 16 week old diabetic rats (*N*=2) and normoglycaemic controls were quantified. A histological evaluation on DRG sections was performed. The neurons were made visible using anti-NeuN antibody (neuron, green) and Hoechst staining was used as a counterstaining for the nucleus (blue). Additionally, an anti-ATF3 antibody was used to stain the nucleus (red) of neurons indicative of neuronal stress. Diabetic animals showed only a slight increase in the amount of ATF3 positive neurons (0,64%) as compared to control group (0,13%) (*P*=0.0604).

Our results show no significant differences in ATF3-positive neurons in DRGs from 16-week-old diabetic rats compared to control group.
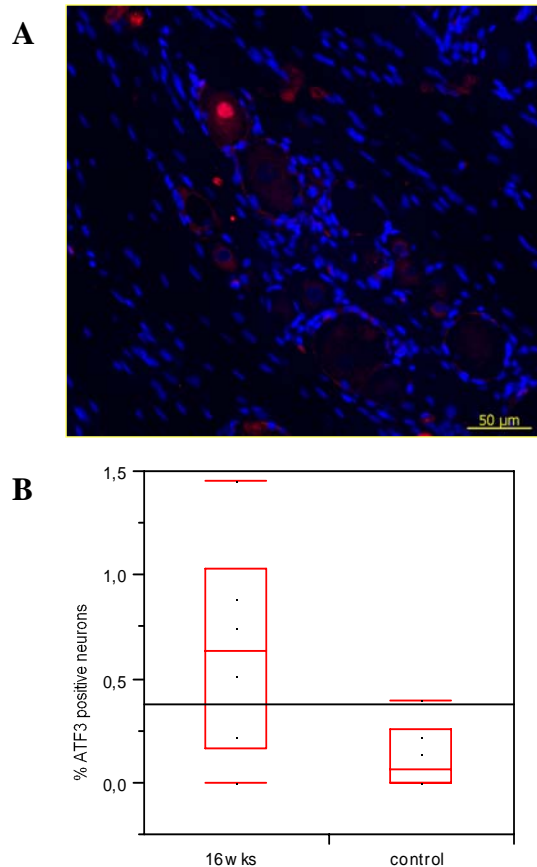
Figure 3-7: ATF3 positive neurons in DRGs of 16w old diabetic rats ($N=2$) versus control animals ($N=2$). **A**. Mozaïk images showing ATF3-positive neurons (red nucleus) in DRGs of 16-week-old diabetic rats. **B** Graphic presentation of ATF3 positive neurons present in DRGs of 16-week-old diabetic rats versus control animals ($N=2$). A slight increase was seen in the amount of ATF3-positive neurons in 16 week diabetic animals versus control rats. Statistical significance between diabetic and control animals was determined using the Wilcoxon/Kruskal-Wallis test (Rank Sums). $P < 0,05^*$, $P0,01^{**}$, $P<0,001^{***}$.

3.5.4 PGP9.5 quantification of nerve fibres

Intraepidermal nerve fibre (IENF) densities are reduced in patients with diabetic peripheral neuropathy. This suggests that epidermal nerves are affected in diabetes and skin biopsy is useful in evaluating small fibre sensory neuropathies of diabetes. However, there is a lack of direct evidence regarding the correlation between epidermal denervation and diabetes duration. Langerhans cells (LCs) are dendritic cells abundant in epidermis, which play a pivotal role in the immune system and could play a role in nerve denervation. Therefore we examined the epidermal nerve fibre density and the presence of LCs in a long-term diabetic model (16 weeks). PGP9.5 immunocytochemical labelling was used to quantify the intradermal, -epidermal and transmembrane nerve fibres (11, 38, 40).

Epidermal nerve fibres in the skin of diabetic ($N=5$) and control animals ($N=5$) are decorated by immunohistochemistry with a neuronal marker, PGP9.5. Panel B of figure 3-8, demonstrates a significant increase ($p=0,0373$) in the amount of Langerhans cells in diabetic animals versus control animals. By quantifying the intradermal (right below epidermis) and transmembrane (basal layer of epidermis) branches in these skin biopsies, no significant difference could be marked

(p=0,3913 and p=0,7133). Also, no difference was seen in intraepidermal nerve fibres of diabetics compared to normoglycaemic controls (p=0,1084).

Our results indicate that the number of Langerhans cells in skin biopsies of 16 week diabetic rats increases significantly in comparison to control animals. The amount of intradermal, transmembrane and intraepidermal nerve fibres in 16 week diabetic rats were not significantly different from control group.
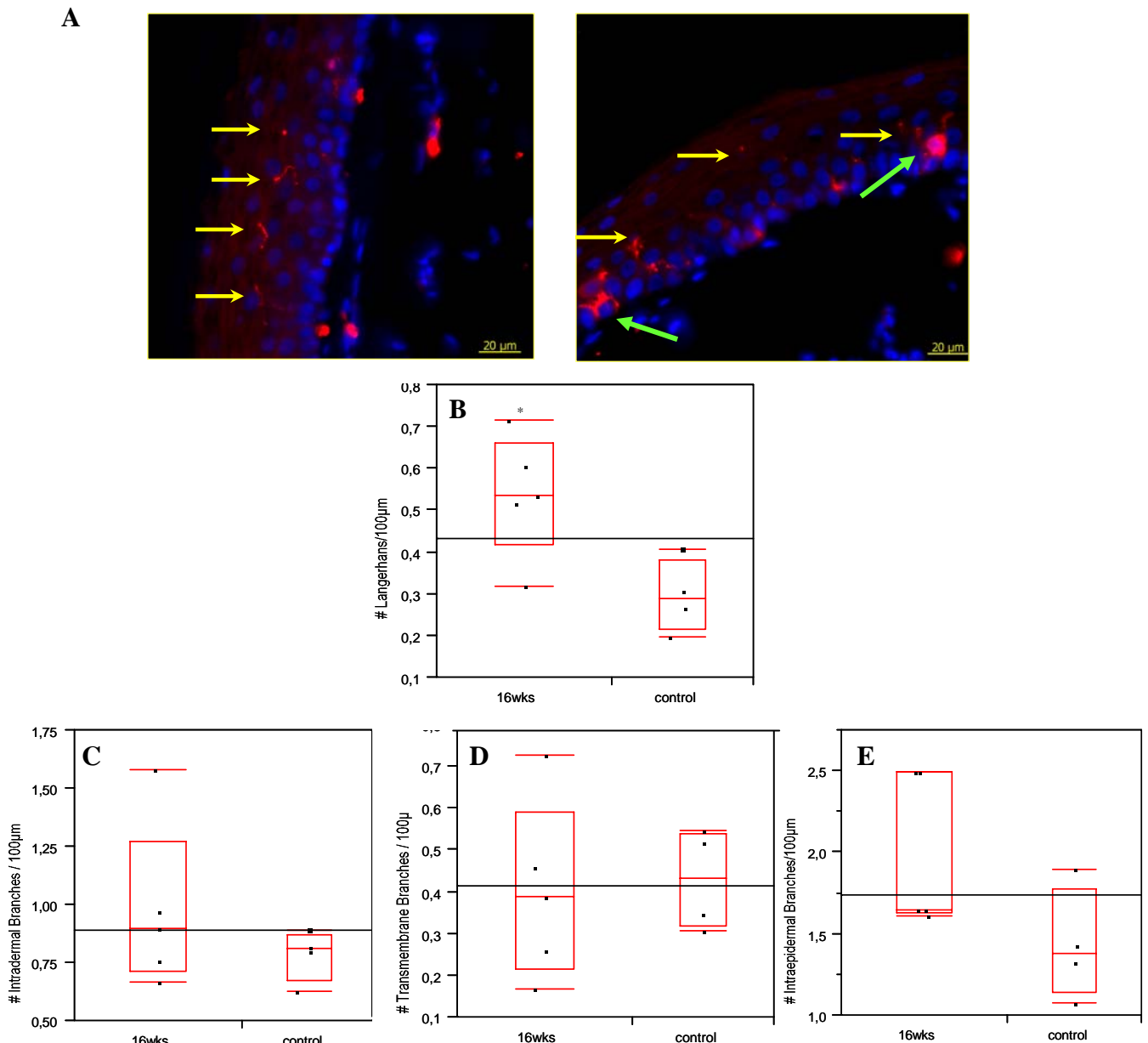
Figure 3-8: PGP9.5 quantification of nerve fibres. **A**. Mozaïk images (20x objective) of PGP 9.5 immunoreactivity in epidermis of control (N=5) (left) versus diabetic rats (N=5) (right). Yellow arrow indicate PGP9.5 stained nerve fibres; green arrow indicate PGP9.5 positive Langerhans cells. **B**. Percentage LCs in epidermis/100µm. A significant higher amount of LCs was present in the epidermis of 16 week old diabetic rats. **C**. Number of intradermal nerve fibre branches/100µm. **D**. Number of transmembrane nerve fibre branches/100µm. **E** Number of intraepidermal nerve fibre branches/100µm. No difference in intradermal, transmembrane and epidermal fibres was seen in 16 week diabetic rats versus control rats. Statistical significance between diabetic and control group was determined using the Wilcoxon/Kruskal-Wallis test (Rank Sums). P <0,05[*], P0,01[**], P<0,001[***].

3.6 Nociceptive tests in diabetic rats after trophic treatment

Previous research had indicated growth factors as potential therapeutic agents for the treatment of diabetic neuropathy. NGF has been shown to promote survival and neuritogenesis in primary neurons of the central and peripheral nervous systems (41-42). Also, intrathecal infusion of GDNF has been shown to prevent and reverse the behavioural expression of experimental neuropathic pain (43). Because of their beneficial effects in neuropathic pain, we examined the effects on allodynia and hyperalgesia after a subplantar injection of GDNF and NGF. The results of these experiments are shown in figure 3-9 and 3-10.

3.6.1 Hind paw mechano-allodynia

In our study, we have investigated the beneficial effects on allodynia in diabetic peripheral neuropathy of GDNF and NGF treatment. Behavioural test were carried out in diabetic rats, which received the first treatment with NGF or GDNF after nineteen days post-STZ injection.

After rats were rendered diabetic with STZ, mechanical thresholds of both hind paws were monitored before and after neurotrophin treatment, using the von Frey test (allodynia). Only the left hind paw was injected with a trophic factor or vehicle (PBS), through which the right hind paw could serve as a control side. As shown in figure 3-8 (left hind paw), NGF injection produced a significant effect of allodynia compared to control and the GDNF group. This increased hypersensitivity started after two days of NGF-injection (D16-NGF: 49,63 ± 2,14g, D20-NGF: 29,90 ± 2,07g; $P<0,001$). No differences in allodynic responses in the left hind paw were observed after the first GDNF injection (D19-PBS: 52,60 ± 2,66g, D19-GDNF: 53,90 ± 2,57g; $P=0,470$), compared to the control group. However, a significant decrease was shown the second day after GDNF-injection ($P=0,023$), but the effect disappeared the following days. The effects of NGF and GDNF were reversible and thus no significant differences of allodynia of both groups versus control groups were visible one day post-treatment. The right hind paw received no injection of growth factor or vehicle. The results showed no significant differences in the two treatment groups compared to the control group. However, on day five of treatment a significant decrease ($P=0,004$) in hypersensitivity threshold was noticed in the NGF group. A significant difference in hypersensitivity was also seen at day 28 after STZ injection in the NGF group ($P=0,049$). One could also notice that the highest decrease in hypersensitivity threshold of both groups was visible after 2 days of trophic treatment compared to the control group (GDNF = 24%, NGF = 48%). Hereafter, the effect in both treatments diminished slowly until it reached the threshold level of the control group at day 28 post-STZ injection.

Our study suggests that a subplantar injection of NGF in the left hind paw of a diabetic rat, increased allodynia after the first injection. In contrast, the right hind paw displayed no significant difference in hypersensitivity throughout treatment period, with the exception of day five. No differences of hypersensitivity threshold in the left hind paw were noticed after five days of GDNF treatment, with the exception of the second day of treatment. The contralateral side of the GDNF injected animals displayed no effect of allodynia after five days of treatment.
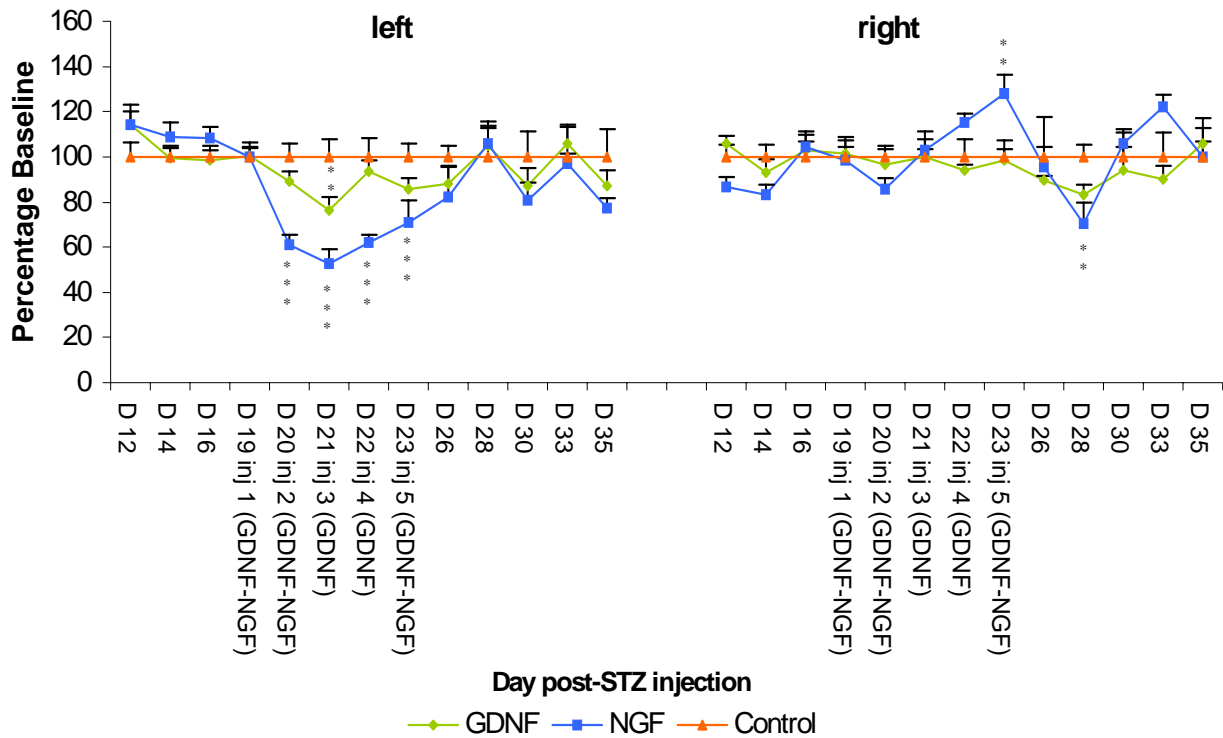
Figure 3-8: Hind paw mechano-allodynia in diabetic rats that received either GDNF injection (N=10), NGF-injection (N=10), or PBS-injection (control, N=8). NGF-injection in the left hind paw showed a significant increase in nociceptive responses during the injection days and reversed after injection days. In contrast, GDNF-injected animals displayed no difference in nociceptive responses during injection days. The right hind paw of both experimental groups displayed no significant differences in nociceptive responses compared to control animals. Data are represented as mean ± S.E.M. (%), after normalisation to body weight and pre-values. Significant differences between GDNF/NGF injected animals versus control animals was determined using two-way ANOVA (Tukey post-hoc); P <0,05[*], P0,01[**], P<0,001[***].

3.6.2 Hind paw mechano-hyperalgesia

Because of the potential role of NGF and GDNF in diabetic neuropathy, we also investigated the beneficial effects of these growth factors towards hyperalgesia, seen with the paw pressure test.

After rats were rendered diabetic with STZ, mechanical thresholds of both hind paws were monitored, before and after neuronal growth factor or vehicle treatment, using the paw pressure test (hyperalgesia) (figure 3-9). Only the left hind paw was injected with a trophic factor or vehicle, through which the right hind paw could serve as a control side. The GDNF group displayed a significant decrease in hypersensitivity, thus increase in percentage baseline, after the second subplantar GDNF-injection (D16: 65,50 ± 5,98g, D20: 74,50 ± 4,25g; P<0,001). The increase versus baseline was visible throughout the entire treatment period. At day 26 post-STZ injection, the effect of GDNF was reversible (P=0,525). The NGF group, however, displayed a significant hyperalgesia effect on the left hind paw after three days treatment (D16: 57,50 ± 3,10g, D21: 39,50 ± 3,45g; P=0,043). Such difference in hypersensitivity was also visible at day 26 and 28

post STZ-injection ($P=0{,}018$, $P=0{,}032$). In the right hind paw, no significant differences of both treatment groups compared to the control group were seen ($P=0{,}027$).

Our results suggest that GDNF improves the hyperalgesia resulting from diabetic neuropathy, immediately after the first injection. In contrast, NGF doesn't have a beneficial effect. It increases the already present hyperalgesia resulting from diabetic neuropathy.
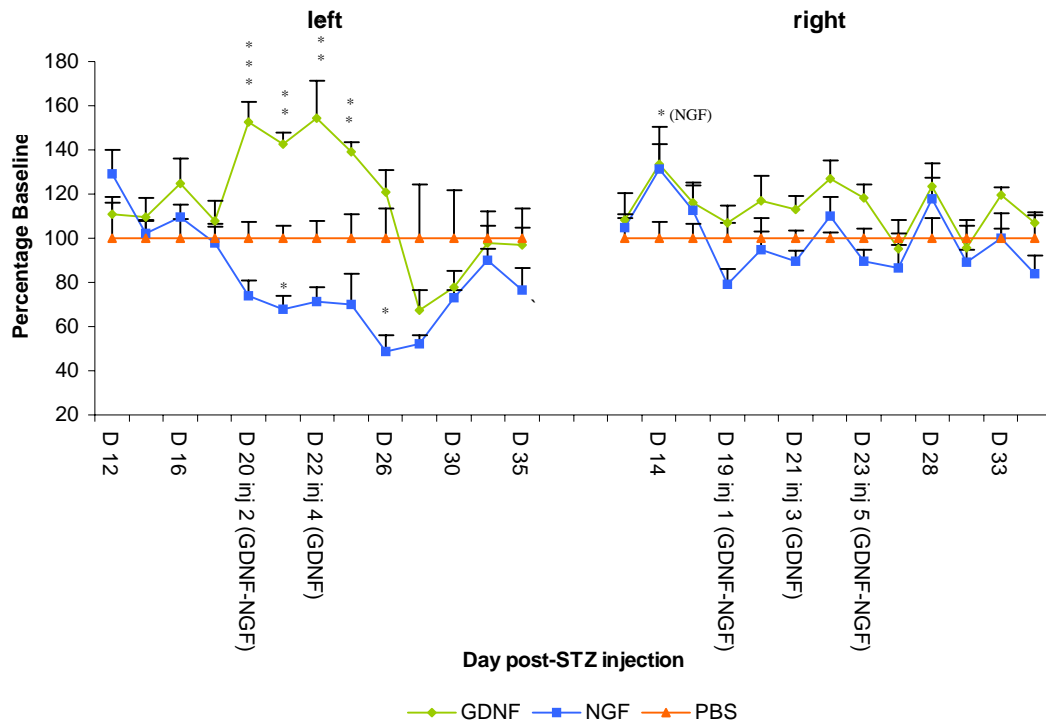
Figure 3-9: Hind paw mechano-hyperalgesia in diabetic rats, receiving GDNF or NGF treatment in the left hind paw, versus control group, receiving PBS injection in the left hind paw. An improvement of the STZ-resulting hyperalgesia during injection days was seen in the GDNF group. The NGF group displayed increases in nociceptive threshold, so increase in hyperalgesia. The right hind paw displayed no significant differences in hyperalgesia. Data are represented as mean ± S.E.M. (%), after normalisation to pre-values. Significant differences between treatment groups versus controls were determined using two-way ANOVA (Tukey post-hoc); $P < 0{,}05^{*}$, $P 0{,}01^{**}$, $P < 0{,}001^{***}$.

3.7 Skin biopsies of diabetic rats after trophic treatment

One day after the last neurotrophin injection, five animals of the GDNF group and five animals of the NGF group were sacrificed and skin biopsies were collected. Consequently, epidermal thickness and inflammatory response was measured.

3.7.1 Epidermal thickness of diabetic rats following trophic treatment

As been said earlier, NGF and GDNF have been proven to be beneficial in the disease model of diabetic neuropathy. Schmidt *et al* have stated that NGF promotes survival and neuritogenisis in primary neurons (41). Another report also shows that the hyperglycaemia induced neuronal loss is reversed by endogenous administration of GDNF (44). Therefore, a first step in this study was the evaluation of the epidermal thickness of growth factor-treated diabetic rats.

Figure 3-10 shows the average epidermal thickness in skin biopsies of diabetic rats treated with neurothrophins (GDNF or NGF) or vehicle (PBS). Skin biopsies from both hind paws of five animals in both treatment groups (NGF and GDNF) were analysed. Also, four animals treated with PBS were sacrificed for obtaining skin biopsies. The average epidermal thickness from GDNF-treated animals didn't differ significantly compared to the control group treated with PBS (GDNF: 69,59 ± 3,72μm, PBS: 80,84 ± 1,57μm; $P=0,155$). Also, no significant difference was seen in epidermal thickness of the NGF group compared to the control group (NGF: 74,38 ± 4,33μm; $P=0,738$). By comparing both treatment groups, also no significant difference in epidermal thickness was shown ($P=0,773$).

These results indicate that there are no differences seen in epidermal thickness in diabetic rats receiving NGF or GDNF treatment when compared to diabetic rats receiving vehicle treatment (PBS).
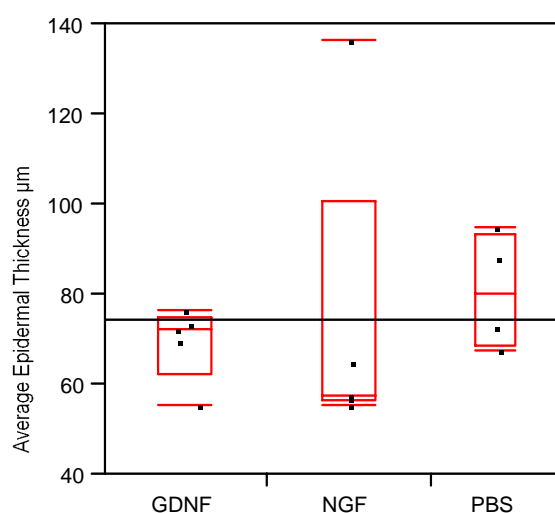

Figure 3-10: Average epidermal thickness of skin biopsies of diabetic rats treated with neurotrophins (GDNF or NGF) or vehicle (PBS). No significant differences in epidermal thickness were seen between the three treatment groups. Significant differences between groups was determined using the Mann-Whitney (Rank Sum) test, $P <0,05^{*}$, $P0,01^{**}$, $P<0,001^{***}$.

3.7.2 Inflammatory response in diabetic rats following trophic treatment

The subplantar administration of a growth factor in the hind paw could have evoked an inflammatory response at the injection site. Therefore, these skin biopsies were morphologically investigated for the presence of inflammatory cells.

Figure 3-11 shows HPS stained sections of the three treatment groups receiving subplantar GDNF, NGF or vehicle (PBS) injection and the contralateral side (right hind paw, receiving no injection). Both groups receiving the subplantar injections of GDNF or NGF, displayed an increased accumulation of inflammatory cells (i.e. neutrophils, macrophages, lymphocytes…) as compared to control group, receiving PBS or compared to the contralateral side. Also the PBS group displayed a minor inflammatory reaction in epidermis following subplantar injection. The contralateral control side (no subplantar injection) displayed no inflammatory response.

Injection of GDNF or NGF resulted an increased inflammatory response compared to the PBS group and the contralateral side. Also the injection of PBS in the plantar surface of the hind paw results in a minor inflammatory response, compared to the results of the right hind paw.
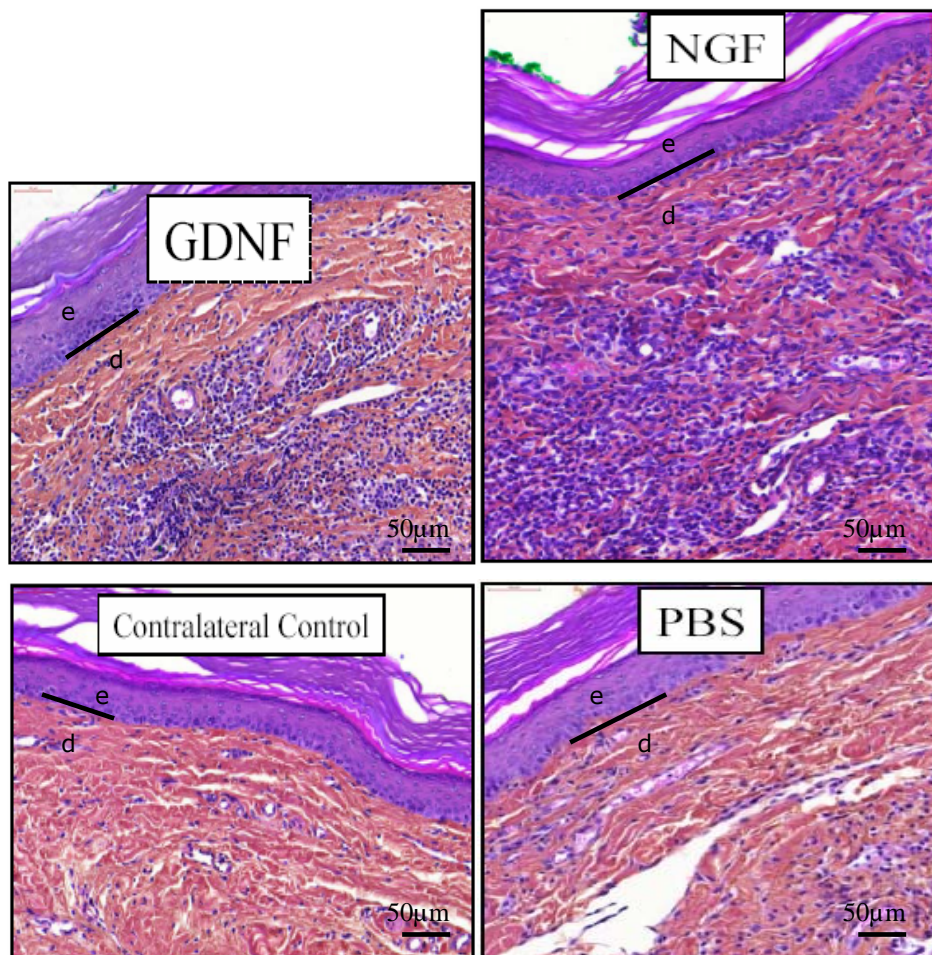
Figure 3-11: Mirax images of HPS stained sections of skin biopsies of the plantar surface of diabetic rats, treated with growth factors (GDNF or NGF) or vehicle (40x objective). A severe inflammatory reaction in the dermis was seen after subplantar injection with GDNF and NGF. The PBS group displayed also a minor inflammatory reaction, following subplantar PBS injection. The contralateral control (normal diabetic) displayed no inflammatory response in the skin. The boundary between the epidermis (e) and the dermis (d) is marked by a line.

4. **DISCUSSION**

4.1 Experimental model

Optimalisation of the treatment regimen with streptozotocin, to induce diabetic peripheral neuropathy, was performed before the current data were produced. After assessing different types of solution (sodium citrate and MilliQ water) and after exploring the right body weight of rats to use at the moment of injection, we came up with an optimal animal model to use in this study. By conclusion, the optimal model for diabetic peripheral neuropathy used in this study consisted of a Sprague-Dawley rat, weighing 275-320g at the time of STZ-injection (dissolved in MilliQ water, 65 mg/ kg and 1 ml/ 100 g rat). This animal model is used in all of the following experiments.

4.2 *In vivo* evaluation of STZ-induced diabetics rats

Tactile allodynia was evident in diabetic rats at one week after streptozotocin treatment, and these responses progressed over the following weeks. Control rats remained stable for a period of eight weeks post STZ-injection. After eight weeks, no significant differences in tactile allodynia were seen in diabetic animals compared to normoglycaemic controls. At this time point control rats displayed increases in hypersensitivity compared to threshold level and remained so for at least twelve weeks. At twelve weeks the allodynic responses in the diabetic group were still evident, but were not significantly different from control group. In contrast, the diabetic group of sixteen weeks post STZ-injection, showed a slight increase in hypersensitivity compared to control animals. These results stand in contrast to other reports (36) that also evaluated control rats for a period of 12 weeks and showed a stable tactile response during the entire testing period. Methodological differences between our study and these reports, including housing conditions, habituation to the testing environment and experimental manipulations in the determination of nociceptive responses may have contributed to the observed differences between our findings and theirs. By performing two test periods every week, the control animals could also got accustomed to the tests and therefore developed a tactile response to the unpainful stimulus. But more likely, the difference in significant allodynic responses could be related to the use of elderly control rats. These control animals were older at the start of the experiment as compared to the diabetic animals. The high body weight, at the end of the study (approximately 450g = ±20 weeks of age), could have contributed to the increase in nociceptive flexion reflexes in the control group. Probably, the age of the animals is related to the spontaneously development of nerve injury.

Mechanical hyperalgesia was evident in diabetic animals one week post-STZ injection. This hyperalgesia progressively increased the following weeks. At four weeks the diabetic animals reached a stable level of withdrawal threshold and this remained until the end of the twelfth week testing period. Control animals remained stable around threshold level for the entire testing period. After sixteen weeks, hyperalgesia was still apparent and significantly different from control animals. In contrast with the von Frey results, control animals did not exhibit changes in

nociceptive threshold. This observation stands linearly with other reports where control animals also didn't display hyperalgesic responses after twelve weeks.

In addition to the hyperalgesic and allodynic responses one week after STZ-injection, hyperglycaemia was present three days post-STZ injection and remained significantly different from control group for at least sixteen weeks post-STZ. However, after three days no change in nociceptive threshold was evident. This finding is in correlation with the findings of previous studies (33-34, 36). Also, a study by Romanovsky *et al*. (45) showed that STZ-treated rats, that did not become hyperglycaemic, also displayed a decreased nociceptive mechanical withdrawal threshold. This suggests that a factor other than hyperglycaemia might trigger mechanical hyperalgesia and allodynia in this animal model. Failure of nerve mechanisms directly regulated by insulin may be an essential part of the pathogenesis of early decrease in mechanical nociceptive threshold observed in diabetic neuropathy. A study by Calcutt *et al* (46) showed that tactile allodynia is not alleviated by rapid normalization of blood sugar, but can be prevented and reversed in diabetic rats by protracted insulin therapy. Also other studies where STZ-injected rats received an insulin implant had responses similar to controls. By instituting insulin treatment, glucose concentrations turned to normal, but not by the rapid and acute lowering of blood sugar levels by a single insulin injection (47). Together, these data suggest that probably impaired insulin action, rather than hyperglycaemia accounts for the late responses of mechanical hyperalgesia and allodynia.

STZ-treated diabetic animals displayed the typical characteristics of this model, i.e. reduced growth, polyuria, diarrhoea and cataracts. As with other published studies using this animal model (35-36), the general health condition of the animals declined extremely fast. As soon as three days post-STZ injection, a decrease in clinical health was noticeable, compared to control group. The first visible factor was the excessive urination, present two days post-injection. This polyuria, creates an extreme weight loss of the animals in the first week after STZ-treatment, and persisted for at least sixteen weeks. In contrast, control animals gained weight during the entire testing period. Due to their severe ill-health the activity of the diabetic animals was greatly reduced, with animals extremely lethargic and exhibiting greatly reduced exploratory behaviour. Cataracts were seen as soon as ten weeks post-STZ injection. After twelve weeks of testing, seven animals of the diabetic group displayed cataract on both eyes. All other symptoms remained or tended to get even more severe. Control animals also had some symptoms similar to that of diabetic rats, such as porphyrins on skin and a desquamated skin. These symptoms are manifested probably through age and the quick onset of the diabetic symptoms was a consequence of their bad clinical health due to STZ-injection. Because allodynic responses could be influenced by body weights, normalisation to body weight was assessed. The stress situation which the animals could have experience during tests, may have interfered with the pain evaluation. Therefore, overtraining the animals circumvented this factor of stress.

The present results provide further evidence that STZ-treated diabetic rats develop a variety of anomalies in pain perception, in particular allodynic and hyperalgesic responses to non-noxious tactile stimulation. These observations extend other reports of streptozotocin-induced alterations in nociception, including both thermal and chemically stimulated hyperalgesia (46, 48).

4.3   Histological evaluation of STZ-induced diabetic rats

Glucose-mediated injury of DRG neurons and their axons result in the development of diabetic neuropathy. *In vitro*, DRG neurons provide a relevant model of diabetic neuropathy. In this study, the expression of ATF3 in DRG neurons was used to investigate neuronal injury. A study by Tsujino *et al* (27), showed an induction of ATF3 expression in virtually all DRG neurons that were axotomized after neuronal injury was induced by transection of the sciatic nerve in rats. They showed that the time course of ATF3 expression was dependent on the distance between the injury site and the cell body. Another report published by Shortland *et al* (49) also found an increase in ATF3 expression in neurons of the axotomised L5 DRG ganglion and in adjacent L4 ganglion after L5 spinal nerve ligation and transection in rats. Similarly, Huang *et al* (50) observed an up-regulation of ATF3 after spinal compression. With the results published in these articles, ATF3 can be considered as a specific neuronal marker of injury in the nervous system.

In the current study, no significant differences of ATF3 positive neurons were observed after histological examination of DRGs from 16-week-old diabetic rats compared to control rats. A previous report by Wright *et al* shows that there is indeed a significant higher level of ATF3 expression in diabetic mice when compared to control mice (25). Our study provides an indication towards increased ATF3 expression in DRGs of diabetic animals, but has probably a weakness in such that only a small amount of animals were used. Nevertheless, it provides an indication of the stress-induced response resulting from the hyperglycaemic conditions in the diabetes-induced peripheral neuropathy. Therefore, the next step in this study was to further explore the mechanisms behind the onset of peripheral neuropathy by performing immunohistochemistry on skin biopsies.

Loss of cutaneous nerve fibres is thought to be partially responsible for the diabetes-induced impairment in the skin's immune defence, wound healing and pain perception. The mechanisms leading to this diabetic neuropathy is still unclear. Skin biopsy has become a new approach to investigate small-fibre sensory neuropathy. The cutaneous nerve fibres are terminal parts of sensory nerves and the skin is the initial part from which nerve fibres start to degenerate in dying-back type polyneuropathy. Nerve endings of small myelinated and unmyelinated fibres in the epidermis of the skin can be demonstrated by immunohistochemistry using various neuronal proteins, particularly PGP 9.5. In the present study we investigated the epidermal thickness and the loss of epidermal nerve fibre densities in the rat model of diabetic neuropathy.

The current study indicates that diabetic rats display a decrease in epidermal thickness compared to control groups after two weeks STZ-treatment. Even after 16 weeks of diabetes, the decrease in epidermal thickness was still apparent. This reduced epidermal thickness could implicate a reduction in epidermal nerve fibres. Also, the decrease in epidermal thickness could also mean that the epidermal nerve fibres are becoming more pronounced at the surface area of the skin, which could have played a role in the increased pain perception seen in diabetic animals.

After evaluation of the intradermal, transmembrane and intraepidermal nerve fibre branches, we concluded that there were no significant differences seen in diabetic animals compared to the normoglycaemic control group. Findings of other reports (38, 51-52) examinating epidermal nerve

fibre density, stated that a decrease in intraepidermal nerve fibres was seen after STZ-injection. Because our data didn't reach significance, it doesn't necessarily mean that there isn't a difference in epidermal nerve fibres in diabetic animals compared to control rats. A report by Chen *et al* (53) has investigated nerve fibre loss in *thy1*-YFP transgenic mice. They concluded that the loss of primary cutaneous nerve fibres did not occur until the 3rd month of diabetes, while functional impairment of the sensory nerve, indicated by the behavioural tests, occurred a few weeks after onset of diabetes. Also, the loss of secondary cutaneous nerve fibres occurred after 6 months of diabetes. This finding indicates that functional impairment preceded the loss of small cutaneous YFP nerve fibres. In contrast, our study included diabetic animals of sixteen weeks of age. Because the work by Chen at al and others (59) who stated that the loss of primary and secondary sensory nerves was only visible after three to six months, this could explain the differences seen with our study. A mean weakness in evaluating nerve fibres was the small test group we used. Therefore, in the future, this experiment should be repeated using larger test groups and probably using animals of different age groups to create a complete perspective of the disease spectrum in diabetic neuropathy.

The present study shows a significant increase in Langerhans cells of 16-week-old diabetic rats. The presence of LCs in a taxol-induced neuropathy model has already been described previously by Siau *et al* (40). They stated that only low levels of PGP9.5 were detectable in Langerhans cells in the skin of normal rats, but a significant increase of PGP9.5 was seen in the skin of paclitaxel-induced neuropathy rats. LCs are dendritic cells abundant in epidermis containing large granules, called Birbeck granules. On infection of an area of the skin, the local Langerhans cells will take up and process microbial antigens to become fully functional antigen-presenting cells. It is conceivable, that in the presence of IENF degeneration, other kinds of dendritic cells and macrophages would invade the skin. Siau *et al* suggested an up-regulation of PGP9.5 in LCs in cases with IENF loss but not in cases without degeneration, which could mean that products of axonal degeneration may be the initiating signal for LC activation. If this was the case, one might expect the largest increase in PGP9.5-positive LCs in cases with the greatest IENF loss. However, this observation isn't compatible with our results. Maybe, the PGP9.5 present within the LCs may represent protein that the LCs phagocytosed from degenerating nerve fibres. In conclusion, our study is the first to demonstrate an increase of LCs in the skin of rats suffering from chronic diabetes.

4.4 *In vivo* evaluation of STZ-induced diabetic rats after trophic treatment

It is meanwhile well established that neurotrophins, in particular NGF, play a fundamental role for sensory neurons, in particular during their ontogenic development. NGF responsive neurons include both C and thinly myelinated Aδ fibres. Physiologically, NGF treatment has been shown to modify the sensitivity of Aδ mechanoreceptors. The mechanisms that underlie this are not clear, but one suggest that the effects of NGF on nerve fibre morphology may be mediated through improved glial-nerve interactions, which are known to be affected early during the course

of diabetes. Studies have already shown that the sensitivity of peripheral sensory receptors, i.e. in skin, can be modulated by NGF administration. It has known by previous studies that NGF injection can result in hyperalgesia (54). Lewin *et al* demonstrated that hyperalgesia induced by NGF in a dose-dependent manner might be caused by a peripheral and a central component, i.e. mast cell degranulation. However, the more recent findings that mast cells release NGF, and that NGF can still elicit hyperalgesia under some conditions after mast cell degranulation, suggest that some of the effect of NGF may be exerted on sensory neurons directly (9, 54). Additionally, GDNF has also been shown to have neuroprotective effects on damaged sensory neurons in a number of experimental models of peripheral neuropathy. Akkina *et al* demonstrated that diabetes mellitus results in significant deficits in the central terminals of GDNF-responsive neurons in the spinal dorsal horn. GDNF administration to diabetic mice reversed these diabetes-induced deficits in the spinal cord (55). A study by Wang *et al*. (43) concluded that GDNF is capable of regulating a variety of gene expression in multiple subtypes of primary afferent sensory neurons. Thus, although the causal relationship of these gene products and the neuropathic pain states remain to be defined, the efficacy of exogenous GDNF in blocking nerve injury-induced pain continues to support GDNF as potential therapeutic intervention for neuropathic pain. Because of their known neuroprotective properties, the effect of NGF and GDNF on diabetes mellitus-induced neuropathy was evaluated in the *in vivo* model of diabetic neuropathy in the present study.

Our findings suggest that NGF induces a hyperalgesic and allodynic response following subplantar injection. The maximum response was reached after two days injection. After injection the increased nociceptive responses were reversed to the stable level after STZ-treatment. A study by Lewin *et al* (54) stated that seven hours after a single systemic injection of NGF mechanical hyperalgesia resulted. This finding correlates with our results after the subplantar injection of NGF. In our study NGF was administered directly into the left hind paw. This could have evoked the severe inflammatory reaction seen after trophic injection in the left hind paw. The inflammatory hypersensitivity could have had influence on the results measured by the von Frey test and explains the slight decrease in allodynic responses in the right hind paw after NGF-treatment.

The present study shows that a subplantar GDNF-injection in the left hind paw improves the hyperalgesia and not the allodynia, following STZ-treatment. In contrast, the right hind paw didn't show significant differences in allodynic nor hyperalgesic responses, suggesting that the subplantar injection does not progress into a systemic treatment. A maximum response was reached after two days GDNF-injection and were reversed GDNF-injection stopped. Other studies, which have shown beneficial effects of GDNF, have used intrathecal administration of GDNF. This intrathecal treatment overcomes the fact that regenerating sensory neurons cannot enter the spinal cord. Surprisingly intrathecal treatment with GDNF or NGF can cause sensory axons to regenerate back into the spinal cord, thereby rescuing sensory function (56). These findings are in correlation with previous findings suggesting a potential role of GDNF in relieving neuropathic pain. Such a study, by Akkina *et al* (55) stated that diabetic mice treated with GDNF exhibit increased cutaneous innervation and axon branching in the flank and footpad skin, compared to non-treated diabetic mice. This study also implicates that the GDNF-responsive sensory axons are particularly vulnerable in diabetic peripheral neuropathy.

Delivery of exogenous neurotrophic factor to treat neuropathies has not yet translated from the experimental to clinical settings with any degree of success. It should also become apparent that the side of growth factor injection should be taken with great considerations. A subplantar injection could have influenced the results of the different behavioural tests, but do not rule out its efficacy. Our results suggest that GDNF has only a beneficial effect during injection days. In the future one could increase the dose or increase the injection days of neurotrophic treatment in order to create a long-lasting beneficial effect. Another approach is the use of small molecule enhancers of endogenous neurotrophic factors, which has site specificity and allows dose ranging to efficacy (57). To evaluate the impact on nerve fibres after growth factor administration, the next step in this study was to evaluate the epidermal thickness and inflammatory response on skin biopsies of these animals.

4.5   Histological evaluation of STZ-induced diabetic rats after trophic treatment

We already pointed out the potential therapeutic effects of NGF and GDNF and their role in diabetic neuropathy with the *in vivo* animal model. Histology of skin biopsies provides another way of evaluating the influence of GDNF and NGF treatment in these animals. The results of this study provide a first step of fully understanding the pathogenic role of growth factors in diabetic neuropathy. Our study evaluated the epidermal thickness and the inflammatory response following injection. Christianson *et al* reported that, similar to human diabetic neuropathy, diabetic mice develop severe reductions in the number and branching of peripheral sensory afferents in hind limb skin. GDNF treatment increases both the number and branching of cutaneous axons, suggesting that GDNF may be effective in improving cutaneous innervation deficits that occur in sensory neuropathies (58).

The morphological characteristics of skin biopsies were evaluated in diabetic rats that received trophic treatment or vehicle. In our study, following growth factor treatment, immunohistochemistry on these skin biopsies revealed no significant differences of epidermal thickness compared to vehicle treated rats. It was also noticeable that following growth factor treatment a significant increase in inflammatory response could be remarked. Comparing to the control group, receiving PBS injection, the inflammatory response was more severe. This means that not the injection accounts for the increased inflammation, but the growth factor itself.

These results could provide functional use in today's treatment view of diabetic neuropathy. However, further studies on these skin biopsies concerning the epidermal nerve fibre densities by PGP9.5 are still to be processed. Together with the data we already have, this should give a better perspective of the influence of subplantar growth factor treatment in diabetes-induced peripheral neuropathy in rats and provides insights how to treat the neuropathy with neurotrophic factors.

4.6   General conclusion

The present data has provided us more information about the events that take place during the development of diabetic peripheral neuropathy. The *in vivo* results indicated an increase in nociceptive thresholds after STZ-injection for at least 16 weeks. The histological results of the skin biopsies and the DRGs showed that diabetes resulted in a decrease in epidermal thickness and increased ATF3 positive neurons. The epidermal nerve fibres are not significant different between diabetic and control rats.

A subplantar GDNF injection resulted in an improvement of hyperalgesia but not allodynia. In contrast, subplantar NGF-injection resulted in an increased hyperalgesia and allodynia at the moment of injection. GDNF improved the neuropathy resulting from STZ-injection. This could serve as a novel potential therapy against diabetic neuropathy. But more research is needed towards injection routes, dose and the endurance of the effect. The skin biopsies of these diabetic animals gained insight in the epidermal nerve fibres and the onset of peripheral neuropathy. Complete histological investigations of the epidermal nerve fibres using PGP9.5 are yet to be performed. If these results are as promising as the *in vivo* results and the already available *in vitro* results, the use of GDNF as a treatment option for diabetic neuropathy will be closer at hand.

As such, the quest for finding an efficient treatment for one of the most threatening complications of diabetes mellitus has revealed new opportunities.

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